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Silver nanoparticles produced by green synthesis using *Citrus paradise* peel inhibits *Botrytis cinerea* in vitro

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ABSTRACT

Purpose: Our objective was to undertake the green synthesis of silver nanoparticles using Grapefruit *(Citrus paradise)* peel extract and evaluate the effects of silver nanoparticles on *Botrytis cinerea.* **Research method:** The silver nanoparticles formation was evaluated at different temperatures and concentrations of $AgNO_3$. The experiment was conducted during 2015 at Science and Research Branch, Islamic Azad University, Tehran, Iran. **Main findings:** Silver nanoparticles were successfully synthesized by Grapefruit's peel through a simple green and eco-friendly route. Aqueous extract of Grapefruit's peel was used synthesize nanosilver. The size of nanoparticle was determined at 5-65 nm, with SPR absorption at 420 nm in UV-Vis spectroscopy. Transmission electron microscopy (TEM) and X-ray diffraction spectroscopy (XRD) revealed that the synthesized nanoparticle was face centered. The silver nanoparticles characterized for their size and shape using scanning electron microscopy and TEM, respectively. XRD was used to determine the concentration of metal ions. Result indicated that nanosilver reduced the growth of *Botrytis cinerea* inviro culture. The highest antifungal effect was seen in the treatment with 40g/l nanosilver. In the other hand, the effect of nanosilver and time on diameter growth of *Botrytis cinerea* was not significant, individually (p≤1%). **Limitations:** No limitations were founded. **Originality/Value:** Green Synthesis of Nano is a reliable method for the nanoparticles synthesis and environmentally friendly approach.

INTRODUCTION

Plant mediated synthesis of nanoparticles has been considered as green route and a reliable technique for the synthesis of nanoparticles due to its eco-friendly approach. Over the past decade, it has become clear that nano-scale materials have useful physicochemical, optical and electrical properties. Nanoparticles synthesis is emerging as one of the fastest growing field due to their physical, chemical and biological properties. Consequently, a steadily increasing number of consumer products employing nanotechnology have become available for purchase (Biswas & Wu, 2005). In green procedure for synthesis of metal nanoparticles, plant extract may act as both reducing and capping agents, due to their specific structures and functional groups (Kouvaris et al., 2012). Nanoparticles defined as considered as particles with a maximum size of 100 nm. They display completely improved properties, which are quite different from those of when particles. These characteristics depend on the size, shape, and surface compared to the larger particles of the bulk material (Gurunathan et al., 2009).
Nano sized silver particles have found tremendous applications in the different field such as high sensitivity, bio molecular detection, antimicrobials, antioxidants, therapeutics and catalysis (Tada et al., 2000). There are three methods for preparation of nanoparticles containing; Physical, chemical and biological. In fact, Nanoparticles can be synthesized by various approaches like chemical and photochemical reactions in reverse micelles, microwave assisted, thermal decomposition, electrochemical, sonochemical process and green synthesis methods (Maribel et al., 2009). The green synthetic approach for nanoparticles formation, using bacteria (Mukherjee et al., 2008), fungi (Mohanpuria et al., 2008) and plants (Dhillon et al., 2012) has already been reported.

The physical and chemical processes for nanoparticles synthesis are very costly; because of that reason, researchers have found the cheapest way by using microorganisms and plant extracts for nanoparticle synthesis. The uses of microorganisms in biological nanosynthesis have been widely established and it is a kind of bottom up approach where oxidation/reduction reaction is the main reaction (Gericke & Pinches, 2006; Kaler et al., 2011). Three procedures of green synthesis are: (a) use of microorganisms like fungi, eukaryotes and Prokaryotes, (b) Use of plant extracts or enzymes and (c) Use of templates like DNA, membranes, viruses and diatoms. .
Metal nanoparticles, such as Ag, Au, and Pt are applied in products that directly are exposed to the human health, such as household items like detergents, soaps, shampoos, cosmetic products, and toothpaste. They find applications in the pharmaceutical and medical area (Ankanna et al., 2010). Therefore, green synthesis of nanoparticles is gaining importance due to its simplicity, cost effective and eco- friendliness (Yogeswari et al., 2012; Farooqui et al., 2010).

Among all the noble metals, silver nanoparticles are most important product in the field of nanotechnology which has gained boundless interests because of their unique properties; such as chemical stability, good conductivity, catalytic antibacterial, anti-viral, antifungal and anti-inflammatory activities. They can be incorporated into composite fibers, cryogenic superconducting materials, cosmetic products, food industry and electronic components (Ahmad et al., 2003; Klaus-Joerger et al., 2001).

Some reports have pointed out to the promising prospect of nano-silver by using various plants. Krishnaraj et al. (2010) synthesized nanosilver by *Acalypha indica* in spherical shape and diameter of 20-30 nm. Green synthesis of silver nanoparticles is currently being considered using *Allium sativum* in spherical shape and 4-22 nm. Chandran et al. (2006) studied one and remarkable effort were spent to develop Green synthesis of silver

nanoparticles by *Aloe vera* in spherical and triangular shape and size of 50-35nm. *Azadirachta indica* is extensively used for Silver nanoparticles synthesis in spherical, tringular and quasispherical shape and size of 7.5-65nm by Kasthuri et al. (2009).

Nanoparticles can be characterized by their size, surface area, and shape and dispersive (Jiang et al., 2009). Some common techniques shown their characterization such as, UV-visible spectrophotometer, scanning electron microscopy (SEM), Transmission Electron Microscopy (TEM), X-ray diffraction (XRD), Fourier transforms infrared spectroscopy (FTIR) and energy dispersive spectroscopy (EDS) (Feldheim & Foss, 2002; Sepeur, 2008; Shahverdi et al., 2011). UV-Vis spectrophotometer allows identification, characterization and analysis of metallic nanoparticles. Generally, 300-800nm light wavelength is used for the characterization of size range from 2 to 100nm (Feldheim & Foss, 2002). Electron microscopy is a common method for surface and morphological characterization. SEM and TEM are used for the morphological characterization at the nanometer to micrometer scale (Schaffer et al., 2009). SEM can provide morphological information on the submicron scale and elemental information at the micron scale; but TEM has a 1000 fold higher resolution compared with the SEM. Characterization of nanoparticle using FTIR is very useful for the understanding of the surface chemistry because the organic functional groups which are attached to the surface nanoparticles can be determined (Chitrani et al., 2006). XRD is used to examine the overall oxidation state of the particles as a function of time, i.e. phase identification and characterization of the crystal structure of the nanoparticles (Sun et al., 2000). EDS is used to determine the elemental composition of metal nanoparticles (Strasser et al., 2010).

Botrytis cinerea is considered as one of the serious diseases of cucumber plants. It can attack several plant tissues and many greenhouse crops, such as pepper, sweet basil strawberry, sweet basil tomato and strawberry. In vegetables, it may infect fruits, stems and leaves (De Cremer et al., 2013). In addition, it causes pre- and post-harvest diseases in at least 200 plant species (Jarvis, 1977). Infection resulting from growth through the infection or petiole of wounds may cause plant death. *B.cinerea* is one of the most comprehensively studied fungal plant pathogens in greenhouses (Van Kan, 2006). When *B. cinerea* infects the host, it can destroy the cell walls by secreting diverse enzymes and proteins (Zhang & Van Kan, 2013). The symptom of Botrytis infection of fruits appears as a gray rot. In the spring, the fungus germinates from small and dark-colored, over-seasoning structures known as sclerotia. Then the fungus produces asexual spores that caused spread the disease.

MATERIALS AND METHODS

Materials

All chemical materials were purchased from Merck Company, Germany. The *Citrus paradise* fruits were collected from Sari, Province of Mazandaran, with longitude 53˚ 5'Eand latitude 36 ◦ 4'Nat 132m above sea level and an average annual temperature of 15°C, which has a temperate climate Caspian. Plant material was identified in Citrus and subtropical research center of Iran. The fruit peel was cut and dried in shade under a stream of air in a chamber room. The dried peel was ground and stored at 4˚C.

Instruments

UV-Vis studies were carried out using a Varian Cary 300 UV-Vis spectrophotometer. Crystallographic studies were carried out using a 3003 PTS Seifert (Germany) X-ray diffraction (XRD) instrument. Morphology the synthesis of silver nanoparticles were performed by transition electron microscopy (TEM) using a PHILIPS EM 208 instrument

at100 Kv. FTIR experiment was used to determine the active functional groups in plant extract using a Nicolet NEXUS 870 FT-IR spectrophotometer.

Preparation of Grapefruit peels extract

Twenty (20) gr of *Citrus paradise* fruit peel was extracted using 100 ml deionized water for 30 min in an 80˚C water bath. The extract was filtered via a No.1 whatman filter paper. The volume of solution was adjusted to 100 ml in a volumetric flask. The concentration of extract in solution was 0.025gr/ml. The solution was stored in 4˚C for further experiments.

Plant mediated synthesis of Ag nanoparticles

Twenty (20) ml of *Citrus paradise* fruit peel extract solution was added to 80 ml of 0.001M solution of $AgNO_3$ at room temperature (25° C). The mixture was shaken in darkness at 200 rpm using a IKA orbital shaker in four pH (4, 7, 8 and 10) and three temperatures (40, 50 and 60° C). The color change of solution from pale yellow to reddish brown after 30 min showed the reduction of Ag^+ ions to Ag^0 nanoparticles.

Characterization of synthesized Ag nanoparticles

The plant mediated synthesized Ag nanoparticles, structurally, was investigated by X-ray diffraction, transmission electron microscopy (TEM) and Ft-IR instrument. The nanoparticles were precipitated by centrifugation at 12000 rpm for 20 min. The obtained pellets were washed twice with deionized water, was further washed with ethanol. It was dried at 60˚C in vacuum oven for 5h. Ft-IR experiments were done by KBr disk method. A 0.5 mg of the sample was ground with KBr in an agate mortar and 15 mm disks were made using 10-ton pressure. The FTIR spectra was achieved using a Nicolet NEXUS 870 FTIR spectrophotometer from 4000-400 cm^{-1}.

XRD analysis was done to determine the structure of synthesized Ag nanoparticles. XRD studies were done by a Sifert XRD 3003 PD (Germany) with a Cu-Kα_1 X-ray tube with λ of 1.54 A° in the range of 2θ from 20° to 90° using dried powdered of Ag nanoparticles. Calculation of particles size of synthesized Ag nanoparticles was done using Debye-Scherer's equation from XRD parameters as shown below:

$$D = K\lambda / ß \cos\theta$$

Where, D is particle size, K is a dimensionless shape factor, with a value close to unity (0.9), λ is wavelength of X-Ray in angstrom (A°), ß is the full width at half the maximum intensity (FWHM) in radian and θ is the diffraction Braggs angle. Elimination of additional instrumental broadening the ß value should be corrected according to blow formula using the FWHM from a large grained silicon sample, which was calculated as 0.15868 A° in our XRD instrument (Ghosh et al., 2012).

$$ß \text{ corrected} = [(FWHM_{sample})^2 - (FWHM_{Si})^2]^{0.5}$$

Morphology of Ag nanoparticles was performed using transmission electron microscopy (TEM) image by means of a Philips EM 208 instrument with 100 kV acceleration voltages. 10-µL solution of dried Ag nanoparticles that were dispersed in deionized water using ultrasonic bath, was located on a carbon coated TEM grid and subjected to TEM instrument.

Preparation of fungal culture and inoculums

The pathogen, *Botrytis cinerea*, was obtained from Plant Pathology laboratory in Islamic Azad University, Science and Research Branch, Tehran, Iran. The cultures were cultured on potato dextrose agar (PDA) medium at 4°C; and fresh cultures were grown on PDA plates at 25°C.

RESULTS AND DISCUSSION

UV-Vis spectral observation

Color change of mixture solution of *Citrus paradise* fruit peel extract and 0.001M $AgNO_3$ from pale yellow to reddish brown was due to the formation of Ag nanoparticles (Fig. 1).

The UV-Vis spectral studies at different time intervals 30 min, 180 min, 24 h and 48 h) as shown in Figure 1. The Surface Plasmon Resonance (SPR) absorption in 430 nm appeared due to colloidal Ag nanoparticles (Fig. 2).

The FTIR spectra of *Citrus paradise* peel extract with three strong absorption peaks at 3410,2929 and 1637cm^{-1} (Fig. 3, A) which related to stretching frequency of OH, C-H and C=O, respectively showed that the active biomaterials such as flavonoids, carbohydrates and phenolic compounds are responsible for reduction and stabilization Ag^{+} ions to Ag^{0} nanoparticles. This could be seen by a reduction in intensity of main peaks after decreasing reduction of Ag+ ions (Fig. 3, B) observed (Fig. 2).

The spectra characterizing the phytochemical fabricated Ag nanoparticles showed a XRD pattern with four main peaks at 37.8431°, 45.9587°, 64.1242° and 76.9911° in the range of 2θ from 20° to 90° related to (111), (200), (311) and (222) HKL values, respectively, which shown that the Ag nanoparticles were synthesized in a face center cubic (fcc) lattice system (Fig. 4).

Fig. 1. Color change of AgNO3 solution + *Citrus paradise* extract from pale yellow (A) to reddish brown (B)

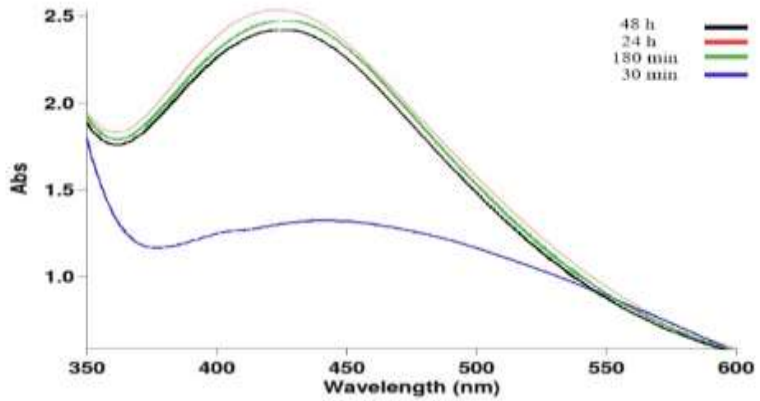

Fig. 2. UV.Vis spectra of plant mediated synthesized Ag nanoparticles

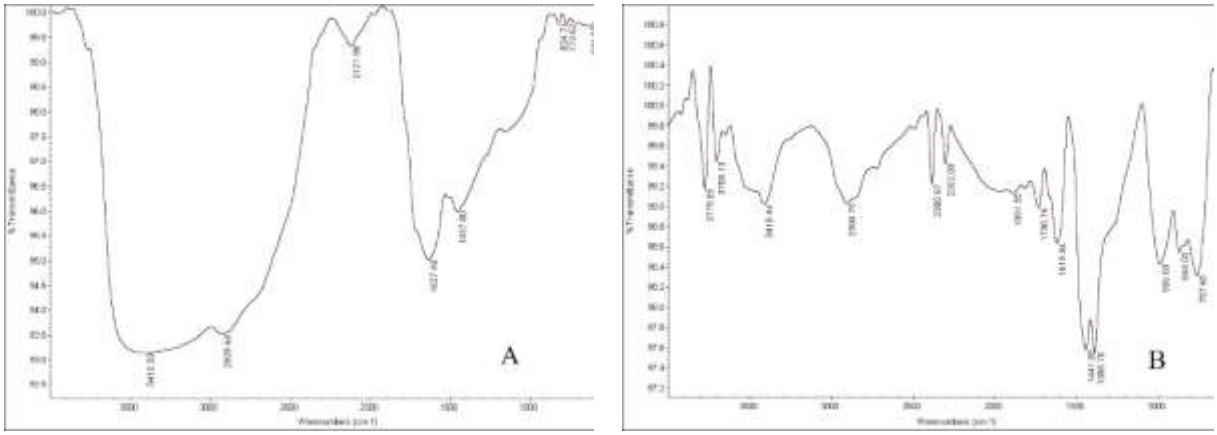

Fig. 3. FTIR spectra of *Citrus paradise* peel extract (A) and synthesized Ag nanoparticles (B)

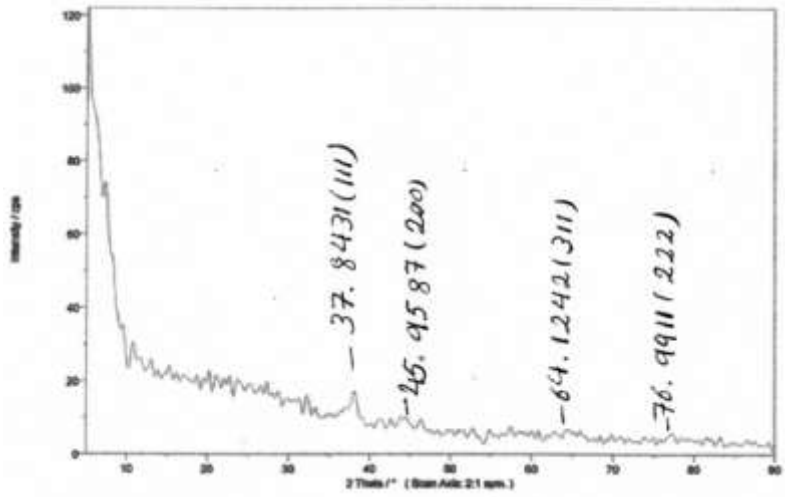

Fig. 4. XRD pattern of Ag nanoparticles using aqueous *Citrus paradise* peel extract

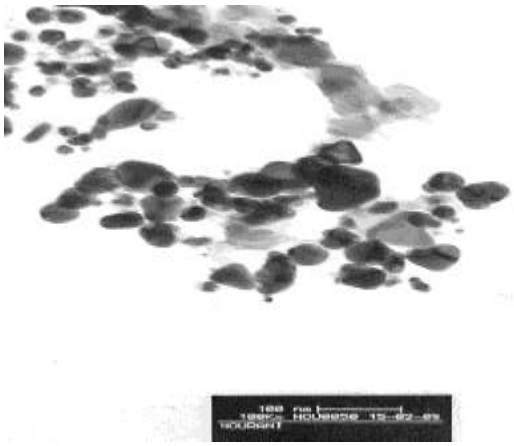

Fig. 5. TEM image of Ag nanoparticles using aqueous *Citrus paradise* peel extract

The average size of Ag nanoparticles was calculated as 55.02 nm according to Debye-Scherer's calculation at 2θ of 37.8431° (111) whit FWHM $_{sample}$ and FWHM $_{Si}$ were 0.2202 and 0.15868, respectively.

TEM image (Fig. 5) revealed that the aqueous *Citrus paradise* peel extract could be fabricated the Ag nanoparticles in a spherical shape with the diameter ranging from 5-65 nm.

The inhibitory effect of nanosilver on the growth of the *Botrytis*

According to Figure 6, the highest inhibitory effects on the growth of the *Botrytis* were related was at 40g/l nanosilver. Also 20 and 30 g/l nanosilver had significant inhibitory effects; nanosilver concentration decreased fungal growth. Increasing nanosilver from 20 g/l to 30 g/l or even up to 40 g/l significantly reduced the growth of microorganisms.

The effect of time on *Botrytis* growth had shown an increase in the diameter of the fungus over time. Figure 7 is shown the lowest *Botrytis* growth rate in the first day (0.92 cm). After 11 days, it reached to 6.81 cm, which was the highest growth.

Result indicated that, 40g/l nanosilver had the highest inhibitory effect. On the other hand, 30 and 20 g/l nanosilver had a considerable rate (Fig. 8).

It showed that the growth of *Botrytis cineara* is inhibited at different concentrations of silver nanoparticles. In addition, it presented in this investigation the inhibitory effect of nanosilver on growth of *Botrytis cinerea.* This agrees with other studies which stated, antimicrobial activity of silver was different depending on microbial species (Galeano et al., 2003).

Nanosilver can significantly delay mycelial growth in a different concentration in vitro (Aguilar-Mendez et al., 2011). It may directly stick to and enter the cell membrane to destroy spores, but this mechanism is not understood (Hwang et al., 2008).

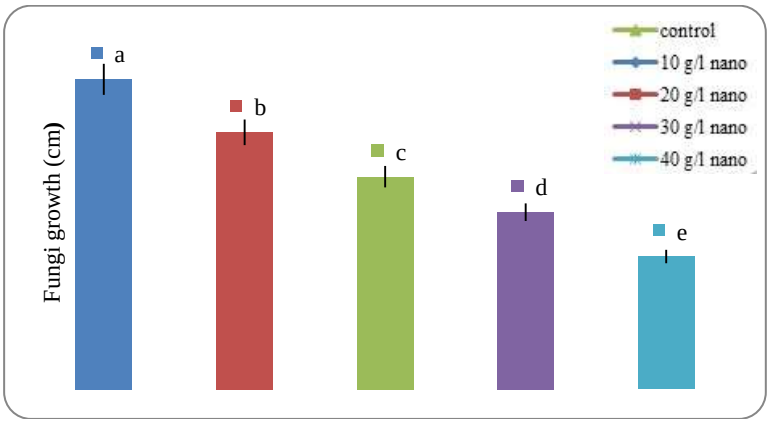

Fig. 6. Effect of treatment on *Botrytis* growth

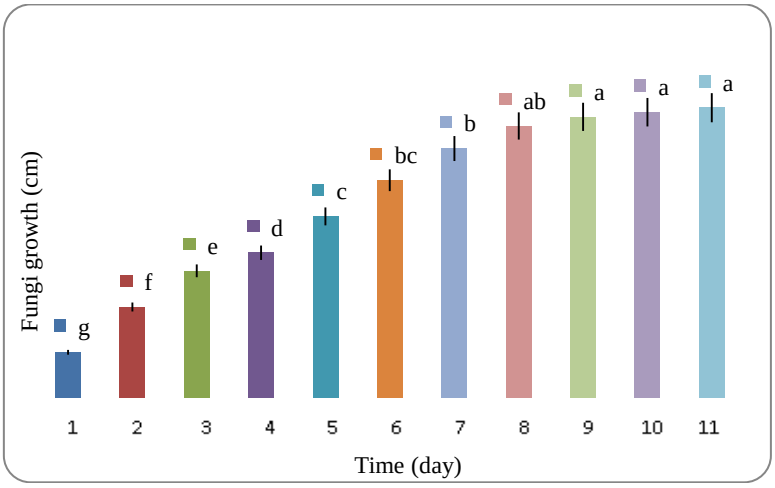

Fig. 7. Effect of time on *Botrytis* growth

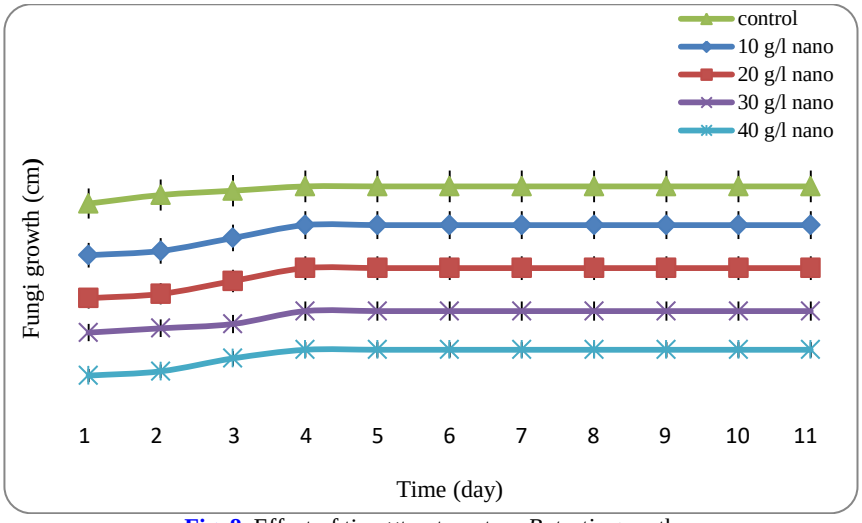

Fig. 8. Effect of time×treatment on *Botrytis* growth

Our results demonstrate that fungal growth was associated with amount of silver nanoparticles, which is consistent with research by Sahar and Ouda (2014), who indicated that nanosilver reduced the growth of microorganisms.

The highest antifungal effect was seen in the treatment with 40g/l nanosilver. It revealed that nanosilver damaged the *Botrytis cinerea* hyphae when compared with control. Qiu et al. (2014) reported that nanosilver caused deleterious effects, not only on fungal hyphae, but also on conidial germination. It showed bacteriostatic action against *Botrytis cinerea*. The principle of bacteriostasis is that nanosilver penetrates the cell membrane of *B. cinerea* and damages it.

CONCLUSION

This study indicated that Silver nanoparticles were successfully synthesized by Grapefruit's peel through a simple green and eco-friendly route. The highest antifungal effect was seen in the treatment with 40g/l nanosilver. In conclusion, results showed that fungi could not be grown on low concentration of nanosilver.

Conflict of interest

The authors declare that they have no conflict of interest.

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Essential oils to control *Botrytis cinerea* in vitro and in vivo on grape fruits

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ABSTRACT

Purpose: The effect of abusing chemical biocides in controlling pests and diseases has drawn the attention of policymakers to the development of methods potentially available in nature for this purpose. **Research method:** In the present study, the inhibitory effects of four different essential oils against *Botrytis cinerea* were tested at various concentrations (0, 200, 400, 600, and 800 µL L^{-1}) in *in vitro* and *in vivo*. **Main findings:** The *in vitro* results showed that the growth of *B. cinerea* was completely inhibited by the application of anise oil at concentrations of 800 µL L^{-1}. The *in vivo* results indicated that treated fruits with marjoram oil had more total soluble solids, and anthocyanin content in comparison to anise, chamomile, and black caraway oil. Furthermore, among essential oils, treated fruits with black caraway essential oils had the lowest pH, while anise, chamomile, and marjoram oil had the highest pH. The highest anthocyanin content and pH were obtained at 200 and 400 µL L^{-1} concentration and lowest values were found at a control treatment (respectively). The most total soluble solids were observed at control treatment and the lowest values were recorded at 600 µL L^{-1}. The application of each essential oil decreased the percentage loss in fresh weight significantly and increased the storage-life of the fruit. **Limitations:** Higher cost of application was a limitation. **Originality/Value:** This research confirms the antifungal effects of anise, fennel, chamomile, and marjoram essential oils both *in vitro* and *in vivo* on grape fruits postharvest. Therefore, these essential oils could be an alternative to chemicals to control postharvest phytopathogenic fungi on grape fruits.

INTRODUCTION

Grape (*Vitis vinifera* L.) is a plant from the family Vitaceae. In this family, there are about 10 genus and more than 600 species. The most important genus of this family is the economically and nutritionally Vitis (Jalili Marandi, 2007). The total grape production in the world in 2016 was reported to be about 77.4 million tons, and Iran was ranked ninth in the world with about 2.45 million tons (FAO, 2016).

Botrytis cinerea, which causes severe damage to many fruits, (gray mold) is a ubiquitous pathogen vegetable, and ornamental crops, both pre and postharvest (Elad, 1997). *B. cinerea* is one of the commonly present fungi causing severe postharvest losses to fruits and vegetables. In recent years and according to its scientific and economic extent, this, the fungus was placed in the second rank in the world top ten pathogens listed (Feliziani & Romanazzi, 2016). It infects the plant both during the growing season and during storage and causes up to 55% loss during post-harvest storage (Martínez-Romero et al., 2007).

Essential oils or the so-called volatile or ethereal oils are aromatic oily liquids obtained from various plant organs which include: flower, bud, seed, leaf, twig, bark, herb, wood, fruit and root (Serrano et al., 2005). The application of essential oil is a very attractive method to control postharvest diseases. The production of essential oils by plants is believed to be predominantly a defense mechanism against pathogens and pests (Oxenham, 2003). Essential oils are volatile, natural, complex compounds, characterized by having a strong odor, and are formed as secondary metabolites by aromatic plants. In nature, essential oils play important roles as anti-bacterial, antiviral, anti-fungal, and insecticidal protectants in plants. They also act against herbivores by reducing the dietary appeal of such plants (Bakkali et al., 2008). Considerable effort has been focused on such plants as sources of potentially useful natural products for use as commercial fungicides (Tian et al., 2011; Tzortzakis, 2009). Generally, the use of plants essential oils has been broadly used as postharvest treatments that are applied to fruits (Maqbool et al., 2011). The exploitation of natural substances such as the essential oils is safer to consumers and the environment for the control of postharvest disease (Isman, 2000). In recent years, numerous studies have documented the antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* (Amiri et al., 2008; Feng & Zheng, 2007; Omidbeygi et al., 2007; Tian et al., 2011). The quality of essential oils depends on several physical parameters such as specific gravity, optical rotation, refractive index, and solubility in different organic solvents, acid number, saponification value, ester value, and phenolic contents (Chowdhury & Kapoor, 2000). However, their data indicates many variations between the same essences.

Antifungal activity of various essential oils from aromatic plants against *B. cinerea* has been documented (Bishop & Reagan, 1998; Chebli et al., 2003; Daferera et al., 2003; Reddy et al., 1998; Wilson et al., 1997) . According to results Mohammadi et al. (2011) application of fennel, anise, peppermint, and cinnamon oils showed that all these essential oils caused an increase in the shelf life of fruits and inhibited *Botrytis cinerea* growth as compared to the controls. Based on the report of Ozcan (2003), antagonistic properties of some essential oils such as salvia, laurel, dill, cumin, fennel, and thyme were found to be in control of *B. cinerea*. Antifungal effects of plant essential oils to control food spoilage fungi *in vitro* and *in vivo* were studied in apple (Amiri et al., 2008), mango (Dubey et al., 2008; Regnier et al., 2008), citrus (Du Plooy et al., 2009), tomato (Omidbeygi et al., 2007), *Malus pumilo* (Shahi et al., 2003), avocado (Sellamuthu et al., 2013) and plum (Aminifard & Mohammadi, 2013). Among post-harvest diseases, *B. cinerea* is an important pathogenic pathogen in a 12-year-old

study in the United States that the causal agent of *B. cinerea* was more than 32.5% of the damage to the fresh grapes (Kulakiotu et al., 2004).

The objectives of this study were to test and to compare the inhibitory effects of the essential oils of anise (*Pimpinella anisum*), fennel (*Foeniculum vulgare*), chamomile (*Matricaria chamomilla*), black caraway (*Carum carvi*) and marjoram (*Origanum majorana*), at different concentrations, in post-harvest control of *B. cinerea* on grape fruits.

MATERIALS AND METHODS

Plant materials and extraction of essential oils

In this study, essential oils of fennel and black caraway were obtained from Mashhad Golfa Shafa Company and Chamomile and marjoram oil from Gorgan Essential Oil Company. Air-dried seeds of anise were supplied from agricultural research fields of University of Birjand, Iran. After the plant seeds parts had been authenticated, a 100 g portion of each was subjected to hydro distillation for 3 h in a Clevenger-type apparatus. The resulting oils were dried over anhydrous Na_2SO_4 and preserved in sealed vials at 4 °C for future analysis (Fatemi et al., 2013).

Experiment I (*In vitro* experiment)

Design of experiments and treatments

In vitro experiments were carried out in a randomized factorial design with two factors; including four essential oils (anise, fennel, chamomile, and marjoram) and five concentrations (0, 200, 400, 600, and 800 µL L^{-1}) with three replications.

Antifungal effects of the essential oils on mycelia radial growth in *in vitro* conditions

Antifungal acting was studied using a contact assay (*in vitro*) that produced hyphal growth inhibition. The test was previously used for essential oil treatment on potato dextrose agar (PDA) medium by the "solution method" (SM) (Özden & Bayindirli, 2002). In this method, each essential oil was dissolved in 5% (v/v) Tween-80 and the required amount was added to each 9 cm Petri plate containing 20 ml PDA-agar at 45ºC. A 0.5 mm disc of *B. cinerea* mycelium was placed on the treated PDA medium and the plate was incubated at 24ºC. Radial mycelia growth was determined each day (up to ten days). The inhibitory percentage (IP) was determined using the formula (1):

$$IP = [(dc \times dt) / dc] \times 100 \quad (1)$$

Where dc was the mycelium diameter in a control Petri dish, and dt was the mycelium diameter in the essential oil-treated Petri dish measured daily (Aminifard & Mohammadi, 2013).

Experiment II (*In vivo* experiment)

Design of experiments and treatments

In vivo experiments were carried out in a randomized factorial design with two factors; including four essential oils (anise, black caraway, chamomile, and marjoram) and five concentrations (0, 200, 400, 600, and 800 µL L^{-1}) with three replications.

Application of essential oils and *B. cinerea* inoculation on grape fruits

The experiment was carried out using grape cultivar Rish Baba that prepared from Birjand local Market. First, remove the grapes from the cluster with scissors with a 1 cm of tail, then rinse the surface and spread on sterile paper to dry. Infected grape fruits were selected and collected from storage to isolate *B. cinerea*. The culture was maintained on PDA at 4 °C. Fresh cultures were grown on PDA plates before usage. Spore suspensions were collected by removing spores from the sporulation edges of a 7–8-day-old culture with a bacteriological loop and suspending them in sterile distilled water. Spore concentration was determined with a hemocytometer and adjusted as required with sterile distilled water (10^5 spore's mL^{-1}). Before infection, fruits were treated with sodium hypochlorite (100 µL L^{-1}) for 5 minutes. They were then sprayed in the prepared suspension and stored at room temperature for 2 h to fix the fungal inoculation (Asghari Marjanlo et al., 2009). In this experiment, three replicates were used for each treatment and 20 experimental units (fruit) for each replicate. We then put the grapes in a zipper pack and sprayed the essential oils on various concentrations (0, 200, 400, 600, and 800). We prepared the essential oil solution from the essential oil mixture with acetone and twin 80 (0.05%) for better solubility and uptake by the fruit. Of course, the solvents were selected according to the experiments performed with acetone, since it did not affect the growth of the fungus. Samples were placed in disposable containers and refrigerated and stored at 4 °C for 10 days.

Anthocyanin contents

Total anthocyanin contents were determined by the differential pH method (Rapisarda et al., 2000). A 1.0 ml aliquot of each grape fruit extract was diluted to 10 ml with a pH 1.0 solution made from 125 ml of 0.2 M KCl plus 375 ml of 0.2 M HCl. A second 1.0 ml aliquot of fruit extract was diluted to 10 ml with a pH 4.5 solution made from 400 ml of 1 M sodium acetate, 240 ml 1 M HCl, and 360 ml H_2O. The absorbance of each solution was measured at 510 nm using a UV spectrophotometer (BioQuest CE 2502; Cecil Instruments Ltd., Cambridge, UK) and the concentration of anthocyanins was calculated using the equation (2):

$$C_{mg\ 100\ g^{-1}} = [(A_{pH1.0} - A_{pH4.5}) \times 484.82 \times 1{,}000/24{,}825] \times DF \quad (2)$$

Where the period in parentheses was the difference in absorbance at 510 nm among the pH 1.0 and pH 4.5 solutions, 484.82 was the molecular mass of cyanidin-3- glucoside chloride, 24,825 was its molar absorption at 150 nm in the pH 1.0 solution, and DF was the dilution factor (Aminifard & Mohammadi, 2013).

Total soluble solids

Total soluble solids (TSS) were determined at 20 °C using a refractometer (RF 10, 0-32° Brix, Extech Co., USA) and reported as °Brix.

pH

The pH of fruit juices was measured at 20 °C using a pH meter (Metro model, manufactured by the Swiss Metro Company).

Weight loss percentage

Weight loss was determined by weighting the whole grape before and after the storage period. Weight loss was expressed as the percentage of loss of weight with respect to the initial weight in the formula (3) (Hosseini & Moradinezhad, 2018).

$$WL = (\text{Initial weight} - \text{Secondary weight}) / (\text{Initial weight}) \times 100 \quad (3)$$

Statistical analysis

The experiment was conducted in a completely randomized factorial design with three replications consisting of twenty fruits each. Data were analyzed using SAS Version 9.1. (SAS Institute, Cary, NC, USA) and means were compared by Duncan's multiple range test at 1 and 5% level of confidence.

RESULTS

***In vitro* experiment**

Effect of essential oils on radial growth of *B. cinerea* in *in vitro* conditions

The results of the analysis of variance on the tenth day showed that the effect of type and concentration of essential oil and their interactions on the growth rate of *B. cinerea* fungi were significant (at 5% level) (Table 1). The highest fungal growth rate was observed in essential oil of the chamomile with a growth rate of 25.70 mm, while the least fungal growth rate was recorded at anise oil (11.06 mm). Marjoram and fennel oil after anise oil showed the greatest inhibitory effect (14.3 and 15.3 mm, respectively) (Fig. 1). The results of the mean comparison of essential oil concentration on the tenth day indicated that the control had the highest growth rate with the growth rate of the fungus 40.6 mm, and the concentration of 800 µL L^{-1} with 5.5 mm had the least growth, and the rest concentrations showed significant differences (Fig. 2).

On the tenth day, the growth rate of *B. cinerea* was significantly different in control treatment and all anise oil treatments (Fig. 3). Concentrations of 200, 400 and 600 µL L^{-1} of fennel oil did not differ significantly on 10 days, but all of them had a significant difference with the control of *B. cinerea* fungus and 800 µL L^{-1} (Fig. 3). The growth rate of *B. cinerea* fungi on day 10 was not significantly different in 400 and 600 µL L^{-1} of chamomile oil, but showed a significant difference with other treatments, there was a significant difference between control treatment and 200 and 800 µL L^{-1} concentration. On the tenth day, the growth rate of *B. cinerea* fungus in the control treatment and all treatments of marjoram oils were statistically significant. Generally, the highest growth rate of fungi was observed in control treatment and the lowest values in 800 µL L^{-1} concentration anise, fennel, chamomile and marjoram oils (Fig. 3).

***In vivo* experiment**

Anthocyanin contents

There was a statistically significant difference in the amount of anthocyanin in treated grapes with *B. cinerea* fungus (at 1% level) (Table 2). The highest amount of anthocyanin in grapes treated with marjoram oil (2216.6 mg $100g^{-1}$) and the lowest amount of it was observed in grapes treated with chamomile oil (936.25 mg $100g^{-1}$). The results of mean comparison of grapes incubated with *B. cinerea* fungus between different concentrations on the amount of grape anthocyanin showed a significant difference. The highest amount of anthocyanin was observed at 200 µL L^{-1} concentration (2084.24 mg $100g^{-1}$) and the lowest amount were recorded at control treatment (1157.7 mg $100g^{-1}$).

Total soluble solids

There was a significant difference between the results of mean comparison of grapes treated with *B. cinerea* fungus between different essential oils on the amount of grape total solids content (at 1% level). The highest amount of total soluble solids in the grapes treated with marjoram oil (32.2 °Brix) and the lowest value in the grapes treated with chamomile oil (22.04 °Brix). The results of mean comparison of grapes treated with *B. cinerea* fungus

between different concentrations showed a significant difference in the amount of total solids content of grape (at 1% level). The highest amount of total soluble solids was observed at control treatment (25 °Brix) and the lowest values are at 600 µL L^{-1} (22.32 °Brix) (Table 2).

pH

The results of mean comparison of grapes treated with *B. cinerea* fungus showed significant differences between varieties of essential oils on grape pH. There was no statistically significant difference between the anise, chamomile, and marjoram oils, but all three showed significant differences with black caraway oil. The lowest amount of pH in grapes treated with black caraway oil (4.44). The results of mean comparison of grapes treated with *B. cinerea* fungi between different concentrations showed a significant difference in grape pH. The lowest pH was found at a control treatment (4.43) and the highest values were recorded of 400 µL L^{-1} (4.53) (Table 2).

Table 1. Analysis variance of for the effect of type and concentration of essential oil on radial growth for *B. cinerea* fungi treatments in *in vitro* conditions

Source of variation	df	Radial growth of fungus (tenth day) (mm)
Type essential oils	3	603.8*
Concentrations of essential oils	4	2347.2*
Type essential oils × Concentrations of essential oil	12	51.2*
Error	40	56.6

*, ** Significant at 5%, and 1% probability level, respectively.

Table 2. Comparison of means of the effect of type and concentration of essential oils on quality factors for *B. cinerea* fungi treatments in *in vivo* conditions

Treatment		Anthocyanin content (mg 100 g^{-1})	TSS (°Brix)	pH	Weight loss percentage (%)
Essential oils	Anise	1227.4^{c}	22.86^{c}	4.50^{a}	3.17^{a}
	Chamomile	936.25^{d}	22.04^{d}	4.50^{a}	0.83^{c}
	Black caraway	2077.7^{b}	23.52^{b}	4.44^{b}	0.81^{d}
	Marjoram	2216.6^{a}	32.2^{a}	4.50^{a}	1.45^{b}
Concentrations of essential oils	Control	1157.7^{e}	25.00^{a}	4.43^{e}	2.53^{a}
	200	2080.24^{a}	22.37^{d}	4.49^{c}	1.55^{b}
	400	1596.37^{c}	23.22^{c}	4.53^{a}	1.29^{d}
	600	1658.4^{b}	22.32^{d}	4.51^{b}	1.34^{c}
	800	1579.8^{d}	24.10^{b}	4.47^{d}	1.25^{e}

Within each column, the same letter indicates no significant difference between treatments at 5% levels.

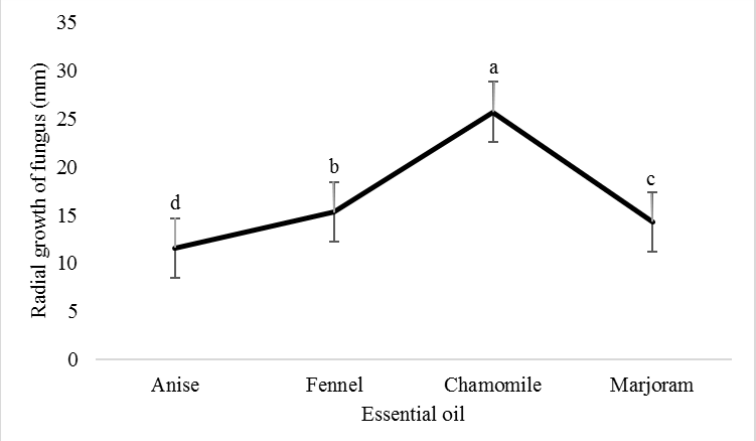

Fig. 1. Effect of four essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

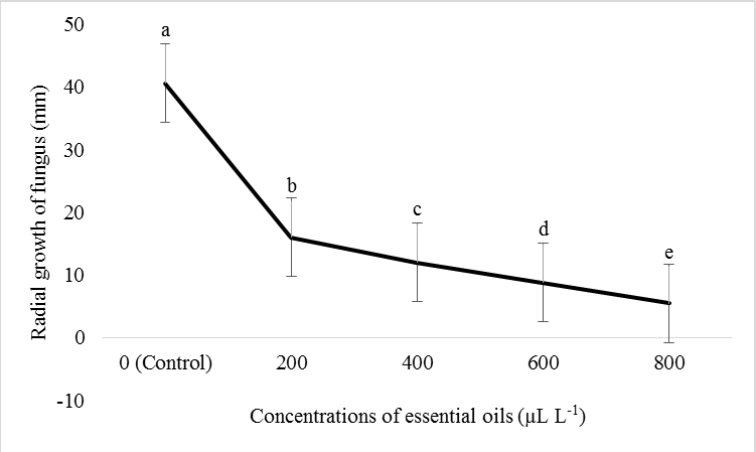

Fig. 2. Effect of different concentrations of essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

Weight loss percentage

Fruit treated with black caraway oil had the lowest weight loss percentage (0.81%), while fruit treated with anise oil had the highest weight loss percentages (Table 2). Furthermore, the weight loss percentage of essential oil-treated fruits was significantly lower than that of control fruits ($p < 0.01$). Fruits treated at 800 µL. L^{-1} of oils showed the lowest weight loss percentage (1.25%), while control fruits showed the highest weight loss percentages (Table 2).

DISCUSSION

The antifungal property of several essential oils on postharvest pathogens of fruits and vegetables under *in vitro* and *in vivo* conditions have been investigated previously (Feng & Zheng, 2007).

The *in vitro* data presented in this study showed that the examined essential oils had a fungicidal effect at higher concentrations, particularly anise essential oil. The results indicated that the control treatment had the highest growth rate of *B. cinerea* and the concentration of 800 µL. L^{-1} had the least growth of *B. cinerea*. Fatemi et al. (2013) reported that the examined black caraway and anise essential oils at 800 µL L^{-1} had a fungicidal effect at higher concentrations, especially anise essential oil. Huang et al. (2010) reported that the antifungal activity of the star anise was related to Trans Antoul. Ćosić et al. (2010) in the study of antifungal activity, several essential oils such as cloves, peppermint, salvia, thyme, cinnamon, anise, black caraway, orange, rosemary, lavender, and pine against some fungal phytopathogens including *Colletotrichum coccodes*, etc., the most antifungal activity was observed in the essential oils of black caraway, thyme, cloves, peppermint, cinnamon, and anise. Similarly, the growth of *B. cinerea* was inhibited by thyme, oregano, dictamnus, and marjoram essential oils (Daferera et al., 2003). Additionally, the mycelia growth of *B. cinerea* was reported by Bouchra et al. (2003) to be inhibited by *Origanum compactum* and *Thymus glandulosus* essential oils. Also, Chebli et al. (2003) reported that the essential oils of *Thymus glandulosus* and *Origanum compactum* inhibited the mycelia growth of *B. cinerea*. The antimicrobial attributes of essential oil and major constituents of fennel can suppress several plant pathogenic fungi (Soylu et al., 2007).

This difference in the antifungal activity may be due to their chemical composition, the structural configuration of these constituents, the activity of their functional groups and possible synergistic interactions between these constituents (Bajpai et al., 2013). Daferera et

al. (2003) investigated the antifungal effects of some essential oils against *B. cinerea* and *Fusarium* Sp. on an artificial culture medium, they reported that marjoram essential oil in low concentrations showed a completely inhibitory effect. Sekine et al. (2007) investigated an antifungal effect of black caraway compounds and 52 herbaceous plants in the study of black caraway and followed by cumin and cardamom strong inhibitory effects against *oxysporum Fusarium*, which is consistent with the results of this study. They also stated that this antifungal activity is related to the composition of the cumin aldehyde in the black caraway.

Treated fruits with marjoram oil had more total soluble solids, and anthocyanin content in comparison to anise, chamomile, and black caraway oil. Furthermore, fruits treated with black caraway oil showed the lowest pH, while treated fruits with anise, chamomile, and marjoram essential oils showed the highest pH. The highest anthocyanin content and pH were obtained at 200 and 400 µL L^{-1} concentrations respectively, and lowest values were found at a control treatment. The highest amount of total soluble solids was observed at control treatment and the lowest values were recorded at 600 µL L^{-1}.

The results weren't in agreement with those of Asghari Marjanlo et al. (2009) who reported that TSS of strawberry infected with *B. cinerea* increased with the application of cumin oil. Mahmoud and Abd El- Salam (2014) reported that essential oils of celery, cinnamon, and coriander, positively, affected postharvest total soluble solids compared with control. Moreover, Abd El Wahab (2015) worked on 'Florda 7.2' nectarine to tested some essential oils to maintain postharvest fruit quality and reported that TSS increased with increasing storage and marketing periods, moreover, Coriander oil during cold storage and market life periods delayed the changes in total soluble solids compared with control. The results disagree with Rattanapitigorn et al. (2006) and Serrano et al. (2005) previous experiments using natural antifungal compounds (eugenol, thymol and menthol vapors) revealed benefits due to reduced weight loss percentage in cherry and grape. Similar weight loss results were obtained when eucalyptus and cinnamon oils were applied to strawberry and tomato (Tian et al., 2011).

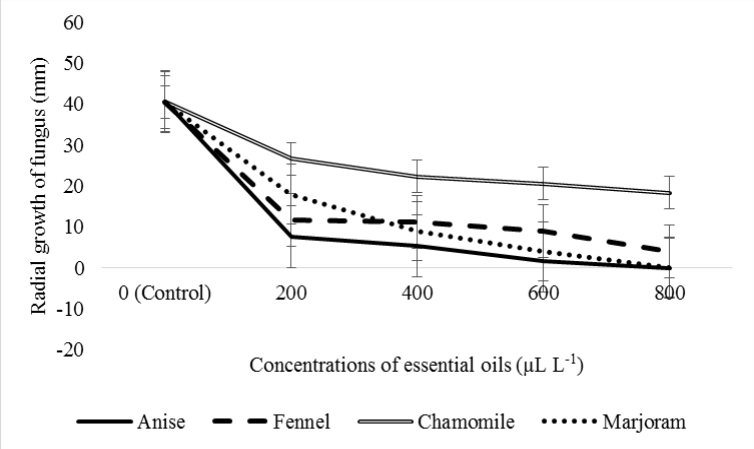

Fig. 3. Interaction effect of different concentrations of four essential oils on radial growth (mm) of *B. cinerea* in *in vitro* conditions

CONCLUSION

Considering the reduction in mycelia growth of *B. cinerea* in *in vitro*, we can conclude that anise oil could be used as possible bio fungicides, as an alternative to synthetic fungicides, against *B. cinerea*. However, more studies are required before these essential oils can be recommended as commercial and natural antifungal agents to increase the postharvest storage life of other horticultural crops.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Effect of different packaging materials on quality and storability of osmotically dehydrated wild apricot fruit under ambient storage condition

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ABSTRACT

Purpose: Wild apricot fruit is a rich source of carbohydrates, minerals and vitamins. Its shelf life is very short due to its perishability characteristics. High perishable nature, high acidity and low sugar content of wild apricot fruits are the major limitations for their utilization as fresh fruits. **Research method:** In this research, Wild apricot fruits were osmotically dehydrated and packed in different packaging materials viz. polyethylene, aluminium laminated and shrink packages were than stored under ambient conditions for six months and analysed for quality and sensory parameters at two months intervals. Osmotic dehydration is a technique where moisture is partially removed and the sweetness is increased by dipping the fruits in concentrated sugar solution, followed by final drying in hot air. Potassium metabisulphite is added in sugar solution as preservative and to improve colour of the final product. After draining the syrup, the fruits are dried in a mechanical dehydrator to a constant weight. **Main findings:** Results showed that aluminium laminated packaging material proved to be best among the all packaging materials in maintaining superior quality up to six months of storage as indicated by higher mean titratable acidity (5.78%), reducing sugars (10.11%), total sugars (37.71%), ascorbic acid (4.77 mg/100g) and lower moisture content (11.14%). All the sensory parameters including colour, taste, texture, flavour and overall acceptability declined significantly during storage period of six months. **Limitations:** There were no limitations to report. **Originality/Value:** It was found that the most suitable packaging material was aluminium laminated whereas the greatest change in sensory parameters was observed in polyethylene packaging.

INTRODUCTION

Wild apricot (*Prunus armeniaca* L.) popularly known as 'chulii' is most common fruit of Jammu and Kashmir, Himachal Pradesh and mid hills of Uttarakhand. The wild apricot plants are drought resistant, salt tolerant, prolific bearers and less susceptible to insects, pests and diseases. Therefore, it can easily be grown with minimum care in the tracts, which are otherwise unfit for cultivation of other temperate fruits. But the high acidity and low sugar content of wild apricot fruits makes them unsuitable for fresh consumption and drying purpose also. Taking into cognizance of the problem, it becomes imperative to develop appropriate technology for the efficient utilization of wild apricot fruits which otherwise go waste. Drying with the help of the sun and wind is one of the oldest methods of fruit preservation known to man, but artificial drying (dehydration) has been developed extensively during the last three decades. Fruit dehydration industry has not shown a satisfactory growth in India due to a variety of reasons like non-availability of promising varieties in adequate quantity, location disadvantage, lack of up-gradation and innovation in the area of product technology (Kapoor, 1998). In recent years more emphasis had been placed upon osmotic dehydration of fruits.

Osmotic dehydration is a technique where moisture is partially removed and the sweetness is increased by dipping the fruits in concentrated sugar solution, followed by final drying in hot air. The osmotic dehydration of wild apricot fruits followed by packing in suitable packaging materials would help to increase in shelf- life of the product and also help the growers to supply their produce according to the market demand and fetches them better prices. The present investigation was conducted to study the effect of different packaging materials on quality and storability of wild apricot under ambient conditions.

MATERIALS AND METHODS

The fruit of wild apricot were harvested at optimum maturity from the experimental orchards of Horticulture Department, Hill Campus, Ranichauri, G. B. Pant University of Agriculture & Technology. For the study healthy and uniform sized fruits were selected, washed and cut into halves to remove the stones. The fruits were lye-peeled in 1.0% NaOH solution in boiling water for 30 seconds followed by immediate cooling and washing in running tap water to remove excess alkali. A 70^{o} Brix sugar syrup containing 0.05% potassium metabisulphite (KMS), with temperature maintained at 50^{o}C was used as osmotic dehydration solution for immersing the lye-peeled fruits of wild apricot for 6 hours in 1:3 ratio (fruit : syrup). During dipping time, the product was agitated periodically at 1 hour interval with a wooden laddle. The dipped fruits were removed from sugar solution and dried in a cabinet dehydrator at 55 $\pm$ 2 ^{o}C temperature up to an almost constant weight. The osmo-dried fruits were packed in three different packaging materials viz., polyethylene pouches (25µ), laminated aluminium pouches (10µ) and shrink packages (20µ) and stored at ambient temperature for a period of six months. The experiment consisted of three treatments and four storage intervals with three replications for each treatment and each storage interval. The change in physico-chemical and sensory parameters were evaluated periodically (0 month, 2, 4, 6 months) at 2 month interval.

Determination of moisture content

The moisture content after each interval of storage was determined by the method of AOAC (2000). The ten grams of sample were kept in a hot air oven at 60 $\pm$ 1^{o}C for drying to a

constant weight. The weight of the sample was taken after cooling it in a desiccator and expressed as moisture percentage (1).

$$\text{Moisture (per cent)} = \frac{\text{Loss in weight on drying (g)}}{\text{Weight of sample taken (g)}} \times 100 \quad (1)$$

The titratable acidity, reducing sugars, total sugars and ascorbic acid were estimated as per standard procedures (Ranganna, 2009).

Determination of titratable acidity

The titratable acidity was determined by the method described by Ranganna (2009). Ten grams of sample were blended and mixed thoroughly in a pestle and mortar with 20-25 milliliters of distilled water. It was then transferred to 100 ml volumetric flask, makeup the volume 100 ml and filtered. Ten milliliters of aliquot was taken and titrated against 0.1N NaOH solution using Phenolphthalein as an indicator. The recorded titratable acidity was expressed in terms of per cent citric acid (2).

$$\text{Acidity (percent)} = \frac{\text{Titre value x Normality of alkali} \times \text{Equivalent weight of acid} \times \text{Volume made} \times 100}{\text{Volume of sample taken} \times \text{Weight of sample} \times 1000} \quad (2)$$

Determination of sugars

The reducing and total sugars were estimated by Lane and Eynon (1923) method as detailed by Ranganna (2009).

Preparation and standardization of Fehling's Reagent

Fehling's reagent was prepared fresh by adding Fehling's solution A and Fehling's B in equal amount with constant stirring and the content was filtered through Whatman filter paper No.2. Ten milliliters of Fehling's reagent were titrated against standard dextrose solution of concentration 2.5 mg/ml using methylene blue as an indicator.

Preparation of sample

A ten gram sample was transferred to a 100 milliliters beaker. It was added with about 50 milliliters of distilled water and the content was neutralized by adding 0.1N NaOH. It was heated to boil. Thereafter, the content was transferred to 250 milliliters volumetric flask using several washings with distilled water. Two ml of 45 per cent neutral lead acetate was added, stirred and allowed to stand for 10 minutes. It was added with predetermined amount (1.9 milliliters) of 22 per cent solution of potassium oxalate and the volume was made up to the mark with distilled water. Thereafter, it was filtered through Whatman filter paper No.1 so as to get the clarified sugar sample.

Reducing sugars

The prepared clarified sample was titrated with freshly prepared and pre-standardized Fehling's reagent using methylene blue as an indicator. The reducing sugars content was calculated as follows (3):

$$\text{Reducing sugar (percent)} = \frac{\text{Dilution} \times \text{Fehling's factor} \times 100}{\text{Titre} \times \text{Volume of sample}} \quad (3)$$

Total sugars

Twenty five milliliters of sample sugar solution were taken in a 100ml volumetric flask and 5 milliliters of HCl (1+1) were added. This was kept for 24 hours at the ambient temperature for the hydrolysis of non-reducing sugars to the reducing ones. Thereafter, the content was neutralized with 1 N NaOH and made up to 100ml with distilled water. It was titrated against freshly prepared and standardized Fehling's reagent as described above.

Non-reducing sugars

The content of non-reducing sugars was calculated by the formula given below (4):

$$\text{Non-reducing sugars (percent)} = (\text{Total sugars - Reducing sugars}) \times 0.95 \quad (4)$$

Results were expressed as per cent of reducing, non-reducing and total sugars on the basis of sample.

Estimation of ascorbic acid

Ascorbic acid content in fresh fruits and in stored jelly products were estimated by using 2, 6-Dichlorophenol Indophenol (DCPIP) Visual Titration Method (Ranganna, 2009). Ten grams of sample was blended with an aqueous solution of metaphosphoric acid (3 per cent) and the volume was made upto 100 milliliters with metaphosphoric acid solution. The content was filtered through a Whatman filter paper No. 1. Ten milliliters of the aliquot were titrated against dye solution till the appearance of light pink colour. Dye was standardized with freshly prepared standard ascorbic acid solution (0.1mg/ml) prepared in 3 per cent metaphosphoric acid solution. This method involves reduction of 2, 6-Dichlorophenol indophenol dye, blue in alkaline solution to a colourless form of ascorbic acid. The ascorbic acid content was expressed in terms of mg/100g and calculated by using following formula (5).

$$\text{Ascorbic acid } \left(\frac{\text{mg}}{100\text{g}}\right) = \frac{\text{Titre} \times \text{Dye factor} \times \text{Volume made up} \times 100}{\text{Aliquot taken} \times \text{Weight or Volume of sample taken}} \quad (5)$$

Organoleptic evaluation

The dehydrated fruits were evaluated by a panel of 7 semi-trained members using 9 point Hedonic scale for colour, taste, texture and overall acceptability i.e. like extremely 9, like very much 8, like moderately 7, like slightly 6, neither like nor dislike 5, dislike slightly 4, dislike moderately 3, dislike very much 2, dislike extremely 1 (Amerine et al., 1965).

Statistical analysis

Statistical analysis of the data pertaining to the sensory evaluation of osmotically dehydrated fruits were analysed according to randomized block design (Mahony, 1985) while, that on physico-chemical characteristics by factorial completely randomized design (Cochran & Cox, 1967). The values were compared at 5% level of significance.

RESULTS AND DISCUSSION

A steady increase in mean moisture content of the osmotically dehydrated wild apricot fruits from 11.32 to 12.70% was observed with the advancement of storage period (Table 1).

Aluminium laminated packaging registered the lowest mean moisture content (11.14%) followed by shrink wrapped packaging (12.68%). The polyethylene packed osmotically dehydrated fruits, on the other hand, had maximum moisture content and registered average moisture content of 12.99 percent at the end of the storage period of six months. The moisture content of the osmotically dehydrated wild apricot fruits during different storage intervals for polyethylene packaging, aluminium laminated packaging and shrink wrapped packaging ranged between 11.32-13.68, 11.32-11.08, and 11.32-13.33 percent, respectively between 0 to 6 months of storage. The gradually increase in mean moisture content in osmotically dehydrated fruits throughout six months of storage might be due to absorption of moisture from atmosphere by the product stored at ambient conditions. Similar results were also reported by Ahmed (2005) in dried apricot. The higher mean moisture content in osmotically dehydrated wild apricot fruits packed in polyethylene packaging during six months of storage was observed which might be attributed to permeability of polyethylene packages to air and water, whereas minimum loss in moisture content in the samples stored in aluminium laminated packages was due to the better moisture barrier properties of the package. A similar trend has also been documented in dried apples (Sharma et al., 2000).

During storage period of six months the titratable acidity of osmotically dehydrated wild apricot fruits experienced a small reduction from an average initial value of 5.82 to 5.66 per cent. The titratable acidity of osmotically dehydrated fruits packed in different packaging materials showed a linear declining trend with the advancement of storage period (Table 1). The highest mean titratable acidity (5.78%) was recorded in the osmotically dehydrated fruits packed in aluminium laminated packages, followed by shrink wrapped packages (5.73%). The lowest mean titratable acidity (5.71%) was recorded in polyethylene packed osmotically dehydrated fruits. A gradual decline in titratable acidity with the advancement of storage period might be due to the utilization of acids during various biochemical reactions occurring in the products during storage. The maintenance of higher acidity in aluminium laminated packages may be due to the decreased hydrolysis of organic acids and subsequent accumulation of organic acids which were oxidized to the slower rate. The delay in the reduction of acidity of osmotically dehydrated fruits packed in aluminium laminated packages confirms the similar findings of Bhardwaj and Kaushal (1990) in dried apples.

The mean reducing sugar content of osmotically dehydrated wild apricot fruits were increased from 9.25 to 10.48 per cent after six months of storage (Table 1), which was probably due to the hydrolysis of non-reducing sugars during storage. Similar findings have also been reported in Papaya powder by Aruna et al. (1998). Among packages, although the mean contents of reducing sugars varied between maximum (10.11%) to a minimum (9.87%) for aluminium laminated packages and polyethylene packages, respectively, but the differences were statistically non-significant. Such results have also been recorded by Khedkar and Roy (1988) in dehydrated mango slices.

The decrease in mean total sugars of osmotically dehydrated wild apricot fruits from 38.46 to 36.89 per cent was observed with the advancement in storage period which might be due to the utilization of sugars in non-enzymatic browning reactions. The decrease in total sugar content in dehydrated products during storage was also observed by Sagar and Khurdiya (1999) in dehydrated mango slices. A negligible effect of packaging on mean total sugars content of osmotically dehydrated fruits was observed. However, the lowest mean total sugars content was observed in osmotically dehydrated fruits packed in polyethylene packages which might be due to higher moisture content in osmotically dehydrated products packed in polyethylene packages which favour faster non-enzymatic reaction during storage. Sharma et al. (2006) also observed similar trend for total sugars content in dehydrated apple products packed in different packages.

Table 1. Effect of different packaging materials on moisture content, titratable acidity, reducing sugar, total sugar and ascorbic acid of osmotically dehydrated apricot fruits during storage

Storage interval (months)	Polyethylene	Aluminium Laminated	Shrink wrapped	Mean
		Moisture (%)		
0	11.32	11.32	11.32	11.32
2	13.46	11.08	12.84	12.46
4	13.52	11.08	13.25	12.61
6	13.68	11.08	13.33	12.70
Mean	12.99	11.14	12.68	
$CD_{0.05}$	Treatment = 0.16	Storage = 0.19	Treatment × Storage = 0.33	
		Titratable acidity (%)		
0	5.82	5.82	5.82	5.82
2	5.75	5.80	5.78	5.78
4	5.67	5.77	5.72	5.72
6	5.60	5.75	5.62	5.66
Mean	5.71	5.78	5.73	
$CD_{0.05}$	Treatment = 0.03	Storage = 0.04	Treatment × Storage = 0.09	
		Reducing sugar (%)		
0	9.25	9.25	9.25	9.25
2	9.98	10.02	10.28	10.09
4	10.20	10.17	10.34	10.24
6	10.07	11.01	10.35	10.48
Mean	9.87	10.11	10.03	
$CD_{0.05}$	Treatment = NS	Storage = 0.56	Treatment × Storage = NS	
		Total sugar (%)		
0	38.46	38.46	38.46	38.46
2	36.84	37.74	36.92	37.17
4	36.66	37.35	36.82	36.94
6	36.59	37.30	36.79	36.89
Mean	37.14	37.71	37.25	
$CD_{0.05}$	Treatment = NS	Storage = 0.63	Treatment × Storage = NS	
		Ascorbic acid (mg/100g)		
0	5.00	5.00	5.00	5.00
2	4.50	4.80	4.55	4.62
4	4.20	4.80	4.28	4.43
6	3.80	4.50	4.10	4.13
Mean	4.37	4.77	4.48	
$CD_{0.05}$	Treatment = 0.13	Storage = 0.15	Treatment × Storage = 0.32	

The ascorbic acid content of osmotically dehydrated fruits followed a decreasing trend from 5.00 to 4.13 mg/100 g during six months of storage which was mostly due to its oxidation with the passage of time and its role as a substrate in non-enzymatic browning reactions (Khedkar & Roy, 1988). It was also observed that the osmotically dehydrated fruits packed in aluminium laminated packages retained maximum (4.77 mg/100 g) mean ascorbic acid content, whereas, osmotically dehydrated fruits packed in polyethylene packages had minimum (4.37 mg/100g) mean ascorbic acid content. The osmotically dehydrated fruits packed in aluminium laminated packages showed highest retention of ascorbic acid content, while, in polyethylene pouches there was maximum loss during six months of storage period which might be attributed to maintenance of lower moisture content in laminated pouches thereby permitting less degradation of ascorbic acid. Similar evidences have been reported by Tripathi et al. (1988) in dehydrated aonla.

Table 2. Effect of different packaging materials on colour, taste, texture, flavour and overall acceptability of osmotically dehydrated apricot fruits during storage

Storage interval	Polyethylene	Aluminium Laminated	Shrink wrapped	Mean
		Colour		
0	8.48	8.48	8.48	8.48
2	6.88	7.84	7.56	7.43
4	6.23	7.82	7.23	7.09
6	6.17	7.66	7.02	6.95
Mean	6.94	7.95	7.57	
$CD_{0.05}$	Treatment = 0.16	Storage = 0.18	Treatment × Storage = 0.31	
		Taste		
0	6.22	6.22	6.22	6.22
2	5.56	5.84	5.55	5.65
4	5.36	5.45	5.42	5.41
6	5.02	5.28	5.22	5.17
Mean	5.54	5.70	5.60	
$CD_{0.05}$	Treatment = 0.19	Storage = 0.23	Treatment × Storage = 0.44	
		Texture		
0	6.45	6.45	6.45	6.45
2	5.94	6.28	6.14	6.12
4	5.53	6.08	5.86	5.82
6	5.23	5.78	5.55	5.52
Mean	5.79	6.15	6.00	
$CD_{0.05}$	Treatment = 0.17	Storage = 0.19	Treatment × Storage = 0.38	
		Flavour		
0	8.23	8.23	8.23	8.23
2	7.82	8.04	7.92	7.93
4	7.14	7.77	7.33	7.41
6	6.50	6.93	6.65	6.69
Mean	7.42	7.74	7.53	
$CD_{0.05}$	Treatment = 0.11	Storage = 0.12	Treatment × Storage = 0.25	
		Overall acceptability		
0	7.05	7.05	7.05	7.05
2	6.13	6.65	6.43	6.40
4	5.71	6.45	6.17	6.11
6	5.47	6.24	5.93	5.88
Mean	6.09	6.60	6.40	
$CD_{0.05}$	Treatment = 0.10	Storage = 0.12	Treatment × Storage = 0.23	

The mean sensory scores for colour of osmotically dehydrated fruits during storage period of 6 month showed gradual decrease from 8.48 to 6.95 (Table 2). Among packages, laminated packages retained maximum (7.95) mean sensory colour score, while, polyethylene packed dehydrated fruits retained minimum (6.94) colour score during 6 month of storage. The change in colour was significantly higher in polyethylene and shrink wrapped osmotically dehydrated fruit and aluminium laminated packages had minimum loss in colour. The decrease in mean sensory score for colour during storage was observed which might be due to occurrence of non-enzymatic browning reactions and oxidation of ascorbic acid to dehydrosascorbic acid and tannins to gallic acid. Similar findings have been reported in different varieties of apricot (Sharma et al., 2004).

The average sensory score for taste decreased from initial level of 6.22 to 5.17 after six months of storage (Table 2). However, on the basis of different packaging materials, the mean sensory scores of taste were found to be higher (5.70) in osmotically dehydrated fruits packed in aluminium laminated packages, whereas lowest (5.54) mean scores were recorded in polyethylene packed osmotically dehydrated fruits during 6 months of storage period. During storage, the mean sensory score for taste showed decreasing trend from initial to 6 months irrespective of packaging, whereas osmotically dehydrated fruits packed in laminated packages had minimum loss in taste during storage.

The mean texture score on 9 point hedonic scale was found to decrease gradually from 6.45 to 5.52 during 6 months of storage. On the other hand, the mean texture scores of osmotically dehydrated apricot fruits packed in different packaging materials varied from 5.79 to 6.15 with the highest score in aluminium laminated packages and lowest in the products packed in polyethylene packages (Table 2). The mean values of texture followed a decreasing trend from initial to six month of storage and samples packed in aluminium laminated packages maintained best texture.

The sensory scores for flavour of osmotically dehydrated wild apricot fruits followed decreasing trend from 8.23 to 6.69 for the mean scores during six months of storage (Table 2). Among packages, aluminium laminated packages retained maximum (7.74) mean sensory score for flavour while, polyethylene packed osmotically dehydrated fruits retained minimum (7.42) flavour score during 6 month of storage.

The overall acceptability mean scores during storage period of 6 months declined from an initial value of 7.05 to 5.88 after 6 months of storage period (Table 2). However, among packages, the mean overall acceptability scores for aluminium laminated osmotically dehydrated wild apricot fruits were found to be higher (6.60), while, polyethylene packed and shrink wrapped packages exhibited mean scores of 6.09 and 6.40, respectively. A general trend was observed in reduction of mean sensory scores during storage period which might be attributed to change in chemical composition of osmotically dehydrated fruits, change in sugar-acid blend and loss of aromatic compounds due to oxidation. Slight change in the texture upon storage was probably due to the degradation of pectic substances during storage. Similar reduction in sensory scores during storage has been reported by Sagar et al. (1998) in dehydrated ripe mango slices. However, the lower mean sensory scores observed in osmotically dehydrated apricot products packed in polyethylene packages which might be due to higher moisture absorption and gas permeability characteristics of the polyethylene, thereby affecting texture and colour of the packed products. The sensory scores were significantly higher in osmotically dehydrated products packed in aluminium laminated packages which might be due to impermeable nature of laminated packages. Similar evidences have also been made by Ahmed and Choudhary (1995) in osmotically dehydrated papaya.

CONCLUSIONS

From the present study it can be concluded that osmotically dehydrated wild apricot fruits can successfully be stored at ambient conditions after packing in aluminium laminated packaging material for a period of six months without any considerable loss in sensory as well as nutritional quality. The successful transfer of such technology after pilot scale testing may open new avenues for the processing industry for the efficient utilization of this fruit which is otherwise being wasted in Uttarkhand state. This may also attract entrepreneurship and may help the youth getting self-employment.

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Conflict of interest

The authors have no conflict of interest to report.

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Improving food security in Iran: quantifying post-harvest rice losses

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ABSTRACT

Purpose: Food is a basic need for human life. Ensuring food security requires guaranteeing adequate food availability, accessibility, and utilization along with the sustainability of the food system. Food availability can be improved by decreasing food losses. The goal of this study is to document the causes and quantify the amount of losses in Iran's rice supply chain. **Research method:** A cross-sectional survey method was used to collect data through interviews with some actors of the rice production chain operating in Mazandaran province, Qaemshahr, in 2015, to approximate percentage weight losses at the different stages in the supply chain. **Main findings:** The rice losses are estimated to be about one-third of the production that could feed 18 million people per year. On the other hand, the rice losses imply an economic loss of 1,403 million U.S. dollars, annually, and losing some scare resources, such as water, in Iran. **Limitations:** It is not easy to apply the ways to collect the useful and more accurate data in order to find the most important reasons of food losses for each stage and process of the food supply chains. **Originality/Value:** It was found that it is necessary to invest in the marketing systems, renew the supply chain and improve knowledge of the actors of the rice chain -from producing to consuming- in Iran's rice system to reduce the physical and economical estimated losses of rice.

INTRODUCTION

Food is a necessity for human life. With a rising population, which is estimated to overtake more than 9 billion by 2050, and a predictable increase in food demand, guaranteeing enough food to feed the world can become an even harder challenge (Dou et al., 2016). According to the World Food Summit, the definition of food security is when all people at all times have physical, social and economic access to sufficient and safe food (World Food Summit, 1996). The issue of food losses overlaps with the issue of food security as it affects the sustainability and resilience of food systems (Ardakani et al., 2019) and their ability to ensure food security (HLPE, 2014). On the other hand, by losing food, all of the resources spent during the supply chains are also missed (Thyberg & Tonjes, 2016). Therefore, reducing food losses across the entire food chain will improve food availability (Godfray et al., 2010; Pearson et al., 2013; Buzby et al., 2014) and will be an important part of any strategy to feed the growing population in the world (Foresight, 2011).

Food losses imply a deduction of the potential quantity of food that might be produced and distributed along the supply chains, from farm to table (Conteh et al., 2015; Routroy & Behera, 2017). The food supply chain is a system of organizations, people, and activities involved in moving food from the producer to the consumer (Beretta et al., 2013) and that losing food is a result of an ineffective operation of these drivers of the supply chain (Abass et al., 2014). Researchers, along with other actors such as farmers, governments and non-governmental organizations, and extension officers might play an important role in tackling the issue of food losses (Abass et al., 2014; Bolarin & Bosa, 2015). Decreasing food losses is considered one of the most favorable measures to improve food security and positively affect the use of resources, such as water (Kummu et al., 2012) to be secured (Alonso-Amelot & Avila-Núñez, 2011).

Despite the many technical growths in production and post-production stages since the 1970s when food losses were first measured by FAO, the problem is still significant and differs in the crops and countries (Kader et al., 2012). The existing studies on food losses have focused on post-production losses (Abass et al., 2014; Bolarin & Bosa, 2015). These studies have played a crucial role in defining the entity of this phenomenon, its causes, and possible solutions. However, further insights might emerge if new contexts and food sectors are explored. According to some studies, food losses are mainly caused by a malfunction in the processing, packaging, and storing of food. The malfunction is due to a combination of inefficient and backward procedures and scarcity of knowledge among food chain actors (FAO, 2013). Inefficiencies in the upstream of the food chains affect more developing countries than developed ones (Pirmoradi et al., 2013).

One-third of food made for human consumption in the world is lost, amounting to 1.3 billion tons annually (Dou et al., 2016). The World Bank estimates that there are between 12 and 16 million tons of post-production grains that are losses worldwide every year. This amount would be enough to feed between 70 million to 100 million people, with a yearly average consumption of 15 kg of grains per person (Basappa et al., 2007). Given the magnitude of the phenomenon, the issue of reducing global food losses has recently received increasing consideration. The FAO and other partner organizations started a global campaign on reducing food losses. The campaign targets all actors along the food chains and provides information on existing initiatives around the world (FAO, 2013). Many studies have highlighted the magnitude of the problem and identified specific targets for reducing food losses as well as policies to meet the targets (HLPE, 2014).

Rice is one of the most important food crops in the world and the basis of the diet (Guenha et al., 2014) especially for the population in Asia where approximately 90 percent of the rice of the world is produced and consumed (Sita Devi & Ponnarasi, 2009; AghaAlikhani et al., 2013). Rice, similar to other agricultural products, has a high proportion of losses that can occur during the various stages of its supply chain (Guisse, 2010; Kazemi et al., 2015). In Iran, Rice production is one of the main food chains with a production of 2,436 thousand tons annually, on average, after wheat, barley and corn. This study seeks to highlight the main figures, challenges, and solutions for the reduction of losses in the rice sector of Iran. In particular, the study focuses on the stages of the rice chain from farming to retailing because this is where most of the losses occur. Moreover, without the presumption of generalization, the results of this study might be relevant to other developing countries being major rice producers and dealing with reducing food losses in this sector.

To this end, this study will measure the scale of rice losses in Iran across the whole food supply chain (Rutten & Kavallari, 2016) and contribute determining the issues involved in food losses in the rice sector. We will do this by investigating the amount of rice lost, and the reasons for it from farming to retailing. The goal of this study is to document the causes and quantify the amount of losses in Iran's rice supply chain. Understanding where and how much rice is lost and the worth of these losses is important evidence that policy-makers can use to reduce rice losses and increase the efficiency of the farm-to-table rice supply chain to feed the growing human population.

The paper is structured as follow. Next section will present the study area, the available data as well as the methods. In section 3, we present and discuss our results. The final section will conclude the study.

MATERIALS AND METHODS

Data and study area

Rice is the second most vital food, after wheat, for the nutrition of much of the population in Iran where per capita consumption of rice is around 40 kg in a year. The latest available figures show that more than 600 thousand hectares area under rice production in Iran. However, more than 80 percent of the land farmed with rice is located in the three Northern provinces. Of this land, 265 thousand hectares are located in Mazandaran and Golestan provinces, while 230 thousand hectares are in Gilan province.

For the present study, a cross-sectional survey method was used to collect data through interviews (Beretta et al., 2013; Kitinoja et al., 2019) with some actors of the rice production chain operating in Mazandaran province, Qaemshahr City in 2015 (Fig. 1). A multistage simple random sampling technique has been used to select the respondents, which included 100 farmers, 20 processors, 20 wholesalers and 20 retailers. According to the data collected, 90 percent of the respondents are males that on an average were around 54 years of age with the most of no (or primary) education. They were asked about the amount of rice losses that they can estimate and experience in each process: harvesting, threshing, drying, milling, winnowing, and packaging, storing and transporting.

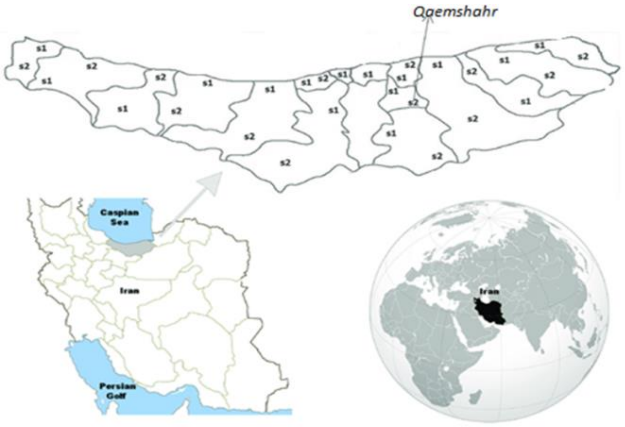

Fig. 1. Study area: Qaemshahr, Mazandaran province, Iran

Table 1. Basic mathematics equations to connect to food security and economic losses

	Item	Basic mathematics equations
1	Consumption quantity	(Production + Import) – Export
2	Losses quantity	Production × Percentage of losses
3	Value of rice lost	Losses quantity × Producer price
4	The value assigned to import the quantity of rice lost	Import quantity × Import price
5	Total missed value	(3) + (4)
6	Number of people can feed with the lost rice	Losses quantity / Consumption per capita

Post-harvest losses calculation

According to Harris and Lindblad (1978), there are several assessment methods available to measure food losses but it is difficult to find which method reveals the actual losses closer and more reliably (Alonso-Amelot & Avila-Núñez, 2011). One method is measuring real losses by following a particular food product from production to consumption, through measuring weight (or quality) losses at each stage. This approach, while difficult for some products, delivers a better estimate of food losses (Harris & Lindblad, 1978). This is mostly the focus on post-harvest losses estimation to date (Alonso-Amelot & Avila-Núñez, 2011; Abass et al., 2014). Another method of measuring food losses is to use estimates by persons who experience food losses, using a defined questionnaire. This method is somewhat easy to apply, but it can only approximate the estimated food losses (Amentae, 2016).

According to the existing possibilities for collecting the data, the method of estimating food losses by persons who experience food losses (Amentae, 2016) was employed in this study. Rice losses were estimated by relying on the traditional knowledge of the respondents (Babarabie et al., 2019) to recall the extent and relative losses happening at each stage of post-harvest handling (Teshome et al., 1999; Abass et al., 2014). They were aimed at gathering data on the losses at different stages of the studied rice supply chain. Comparisons can provide quantitative information to estimate the quantity of rice lost along the supply chain by calculating the percentage (Beretta et al., 2013) of rice lost in each stage of the supply chain and per each process.

In the present study, rice lost is calculated as the difference between the amounts (initial weights) of rice reaching (i.e., input) to a certain process (harvesting, threshing, drying, milling, winnowing, and packaging, storing and transporting) minus the amount (final weights) of rice leaving (i.e., output) the same process by interview of the actors to estimate

the amount of losses in the processes. While the percentage of the rice lost is given by dividing the amount of rice lost by the amount (initial weights) reaching the process as input (Reed, 1987; Guenha et al., 2014) in formula 1.

$$\text{Percentage Weight Losses} = \{(\text{Initial Weight} - \text{Final Weight}) / \text{Initial Weight}\} * 100 \quad (1)$$

The data collected were exposed to statistical analysis including tabular and graphical presentation techniques (Hollingsworth et al., 2006).

Food security and economic losses assessment

In addition, the study seeks to broaden its findings by estimating the quantity of rice losses and their economic value (Kitinoja et al., 2019) beyond the studied case. Basic mathematic equations are used to combine the data gathered about the losses of rice in some rice chains located in Iran using the latest available figures retrieved from the FAO website for the total production, imports and exports, producer prices, and per capita consumption of rice in Iran. Table 1 report the basic mathematics equation used in the current study.

RESULTS AND DISCUSSION

An important component in the food systems to be more efficient is the reduction of food losses across the entire food supply chains (Abass et al., 2014; Affognon et al., 2015; López-Castillo et al., 2018). Furthermore, economically avoidable food losses are of high importance in the efforts to combat hunger and to improve food security, not only in developing but also in developed countries. The analysis in this study covers the entire food supply chain that is associated with rice consumption, to recognize the causes and quantify the amounts of rice losses over all stages from harvesting to intake.

Figure 2 summarizes the percentage of rice lost per each process in the different stages of the rice chain. These stages include farming, processing, wholesaling and retailing. For each stage, there are different processes. Farming includes harvesting, threshing, drying, storing and transporting. Milling, winnowing, packaging, storing and transporting are processes of the processing stage. In the wholesaling stage, there exists winnowing, storing and transporting processes; there are storing and transporting processes in the retailing stage also.

As for the farming stage, around 5 percent of losses happen during the threshing, close to 3 percent occurs in the drying process and around 1.5 percent happens during the harvesting. This data shows that threshing, drying, and harvesting are the main causes of rice losses in the farming stage while storing and transporting in the farming stage show a very small percentage of losses as shown in Figure 2. During harvesting and drying of rice, to compare, Calverley (1996) estimated about 7 percent losses in Madagascar, 4.3 percent losses in China and 4 percent for several Asian countries. Losses for rice in threshing were 6.5 percent and 6 percent in Madagascar and Ethiopia; respectively (Abass et al., 2014).

Moving to the processing stage, see Figure 2, most of the losses take place during milling with a share of more than 10 percent. During winnowing, losses are close to 4.5 percent of total losses. The losses of rice during the storing, packaging and transporting processes represent respectively around 1.01, 0.51, and 0.01 percent of the total losses in the processing stage. From this data, it can be derived that milling and winnowing are the most ineffective processes with respect to losses in the processing stage of the rice chain. Losses for rice in Madagascar and Ethiopia were 2.5 percent; respectively and 5 percent in winnowing (Abass et al., 2014).

If we look at the wholesaling stage (Figure 2), most of the losses happen during winnowing. They amount to 3.23 percent while losses during storing and transporting amount to the 0.02 and 0.01 percent, respectively. Consequently, rice winnowing is the most inefficient process in the wholesaling stage of the rice chain with respect to product losses. Finally, in the retailing stage, 0.01 percent of rice losses derive from storing, and 0.01 percent is due to the transporting processes.

Table 2 reports the results of the estimated quantitative post-harvest rice losses (calculated through formula 1) in the different phases of the supply chain as the sum of the percentages of rice lost in the processes that constitute each stage shown in Figure 2. As Table 2 shows, around one-third (29 percent) of rice production is lost going from fields to forks. It can be compared to post-harvest losses in Africa which are frequently estimated to be between 20 and 40 percent (World Bank et al., 2011). Also, this agrees with the range of 20 percent to 30 percent losses by weight; estimated for grains by Tefera et al. (2011). Most of the losses occur in the processing stage, which equals 16.39 percent of the products that reach this stage. At the stages of farming, around 9.48 percent of rice is lost. In the wholesaling and retailing stages, respectively, around 3.26 and 0.02 percent of rice that reaches these stages are lost. Another study in Iran, Amol city, by Salmani et al. (2013) agrees with these results as the rice losses have been estimated 33 percent with a most share of processing and farming, respectively. In Tanzania, another developing country, quantitative post-harvest losses occur in the field with a share of 15 percent and during processing with a share of between 13 percent and 20 percent (Abass et al., 2014).

Figure 3 reports the total amount of rice losses in the different processes of the supply chain, regardless of the stage. These amounts result from summing up the percentages of rice lost in the same process over the different stages of the supply chain. The main contribution to rice losses happens in the milling and winnowing processes, by a share of 10.43 and 7.69 percent of the total losses. According to the respondents participated in the interviews, traditional methods and old-fashioned machinery used in the milling and winnowing actions of rice in Iran might be behind the high percentage of losses. Also, zinc deficiency and high air temperature at harvest time can increase the rice losses in the milling process.

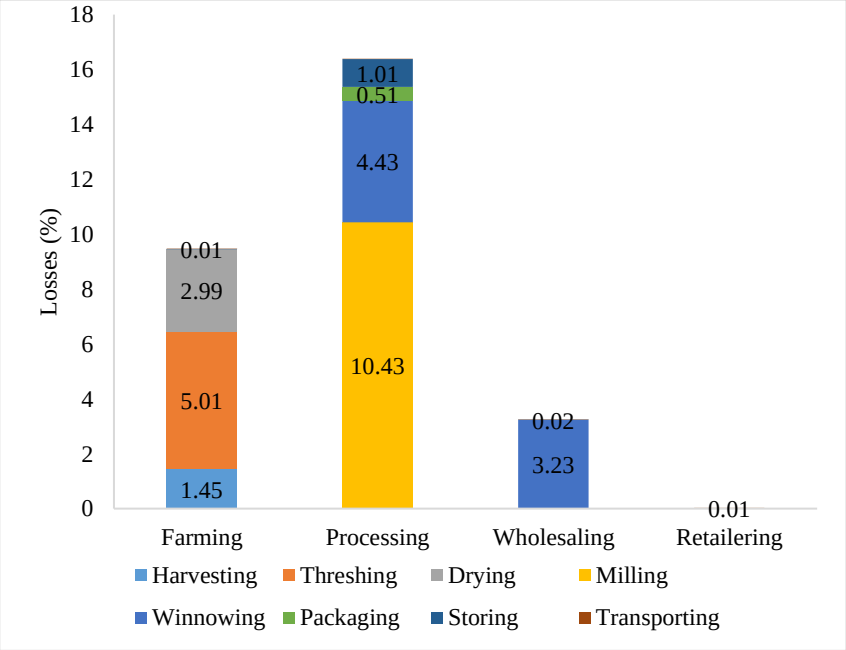

Fig. 2. Percentage of rice lost per each process in the different stages of the chain

Table 2. Percentage of rice lost at the different stages of the supply chain

Stages	Rice losses (%)
Farming	9.48
Processing	16.39
Wholesaling	3.26
Retailing	0.02
Total	29.15

The second-largest contribution to rice losses is relative to the threshing that follows with 5.01 percent of losses. The threshing is implemented during the farming stage only. Hence, all the losses due to threshing are imputable to this phase. The main reason for the rice losses during threshing is the use of combines that are not suitable for rice threshing. It is common to see farmers using combines that are meant for the threshing of other kinds of grains or rice varieties, which are different from the ones that are threshed. The farmers participated in the interviews have the same interpretation.

Drying and harvesting are the third and fourth most inefficient processes if we look at the rice losses they generate. They are also processed typically of the farming stage only and show shares of 2.99 and 1.45 percent of rice losses respectively. Based on the interviews, drying is done following both traditional practices and using modern machines. When implemented in the traditional way, losses of rice are mainly due to the action of birds and insects that eat the rice grains. When modern machines are used for drying rice, losses are due to their misuse. It is common to witness mistakes in the setting of appropriate timing, temperature, capacity, and humidity. As far as it concerns the causes of losses during harvesting, similar to threshing, there is the use of machinery that is not suitable for the kind of rice harvested.

Storing shows a share of 1.05 percent of rice losses. The respondents in the current survey summarized that structural characteristics of the garners are behind the losses of rice during the storing. Sometimes garners are too small to contain all the packed rice; as a consequence, the packs of rice are stored without respecting the minimum required distance between one package and the other. In some other garners, lack of air conditioning systems facilitates the attacks from insects and parasites. For either short-term or long-term storage, rice should be given some air to breathe in order to prevent spoilage.

The packaging is the other most inefficient process with respect to rice losses. This process takes place just in the processing stage and it generates 0.51 percent of rice losses. The lowest losses occur in transporting with 0.05 percent of rice losses. An old-fashioned packing industry is behind the losses of rice during packaging and transporting. Rice is mostly packed in cotton and plastic bags and they are not vacuumed. Various modifications including aluminum foil, lamination or paper bags within the rice packaging design can be added to the rice packaging, making it more convenient and efficient in reducing losses.

The last part of this section aims at broadening the findings of this study by estimating the quantity of rice losses and their economic value beyond the studied case. The data discussed below combine the figures emerging from this study about the losses of rice in some rice chains located in Mazandaran province; Qaemshahr city with figures retrieved at the FAO website concerning the total production, imports and exports, producer prices, and per capita consumption of rice in Iran. Table 3 reports figures from FAOSTAT, while Table 4 combines these figures with some of the data gathered by this study using the basic mathematics equations reported in Table 1.

Annually, around 3,872 thousand tons of rice is consumed in Iran (Table 4). This figure is derived as the sum of the tons of rice imported and produced minus the tons of rice exported on a yearly basis (Table 3). Around 797 thousand tons of produced rice is annually lost in Iran (Table 4). This figure results from multiplying the tons of rice produced in Iran per year (Table 3) by the percentage of rice losses (0.29%) estimated by this study for the main rice production area. As this percentage is close to the 0.30 percent of grain production losses estimated by FAO for the southwest of Asia, therefore, we consider our estimation of losses as an appropriate proxy of the percentage of rice losses for the entire country. This amount of losses of rice corresponds to an economic loss of 714 million U.S. dollars, which is derived by multiplying the tons of rice lost in Iran times the price paid to rice producers. On the other hand, we estimate that 689 million U.S. dollars are paid to import rice for compensating the excess demand in the country. This amount is derived by dividing the import value by the imported quantity, which gives back the price of the rice imported. The final economic value for imported rice is obtained by multiplying its price by the tons of rice imported. Summing up, 1,403 million U.S. dollars are missed annually in Iran because of rice losses. In the Eastern and Southern Africa only, post-harvest losses are valued at 1.6 billion U.S. dollars for every year (Obeng-Ofori, 2011). Finally, from the food security aspect, if we consider the amount of rice losses divided by the rice supplied per capita, we obtain that 18 million people per year could be fed with the amount of lost rice.

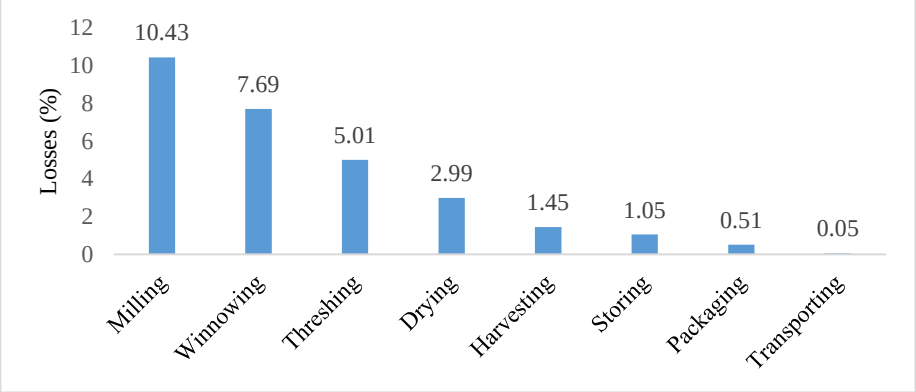

Fig. 3. Percentage of rice lost during different processes in the rice supply chain

Table 3. Figures about rice production, import, export, and producer price in Iran

Items	Quantity	Units
Production quantity	2,75	1000 tons/year
Import quantity	1,13	1000 tons/year
Export quantity	110	tons/year
Import value	973,29	1000 US$/year
Producer price	896	US$/tons
Import price	865	USD$/tons
Rice supply	43.80	kg/capita/year

Table 4. Estimated quantity and economic value of rice losses in Iran

Item	Quantity	Units
Consumption quantity	3,87	1000 tons/year
Losses quantity	797	1000 tons/year
Value of rice lost	714	million US$/year
The value assigned to import the quantity of rice lost	689	million US$/year
Total missed value	1,403	million US$/year
Number of People can feed with the lost rice	18	million/year

Summing up, according to what emerges from the data gathered in this study, the supply chain of rice in the most important rice production areas in Iran is inefficient in producing and supplying rice as around one-third of the produced rice is lost from farming to retailing. Most of the losses occur at the farming and processing stages of the chain. Traditional and inappropriate technologies to produced rice, lack of knowledge on how to use appropriate technologies, and lack of financial resources are behind rice losses in the rice chain in Iran. Hence, the actions that can be taken to tackle rice losses should mostly consider investing in agricultural technologies especially at the farming and processing stages, and improving knowledge sharing among the actors involved in the rice supply chain.

CONCLUSION

Food losses affect the sustainability of food systems and their ability to ensure food security. This paper has highlighted some relevant figures about rice losses in Iran. It has been found in this study that approximately one-third of the total rice production is lost from producers to consumers because of inefficient delivery from producer to consumer. This amount could feed 18 million people per year. On the other hand, it requires the use of resources, such as water, which are limited in Iran. The rice losses imply an economic loss of 1,403 million U.S. dollars hence, the potential benefits of reducing rice losses are, at least, threefold. It would allow improving food security as food availability would be increased. At the same time, it will reduce the environmental impact of rice production by reducing the needlessness of natural resources, which are already scarce, to produce food that is not consumed. Last, but not least, it would allow for the more economically efficient rice supply chain as it would avoid investing economic resources in producing, distributing, and retailing product that will not reach consumers. Mainly, decreasing food losses is an effective way to rise the efficiency and sustainability of food systems and reduce the environmental effect of food consumption. To reduce losses in order to increase the sustainability of food systems and improve food security, policy-makers have to consider useful innovations throughout the entire food chain involving all the actors. Economic and social innovations in the supply chains appear to be the most important essential. Financial support of the actors as an economic innovation will help the actors to improve the technologies and infrastructures used in the entire supply chains and then will improve the sustainability of the food systems to achieve food security. On the other hand, educating all the actors who are active in the food supply chains about how to use the new technologies, how to save food, etc. must be noticed, as social innovation. It is received from the literature on the issue of food losses that there is a lack of useful data to estimate the scale of food losses and identify the real causes. In addition to suggestions of Sheahan and Barrett (2017); future studies should focus on the ways to collect the useful and more accurate data in order to find the most important reasons of food losses in each stage and process of the food supply chains considering economic, environmental, and social issues.

Conflict of interest

The authors have no conflict of interest to report.

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Effect of storage conditions and packaging material on postharvest quality attributes of strawberry

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ABSTRACT

Purpose: Strawberry (*Fragaria* × *ananassa*) is highly perishable fruit with a limited postharvest life at room temperature and is vulnerable to postharvest decay due to its high respiration rate, environmental stresses and pathogenic attacks. **Research method:** To increase the postharvest life of strawberries, a combination of packaging material (polyethylene and perforated polyethylene) along with control and storage conditions {zero energy cool chamber (ZECC) and ambient conditions in laboratory} were tested. **Main findings:** Mass loss (1.59%) and internal temperature (22.24°C) were significantly reduced while shelf-life (more than 3 days) was enhanced in ZECC as compared to ambient conditions in laboratory with 6.46% mass loss, 23.04°C internal temperature and less than 3 days shelf-life. Packaging material significantly influenced mass loss (%) and electrical conductivity (S/m) of strawberry juice irrespective of its interaction with storage conditions and storage durations. Maximum mass loss (9.11%) and EC (3.74 S/m) were recorded in control samples while, minimum mass loss (1.24%) and EC (3.52 S/m) was recorded in polyethylene enclosed fruit. Irrespective of storage conditions and packaging material pH, TSS, titratable acidity (%) and ascorbic acid (mg100 ml^{-1}) decreased while electrical conductivity (S/m) increased during storage. **Limitations:** In future study storage duration should be extended by adding more removals to get clear difference in fruit quality and shelf-life under various treatments. **Originality/Value:** In conclusion ZECC can be used for short term storage of strawberry.

INTRODUCTION

Strawberry (*Fragaria* × *ananassa*) is well-known as the most appetizing and very nutritive fruit. It is the richest source of bioactive compounds with antioxidant activity (Yang et al., 2016) which provide protection against harmful free radicals. In Pakistan, it is consumed in fresh as well as in processed form for making squashes, jams and jellies which may be used throughout the year. Strawberry mainly growing in northern areas of the country like Swat, Charsadda, Mansehra, Haripur, Abbottabad, Mardan, Peshawar and some parts of central and south Pakistan like Gujrat, Sialkot, Jhelum, Chakwal, Multan and Karachi (Murtaza, 2014). Varieties like 'Chandler', 'Corona' 'Douglas','Tufts', 'Gorella'and 'Toro' are locally cultivated in various parts of Pakistan (Memon, 2014).

Strawberry is a highly perishable fruit having high respiration rate (50-100 ml CO_2 per kg of fruits per hour at 20°C), and can be stored only for four days (Panda et al., 2016). In Pakistan postharvest losses in strawberry are pretty high over 40%, of which 10% at farm level, 14% during transport and 23% at retail and these losses are mainly due to non-selective harvesting, poor packaging, transportation and almost absence of cold chain (Rajwana et al., 2016). To slow metabolic processes and reduce deterioration prior to transport, low temperatures are widely used to reduce spoilage and extend the shelf-life of fresh produce (Van, 2013). Storage temperature had significant influence on quality and bioactive compounds of strawberry. Strawberries could be kept for acceptable period of time at 10°C and could be stored for longer duration at 0.5°C (Shin et al., 2007). Higher level of total phenolics and total anthocyanin contents were found when strawberries were kept at 10°C than those stored at 0°C or 5°C (Jin et al., 2011). Mechanical refrigeration is expensive and requires power supply which is not easily available in Pakistan. Mechanical refrigeration is also not environment friendly because it releases chlorofluorocarbons and hydro chloroflorocarbons in the environment that is responsible for ozone layer depletion and global warming (Xuan et al., 2012).

Evaporative cooling is an efficient and economical method for reducing produce temperature and increasing relative humidity to decrease the physical mass loss and diseases incidence (Odesola & Onyebuchi, 2009). The ZECC work on the principle of evaporative cooling and can help to save fresh fruits and vegetables for a reasonable period of time (Jha & Kudos, 2006). The greatest importance of this low cost cooling technology lies in the fact that it does not require any electricity or power to operate and all the material requires to make the cool chamber is cheap and locally available.

The high postharvest losses has been attributed to several factors among which lack of packaging and storage facilities and poor means of transportation are the major ones (Kebede, 1991; Wolde, 1991). Packaging fruits is one of the most commonly used postharvest practice as unitized volumes are easier to handle achieve protection from hazards associated with transportation and storage (Burdon, 2001). Packaging of fruits with polymeric films is often used to prevent moisture loss, to protect against mechanical damage, and to achieve a better appearance (Hening & Gilbert, 1975). Packaging of fruits in polyethylene films creates modified atmosphere conditions around fruits which trigger the rise of CO_2 and fall in O_2 concentration inside package resulting in reduced rate of respiration, transpiration and other metabolic processes of fruit (Singh et al., 2018). Wrapping of strawberries in plastic film reduced ascorbic acid loss by 5-folds at 1 and 10°C and 2-folds at 20°C (Nunes et al., 1998).

The evaporative cooled storage combined with packaging improved the shelf-life of papaya fruits by more than two folds (Azene et al., 2014). Pear fruit individually packed in polyethylene bag (0.05 or 0.01 mm) and stored at ZECC efficiently preserved fruit quality

parameters (Singh et al., 2017). In an experiment conducted in Gujarat, India Kanak and Sanjay (2013) reported that jamun (cv *Goma priyanka*) fruits packed in perforated polythene bag and stored in ZECC performed best and displayed 4 days shelf-life, while fruit kept at ambient conditions had one day shelf-life. Prasad et al. (2015) reported that packaging of banana fruits in high and low density polyethylene bags resulted in longer shelf-life and improved produce quality. Individual packing of pear fruit in polyethylene bags of 0.01 mm and storage under ZECC proved to be most effective treatment in reducing physiological losses in mass (Singh et al., 2018). The shelf-life of custard apple fruits was 9 days in ZECC when wrapped with tissue papers and kept in cardboard boxes as compared to 6 days under ambient storage (Patil et al., 2011).

Therefore, present investigation was conducted to study the effect of packaging materials on strawberry quality attributes under ZECC storage.

MATERIALS AND METHODS

Plant material and experimental site

The experiment was conducted at Department of Environmental Sciences, COMSATS University Islamabad, Vehari Campus. Strawberry cv. 'Chandler' was harvested at red ripe stage from farmers field located at Arien vhin Mailsi, Punjab, Pakistan.

Experimental procedure

Fruit were brought to laboratory, sorted and divided into 36 lots comprising of 8 fruit per lot. Each lot of fruit was kept in styrofoam clamshell container and each container was individually wrapped in polyethylene and perforated polyethylene bags, control containers were kept unwrapped. A factorial experiment was conducted in completely randomized design and different packaging material (wrapped in polyethylene and perforated polyethylene bag) along with no-wrapping as control, storage conditions (ZECC and ambient conditions) and also different storage durations (at harvest, middle and end of storage period) were considered as experimental factors with three replications (3×2×3×3=36). The thickness and mass of polyethylene bag was 0.016 mm and 4.10g and perforations were at 4×3.5 cm distance in case of perforated polyethylene bag.

Construction of ZECC

The ZECC was constructed as described by Pal and Roy (1988). On rectangular floor a double walled structure was erected with bricks having a cavity which is filled with fine sand. Inside the cavity a frame of rectangular plastic pipe having small holes was laid on the sand bed. Rectangular frame was connected with water tank kept on raised stand. Water is applied in the form of small droplets to moisten the sand filled in the cavity. Plastic crates were used to keep strawberry inside the ZECC and top of which were covered with wet gunny bags. The whole structure was covered under shed to prevent sunlight and rain (Fig. 1).

Temperature (°C) and relative humidity (%)

Temperature (°C) and relative humidity (%) was determined with the help of thermo-hygrometer (TFA Dostman/D-97877 Wertheim) three times a day of both ZECC and Laboratory.

Physical quality parameters

Mass loss (%) and shelf-life (days)

Mass of strawberries was determined at start, middle and end of the experiment. The mass loss percentage was calculated by following formula (1):

$$\text{Mass loss (\%)} = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100 \quad (1)$$

Shelf-life of strawberries was determined by observing the shriveling which was due to physiological loss in mass. Five per cent loss in mass was considered as an index of end of shelf-life.

Fruit internal temperature (°C)

Fruit internal temperature was measured with the help of probe thermometer by inserting its needle inside the fruit.

Disease (%)

Disease (%) was determined by counting the diseased fruit out of total fruits and expressed as percentage.

Biochemical quality parameters

Chemicals and reagents

All chemicals and reagents were of analytical grade. Sodium hydro oxide (NaOH), oxalic acid, 2, 6-dichlorophenolindophenol dye, methanol, hydrochloric acid (HCl, acetone, sodium bicarbonate (Na_2CO_3) and gallic acid 1-hydrate were purchased from Sigma Aldrich. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Alfa Aesar.

pH

pH was determined after each removal. Strawberry juice was extracted after mashing it in pastel and mortar. The pH of the juice was measured with digital pH meter (Milwaukee pH55).

Electrical Conductivity (EC) (S/m)

The EC was calculated after extracting the juice of strawberries. The Lovibond Senso Direct Con 110 Digital EC meter was used for its determination. Taking the juice in a beaker and dipping the electrode of EC meter, the reading was noted down when it appears on the screen of the meter.

Total soluble solids (°Brix)

Strawberries juice was extracted and analyzed by using digital refractometer (ATAGO PAL-1). Two drops of clear juice was placed on surface of prism and reading was taken. Data was taken at each removal of both lots and expressed in ºBrix.

Titratable acidity (%)

Strawberry juice titratable acidity (%) was determined by following the method described by Hortwitz (1960). Samples were titrated against 0.1 N NaOH using two to three drops of phenolphthalein as an indicator, and the results were expressed in percentage.

Ascorbic acid (mg100mL^{-1})

Juice ascorbic acid was determined by following the method reported by Ruck (1969) with some modifications. 2, 6-dichlorophenolindophenol dye solution was used to titrate 5ml of aliquot (containing 10 mL of juice and 90 mL of 0.4% oxalic acid solution).

Total phenol contents (µg ml^{-1})

Total phenolic contents were determined by Folin-Ciocalteu (FC) method as described by Ainsworth and Gillespie (2007) with some modifications. Take 0.2 g of pulp of sample and homogenize it in pestle and mortar by adding 8 ml of methanol: acetone: HCl solution (90:8:2). Centrifuge the samples (13000 g for 3 min at room temperature) and collect the supernatant in a fresh falcon tubes. Add 100µl of sample supernatant and blank (methanol: acetone: HCl) in a fresh eppendrof tube add 200µl 10% FC reagent and vortex thoroughly for few seconds. Add 800µl 700mM Na_2CO_3 in each tube and again vortex for few seconds and incubate tubes at room temperature for 1h. Transfer one ml sample and one ml of distilled water in cuvette and read the absorbance at 765 nm.

Antioxidants (IC_{50} µg ml^{-1})

Antioxidant activity was determined by the method of Noor et al. (2014) with some modifications. 50ul extract was added to 5 ml 0.004% (4mg/100ml) of methanol solution of DPPH. After 30 minutes in incubation period at room temperature absorbance was measured at 517 nm. Then same procedure was repeated for 100 µl extract and 150 µl extract. Inhibition (%) was calculated as follows (2):

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (2)$$

Where $A_{control}$ was the absorbance of DPPH and A_{sample} was the absorbance of free radical DPPH after adding a sample extract. Inhibition concentration$_{50}$ (IC_{50}) values represent the concentration of sample, which was necessary to scavenge 50% of DPPH free radicals. The higher the antioxidants activity, the lower will be the IC_{50} value.

RESULTS

Temperature (°C) and relative humidity (%)

Maximum temperature in ZECC was 23.3°C and at ambient conditions in laboratory was 26.4°C. Relative humidity in ZECC was high (86%) as compared to laboratory (43%). Temperature difference of 3.41°C and relative humidity difference of 38.22% was found between ZECC and ambient conditions during the day (Fig. 2).

Physical quality parameters

Mass loss (%) and shelf-life (days)

Effect of treatment (Table 7), storage conditions and treatment × storage conditions (Table 1) had statistically significant influence on mass loss (%) of strawberry fruit while, all other factors and their interactions had statistically no significant (data not given) influence. Mass loss was lower (1.59%) in ZECC and higher at ambient conditions (6.46%) (Table 1). Control had maximum mass loss (9.11%) whereas polyethylene packed fruit had minimum mass loss (1%) after storage (Table 7). Five percent mass loss was considered as end of shelf-life. Shelf-life of strawberry was less at ambient conditions (less than 3 days) and higher (more than 3

days) in ZECC (Table 1). Interaction of storage conditions and treatment revealed that shelf-life at ambient conditions in all treatments was lower than ZECC (Table 1). Control had less than three day's shelf-life while polyethylene packed fruit had more than three days shelf-life (Table 7).

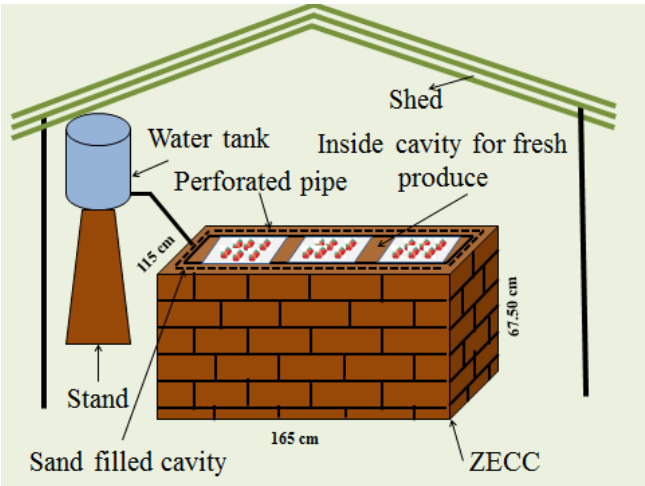

Fig. 1. Schematic view of zero energy cool chamber

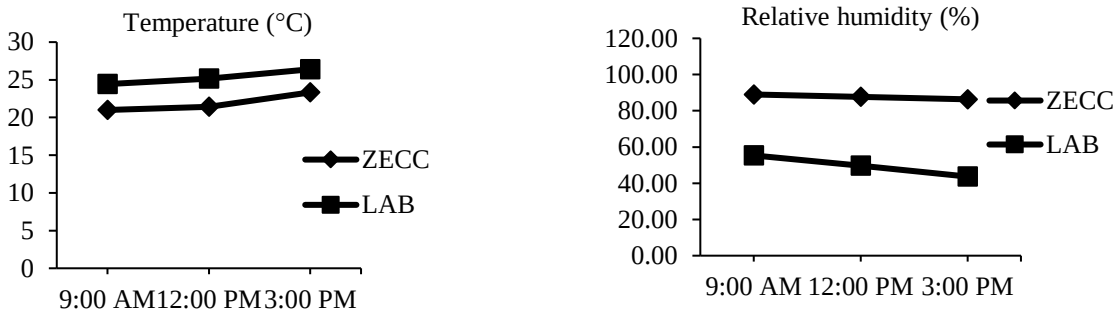

Fig. 2. Temperature (°C) and relative humidity (%) during the study

Table 1. Interaction of storage conditions and treatment on mass loss (%) and shelf-life, internal temperature (°C) and disease (%) of strawberry fruit

Storage conditions	Control	Polyethylene	Perforated Polyethylene	Mean
	Mass loss (%) and shelf-life (days)†			
ZECC	2.95b (> 3 days)	0.68c (> 3 days)	1.15c (> 3 days)	1.59b (> 3 days)
Ambient conditions	15.26a (< 3 days)	1.79bc (> 3 days)	2.32bc (> 3 days)	6.46a (< 3 days)
p-value	0.000			0.000
	Fruit internal temperature (°C)			
ZECC	22.17	22.29	22.25	22.24b
Ambient conditions	22.93	23.09	23.09	23.04a
p-value	0.9471			0.000
	Disease (%)			
ZECC	4.11	0	4.11	2.74
Ambient conditions	1.33	4.11	2.78	2.74
p-value	0.3629			1.000

†Shelf-life was determined on mass loss basis. Five percent loss in mass was considered end of shelf-life. ZECC: Zero energy cool chamber

Table 2. Interaction of storage conditions and treatment on pH, EC, TSS and TA (%) of strawberry juice

Storage conditions	Control	Polyethylene	Perforated Polyethylene	Mean
	pH†			
ZECC	3.91	3.92	3.96	3.93
Ambient conditions	3.94	3.97	3.97	3.96
p-value	0.7931			0.1528
	EC (S/m)			
ZECC	3.71	3.48	3.55	3.58
Ambient conditions	3.77	3.56	3.59	3.64
p-value	0.9648			0.3567
	TSS (°Brix)			
ZECC	6.35	5.82	5.81	6
Ambient conditions	6.03	5.96	5.88	5.96
p-value	0.5401			0.8217
	TA (%)			
ZECC	4.06	4.42	4.02	4.17
Ambient conditions	4.08	3.96	3.97	4.01
p-value	0.7780			0.5974

†EC: Electrical conductivity; TA: Titratable acidity; TSS: Total soluble solids; ZECC: Zero energy cool chamber

Fruit internal temperature (°C)

Fruit internal temperature was significantly lowered in ZECC (22.24 °C) and higher at ambient conditions in laboratory (23.04 °C) while other factors and their interactions had statistically non-significant effect on internal temperature of fruit (Table 1, 4 and 7).

Disease (%)

Storage duration and treatment had no significant influence on disease (%) (Table 1 and 7). Storage duration had significant influenc on disease (%). Maximum disease (8.22%) was recorded at the end of experiment (Table 4). All interaction effects had no significant influence on disease (%) during the study (Table 1, 4 and 7).

Biochemical quality parameters

pH

Storage duration had significant influenc on pH of strawberry juice. The pH reduced during storage duration (Table 5). All factors and their interactions had statistically no significant effect on pH of strawberry juice (Table 2, 5 and 8).

Table 3. Interaction of storage conditions and treatment on ascorbic acid (mg 100mL^{-1}), total phenols (µg mL^{-1}) and antioxidants (IC_{50} µg mL^{-1}) of strawberry juice

Storage conditions	Control	Polyethylene	Perforated Polyethylene	Mean
	Ascorbic acid (mg 100mL^{-1})			
ZECC	124.82	117.94	123.23	122
Ambient conditions	113.71	113.18	139.10	122
p-value	0.2225			1.000
	Total phenols (µg mL^{-1})			
ZECC	0.69	0.72	0.72	0.72
Ambient conditions	0.72	0.75	0.71	0.71
p-value	0.7793			0.6182
	Antioxidants (IC_{50} µg mL^{-1})†			
ZECC	0.73	0.61	0.89	0.74
Ambient conditions	0.91	0.47	0.59	0.65
p-value	0.8096			0.7721

†Antioxidants are represented as IC50 value. Larger IC50 value means less antioxidant activity. IC: Inhibition concentration

Table 4. Effect of three-way interaction on mass loss (%), internal temperature (°C) and disease (%) of strawberry fruit

Conditions	Treatments	Mass loss (%)		Fruit internal Temperature (°C)			Disease (%)		
		Storage duration		Storage duration			Storage duration		
		Mid	End	At harvest	Mid	End	At harvest	Mid	End
ZECC	Control	2.51	3.40	22.24	21.83	22.44	0	0	12.33
	Polyethylene	0.51	0.85	22.24	22.07	22.56	0	0	0
	Perforated polyethylene	1.10	1.20	22.24	22.07	22.44	0	0	12.33
Ambient conditions	Control	14.33	16.20	22.24	23.17	23.39	0	0	4.00
	Polyethylene	2.26	1.32	22.24	23.61	23.44	0	0	12.33
	Perforated polyethylene	1.49	3.14	22.24	23.41	23.65	0	0	8.33
Mean storage duration		3.70	4.35	22.24c	22.69b	22.99a	0b	0b	8.22a
P-value storage condition × treatment × storage duration		0.1953		0.9494			0.3989		
P-value storage duration		0.465		0.000			0.0025		

Electrical conductivity (EC) (S/m)

Treatments had significant effect on EC of strawberry juice (Table 8) while other factors and their interactions had statistically no significant influence on juice EC (Table 2 and 5). Maximum EC (3.74 S/m) was found in control and minimum EC (3.52 S/m) was found in strawberries packed in polyethylene bags. The EC significantly increased during storage duration (Table 5).

Total soluble solids (TSS) (°Brix)

The TSS significantly decreased during storage duration (Table 5). All factors and their interactions had statistically no significant effect on TSS of strawberry juice (Table 2, 5 and 8).

Titratable acidity (%)

The titratable acidity (%) of strawberry juice significantly decreased during storage duration (Table 6). All factors and their interactions had statistically no significant effect on titratable acidity (%) of strawberry juice (Table 2, 6 and 8).

Ascorbic acid (mg100mL^{-1})

Ascorbic acid concentrations significantly declined during storage duration (Table 6) irrespective of treatment and storage conditions. Ascorbic acid concentration of the fruit remained statistically similar under different storage conditions and interaction of storage conditions and treatment (Table 3), with interaction of storage conditions, treatment and storage duration (Table 6) and with various treatments (Table 8).

Total Phenols (µg mL^{-1})

Total phenols of the fruit were not affected by storage conditions and interaction of storage conditions and treatment (Table 3), storage duration, interaction of storage conditions, treatment and storage duration (Table 6) and with various treatments (Table 8).

Antioxidants concentrations (IC_{50} µg mL^{-1})

Antioxidant concentrations of the fruit remained statistically at par under different storage conditions and interaction of storage conditions and treatment (Table 3) and with various treatments (Table 8).

Table 5. Effect of three-way interaction on pH, electrical conductivity (S/m) and TSS (°Brix) of strawberry juice

Conditions	Treatments	pH			Electrical conductivity (S/m)			TSS (°Brix)		
		Storage duration			Storage duration			Storage duration		
		At harvest	Mid	End	At harvest	Mid	End	At harvest	Mid	End
ZECC	Control	4	3.80	3.93	3.32	3.91	3.90	6.77	6.37	5.93
	Polyethylene	4	3.90	3.87	3.32	3.42	3.70	6.77	5.27	5.43
	Perforated polyethylene	4	3.97	3.90	3.32	3.73	3.60	6.77	5.70	4.97
Ambient conditions	Control	4	3.93	3.90	3.32	3.79	4.20	6.77	5.37	5.97
	Polyethylene	4	4	3.90	3.32	3.64	3.72	6.77	5.93	5.17
	Perforated polyethylene	4	3.97	3.93	3.32	3.54	3.91	6.77	5.57	5.30
Mean storage duration		4a	3.93b	3.91b	3.32c	3.67b	3.84a	6.77a	5.70b	5.46b
P-value storage condition × treatment × storage duration			0.5753			0.4099			0.3997	
P-value storage duration			0.0014			0.000			0.000	

Table 6. Effect of three-way interaction on TA (%), ascorbic acid (AA) (mg 100mL^{-1}), and total phenols (µg mL^{-1}) of strawberry juice

Conditions	Treatments	TA (%)			Ascorbic acid (AA) (mg 100mL^{-1})			Total phenols (µg mL^{-1})	
		Storage duration			Storage duration			Storage duration	
		At harvest	Mid	End	At harvest	Mid	End	Mid	End
ZECC	Control	6.18	3.73	2.26	142.80	103.13	128.52	0.74	0.65
	Polyethylene	6.18	4.74	2.32	142.80	109.48	101.55	0.78	0.66
	Perforated polyethylene	6.18	4.33	1.56	142.80	123.76	103.13	0.76	0.68
Ambient conditions	Control	6.18	4.54	1.54	142.80	96.79	101.55	0.76	0.68
	Polyethylene	6.18	4.22	1.47	142.80	103.13	93.61	0.67	0.83
	Perforated polyethylene	6.18	4.20	1.54	142.80	152.32	122.17	0.65	0.77
Mean storage duration		6.18a	4.29b	1.78c	143.80a	114.77b	108.42b	0.72	0.71
P-value storage condition × treatment × storage duration			0.8834			0.7703		0.1210	
P-value storage duration			0.000			0.0002		0.6053	

Table 7. Treatment effect on fruit physical quality parameters of strawberry

Treatment	Mass loss (%)	Shelf-life (days)†	Fruit internal temperature (°C)	Disease (%)
Control	9.11a	< 3 days	72.59	2.72
Polyethylene	1.24b	> 3 days	72.85	2.06
Perforated polyethylene	1.73b	> 3 days	72.82	3.44
P-value		*	0.5081	0.8591

†Shelf-life was determined on mass loss basis. Five percent loss in mass was considered end of shelf-life.

Table 8. Treatment effect on fruit biochemical quality parameters of strawberry

Treatment	EC (S/m)	TSS (°Brix)	pH	Acidity (%)	Ascorbic acid (mg 100mL^{-1})	Total phenol (µg mL^{-1})	Antioxidants (IC_{50} µg mL^{-1})
Control	3.74a	6.19	3.93	4.07	119.26	0.71	0.83
Polyethylene	3.52b	5.89	3.94	4.19	115.56	0.73	0.54
Perforated polyethylene	3.57b	5.84	3.96	4.00	131.16	0.71	0.74
P-value	0.0181	0.2360	0.4155	0.8740	0.1373	0.7377	0.7330

DISCUSSION

The study showed a difference of temperature and relative humidity between ZECC and ambient conditions (Fig. 1). This might be due to evaporation of water applied to the sand. Water absorbs heat from its surroundings to evaporate resulted in reduction of temperature inside ZECC. As water evaporates it raises the relative humidity and at the same time reduces temperature of the surroundings (Lal Basediya et al., 2013). In New Dehli, Verma (2014) reported a 15-18°C fall in temperature and more than 90% rise in relative humidity inside cool chamber. Similarly, Burbade et al. (2017) found a reduction of 8-9°C temperature and an increment of 10% relative humidity in ZECC as compared to ambient conditions.

Mass loss was lower in ZECC and higher at ambient conditions. Mass loss is an important factor for determining quality of fresh produce (Nunes & Emond, 2007). It depends upon rate of respiration and transpiration (moisture loss) of the commodity. Both of these factors are influenced by temperature and relative humidity. Moisture loss from the fresh produce depends upon vapor pressure deficit in the surroundings (Aked & Jongen, 2002). In ZECC, due to evaporative cooling, humidity increased and temperature decreased. The lower temperature and high relative humidity inside the ZECC might reduce respiration and transpiration from fresh produce. Respiration causes mass loss because a molecule of water is produced with a loss of each carbon atom (Sharma et al., 2018). This could be the reason for less mass loss in produce stored in ZECC as compared to laboratory where temperature and humidity were comparatively high. Similar results were also reported by Singh et al. (2010) (Indian gooseberry); Rayaguru et al. (2010) (tomato, potato, brinjal, banana and leafy vegetables), Islam and Morimoto (2012) (tomato and eggplant) and Islam et al. (2013) (tomato). In both ZECC and at ambient conditions mass loss was higher in control and lower in strawberries packed in polyethylene bags. This could be due to the fact that packaging creates an atmosphere of low oxygen and high carbon dioxide around the produce which might reduce the respiration rate and inhibit senescence of fresh produce and hence reduced mass loss. Moreover packaging also increased moisture and reduced vapour pressure deficit around the produce which resulted in reduced transpiration from fresh produce and hence reduced mass loss. The results are in conformity with the findings of Nunes et al. (1998) who reported that more mass loss was found when strawberries were unwrapped and when storage temperature and storage time increased.

Electrical conductivity increased with increase in storage duration. Electrical conductivity can successfully be used as a physical maturity index, and it is a appropriate index of storage quality (Feng et al., 2005). High conductivity is indicative of leakage of intracellular ions and, therefore, damage to membranes (Ade-Omowaye et al., 2003). According to Sarang et al. (2008) electrical conductivity might increase due to increase in senescence of fruit tissue. The electrical conductivity (EC) of the fruit tissue constantly increased after harvest, suggesting a gradual loss of cell membrane integrity (Ahmed et al., 2010). Similarly, increased in electrical conductivity with increased in storage duration was also reported by Ahmed et al. (2010) in avocado. Electrical conductivity of strawberry juice was significantly higher in control (unpacked) as compared to polyethylene and perforated polyethylene packed fruits. Similarly, Sharma et al. (2018) reported reduced electrolyte leakage in basil leaves packed in low density polyethylene bags as compared to control. The increased in electrical conductivity in control unpacked fruit might be due to more availability of oxygen for respiration which resulted in more senescence as compared to polyethylene packed fruits which had limited oxygen availability for respiration and hence reduced senescence and electrical conductivity.

Irrespective of treatment and storage duration titratable acidity, pH, ascorbic acid and TSS decreased with increased in storage duration. Similar results for reduction in titratable

acidity and ascorbic acid were reported by Singh et al. (2017) in pear during storage. Storage conditions had no significant influence on biochemical quality attributes of strawberry during storage. Likewise Shin et al. (2007) also reported that temperature and relative humidity had no significant influence on pH and acidity of strawberry during storage.

Fruit packed in polyethylene bags and kept in ZECC had lowest mass loss and EC of juice and can give maximum return to the grower. Other physicochemical parameters remained statistically similar in both ZECC and ambient conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Assessment of genetic diversity and relationships among tea genotypes in Iran based on RAPD and ISSR markers

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ABSTRACT

Purpose: Tea plant (*Camellia sinensis* L., O.Kuntze) is one of the most popular non-alcoholic beverage crops worldwide. Although tea is important in Iran's economy, little is known about the pattern of genetic variation among the various tea genotypes grown in Iran. **Research method:** The relationship and the genetic diversity of 20 genotypes of the tea germplasm belonging to three regions were analyzed to provide guidance for the breeding of tea tree using 10 RAPD and 8 ISSR markers. **Main findings:** Polymorphism percent was 78.6 in RAPD and 68.06 in ISSR fingerprinting. The results of the PIC analysis were in the range of 2 0.44 to 0.49 and 0.34 to 0.50 for RAPD and ISSR respectively. From these results, it can be seen that these primers can detect genetic differences very well. The pairwise similarity coefficient between the genotypes varied from 0.37 to 0.68 for RAPD and from 0.59 to 0.96 for ISSR. The 20 tea genotypes from genetic resources were grouped into three main groups by UPGMA cluster analysis based on RAPD data, and to five main groups by UPGMA cluster analysis based on ISSR data. Both molecular analyses showed a high degree of variation among the genotypes. **Limitations:** Application of others molecular markers such as AFLP, SSR and (cpDNA investigation can help to found the genetic relationships of samples better. **Originality/Value:** The present study revealed that RAPD and ISSR methods could be successfully utilized to identify genetic diversity and relationship of tea group and this will provide valuable information to assist parental selection in current and future tea breeding programs.

INTRODUCTION

Tea belongs to family Theaceace, genus *Camellia*, section *Thea* and usually involves one species (*Camellia sinensis*), including two or three botanical varieties: *C. sinensis* var. assamica, *C. sinensis* var. Pubilimba (Chen et al., 2000), and sometimes *C. sinensis* var. Kucha (Chang, 1984). Three seed varieties (Dhonjan, Rajghu and Betjan) made the foundation of tea genetic in Iran. By applying different molecular markers such as RAPD, ISSR, RFLP, AFLP and SSR, beneficial information has been achieved about tea genetics. However, in Iran these sorts of studies have been carried out in small cases and most assays were based upon morphological characters. The effects of environmental factor fewer concerns morphological parameters. Whilst molecular markers (DNA based and isozymes) are not affected by environmental factors and many kinds of them are available, their application for identifying and investigating the genetic relationship in plants is very common. Molecular markers were used for genetic relationship assessment in tea in numerous kinds of studies such as genetic diversity, cultivar identification, phylogenetic relationship, parentage identification and QTL (Balasaravanan et al., 2003; Beris et al., 2016; Jahangirzadeh et al., 2020; Kafkas et al., 2009; Lai et al., 2001; Liu et al., 2009; Ma et al., 2014; Paul et al., 1997; Rani et al., 2012; Roy & Chakraborty, 2009; Ueno & Tsumura, 2009). Paul et al. (1997) investigated genetic diversity and differentiation among populations of Indian and Kenyan tea by AFLP markers and reported that these markers were very useful and efficient for tea population identification (Paul et al., 1997). Kaundun et al. (2000) evaluated the genetic diversity among elite tea accessions from Korea, Japan and Taiwan with RAPD-PCR. Accessions from Korea showed higher level of diversity than accessions from Japan and Taiwan. This high level of diversity can be related to huge genetic pool in Korea (Kaundun et al., 2000). In another study, Chen and Yamaguchi (2002) assayed genetic diversity and phylogeny of tea plant and its related species and varieties in the section *Thea* genus *Camellia* by RAPD analysis, and their results showed that RAPD could reveal high level of diversity in tea and its related species. According to their result, it became known that cultivated species and wild related species had narrow genetic relationship (Chen & Yamaguchi, 2002). Beris et al. (2016) used ISSR markers for investigation of genetic diversity in cultivated tea clones (*Camellia sinensis* (L.) kuntze) in Turkey and found similarity range between 0.456-0.743 and clustered samples into two groups. Totally markers (like RAPD and ISSR) were widely used, mainly at the beginning of studies which no information was available about the amount of diversity; because of low DNA requirement, low cost, being fast in analyzing and other advantages. Jahangirzadeh et al. (2020) used SRAP marker to identification of genetic diversity and relationships of some Iranian tea genotypes. Reported similarity ranges were 0.393 to 0.933 and classified samples in five groups. Their results showed that SRAP marker could be useful in identifying polymorphic regions and estimating genetic distances and germplasm management in tea plants.

In the present study, RAPD and ISSR were used to determine genetic relationships among 20 tea genotypes from Iran to provide valuable information about tea genetic diversity.

MATERIALS AND METHODS

Plant material and DNA extraction

Fresh young leaf samples were taken from 20 tea genotypes collected from three locations on the north of Iran (Table 1). The leaves were stored at -80 ºC until being used for DNA extraction.

Total DNA was isolated using the procedure described by the Diversity Arrays Technology Pty Ltd (DArT P/L) company (Diversityarrays, 2007). Approximately 0.2 g of plant material was ground to a fine powder by mortar and pestle under liquid nitrogen. They were transferred to a 2 ml tube and 1 ml of fresh buffer (Table 2 and 3) was added and incubated at 65°C for 1 hour. It was then allowed to cool down for 5 minutes on ice and 1 ml of chloroform: isoamyl alcohol (24:1) mixture was added. This suspension was well mixed for 30 minutes. After centrifugation at 10000 g for 20 minutes, the water phase was transferred to the fresh tube and the same volume of ice-cold isopropanol was added. Then tubes were inverted until nucleic acids became visible. Following centrifugation at 10000 g for 30 minutes, the supernatant was discarded and the pellet of DNA washed with 2 ml 70% ethanol. Ethanol was discarded, and pellet dried and dissolved in 250 µl of 1 X TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Molecular Analysis

For RAPD amplification, thirty RAPD primers and for ISSR 20 primers were screened using four DNA samples. both markers (RAPD and ISSR) amplifications were performed in 20µl of reaction mixture containing 50ng of total DNA, 10 ng of primer pair, 200µM of each four dNTPs, one-unit Taq DNA polymerase, 2mM of $MgCl_2$ and 1X PCR buffer. The PCR was carried out with a Bio-Rad thermocycler in a condition that initial denaturation at 94°C for 4 min, 35 cycles of 1 minute at 94°C, 45 seconds at 38°C for RAPD and 54°C for ISSR, 2 minutes at 72°C and a final extension for 7 minutes at 72°C then brought down to 4°C. The amplification of the screened primers was repeated two to three times independently with the same procedure in order to verify the reproducibility of the RAPD and ISSR marker.

Gel scoring and Data Analysis

The amplification fragments were fractionated by 1.5% agarose gel electrophoresis in 1X TBE buffer at a constant voltage (70V), for 4 hours. Each mixture that loaded in agarose gel contained 10µl of PCR mixture, 3µl of loading buffer and 2µl safe stain. After it, fragments visualized under the UV light. The DNA size marker of 1 kb was used for analyzing the size of polymorphic bands.

Table 1. Tea samples used in RAPD and ISSR analysis

Row	Plant code	Sampling location	Row	Plant code	Sampling location
1	G1	Shahid Eslami Tea Research Station	11	G11	Shahid Eslami Tea Research Station
2	G2	Shahid Eslami Tea Research Station	12	G12	Kobijar village
3	G3	Sheykhzahed region	13	G13	Shahid Eslami Tea Research Station
4	G4	Shahid Eslami Tea Research Station	14	G14	Tonekabon, Garmaposhteh village
5	G5	Sheykhzahed region	15	G15	Kobijar village
6	G6	Sheykhzahed region	16	G16	Kobijar village
7	G7	Shahid Eslami Tea Research Station	17	G17	Shahid Eslami Tea Research Station
8	G8	Shahid Eslami Tea Research Station	18	G18	Shahid Eslami Tea Research Station
9	G9	Shahid Eslami Tea Research Station	19	G19	Shahid Eslami Tea Research Station
10	G10	Shahid Eslami Tea Research Station	20	G20	Sheykhzahed region

Table 2. Extraction buffers and lysis buffer stocks

Extraction buffer stock		Lysis buffer stock	
Sorbitol	0.35 M	TrisHCl pH 8.0	0.2 M
TrisHCl pH 8.0	0.1 M	EDTA pH 8.0	0.05 M
EDTA pH 8.0	5 mM	NaCl	2M
		CTAB	2% (w/v)

Both buffers should be volumed to 500 ml with distilled water.

Table 3. Preparation of fresh buffer

Chemical materials	Required amount	Example for 30 ml
Extraction buffer	The necessary amount based on the number of samples	12.5 ml
Lysis buffer	The necessary amount based on the number of samples	12.5 ml
Sodium dodecyl sulfate	0.5% (v/w)	0.15 g
Polyvinylpyrrolidone	2% (v/w)	0.6 g
Sarcosyl (5%)	0.4 (v/w)	5 ml

The buffer may be divided into two separate phases; therefore it should be heated till 65 °C and shaken before use.

Polymorphic bands were scored as either presence (1) or absence (0). These data were used to calculate the genetic similarity matrix with the Pearson correlation using Jaccard's coefficient for RAPD marker and Dice's coefficient for ISSR marker with the unweighted pair group method using the arithmetic average (UPGMA) as a clustering algorithm. The dendrogram was drawn using the SAHN module in NTSYSpc software (Rohlf, 1998). Polymorphic Information Content (PIC) was calculated for each primer according to PICi = 1-[fi^2+ $(1-fi)^2$]; where PICi is the PIC of primer ith, fi the frequency of ith primer fragment when present and 1 - fi is the frequency of *ith* primer when absent (Roldain-Ruiz et al., 2000).

RESULTS AND DISCUSSION

RAPD analyses

Ten RAPD primers were finally selected according to their diversity and reproducibility (Table 4), and were used for the amplification of the 20 Iranian tea genotypes. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded. The size of amplified fragments ranged from 100 to 3500 bp with the scorable region were from 250 to 2500 bp. Figure 1 showed some samples that amplified by RAPD primer. The number of fragments per primer ranged from 7 (P6: ACTGAACGGC) to 15 (P7: CACAGACACC).) as shown in Table 4. The number of scored polymorphic fragments ranged from 7 with (P8: GGTGAGGTCA and P6: ACTGAACGGC) to 12 with (P7: CACAGACACC) with an average of 8.8 per primer (Table 4). Of the total 112 scorable fragments, 78.60 percent were polymorphic among the samples. Primer P2 (TGAGCCTCAC) and primer P6 (ACTGAACGGC) showed minimum and maximum percentage of polymorphism (66.7 and 100 respectively) (Table 4).

Chen et al. (2005) investigated genetic diversity, relationship and molecular identification of Chinese elite tea genetic resources [*Camellia sinensis* (L.) O. Kuntze] preserved in a tea germplasm repository, calculated these items and report 100% as maximum and 66% as the minimum of polymorphic percent. Roy and Cakraborty (2009) studied genetic diversity and relationships among tea (*Camellia sinensis*) cultivars by RAPD markers, and they reported the similar result for the polymorphic percentage (Max= 90% and Min=80%). Other studies also reported same result (Devarumath et al., 2002; Mishra & Sen-Mandi, 2004).

Table 4. Details of amplified bands generated in 20 tea genotypes based on ten RAPD primers

No.	Primer	Sequences	Total no. of amplified bands	No. of polymorphic bands	% of polymorphism	PIC value
1	P1	TCTCCGCTTG	13	9	69.2	0.48
2	P2	TGAGCCTCAC	12	8	66.7	0.48
3	P3	CTCACGTTGG	14	10	71.4	0.44
4	P4	ACTCCTGCGA	9	8	88.9	0.45
5	P5	GGTACTCCCC	11	8	72.7	0.49
6	P6	ACTGAACGGC	7	7	100	0.49
7	P7	CACAGACACC	15	12	80	0.47
8	P8	GGTGAGGTCA	10	7	70	0.48
9	P9	TCGCACAGTC	11	10	90.9	0.48
10	P10	GTGCTCCCTC	10	9	90	0.47
Total	-	-	112	88	-	0.48
Average	-	-	11.2	8.8	78.6	

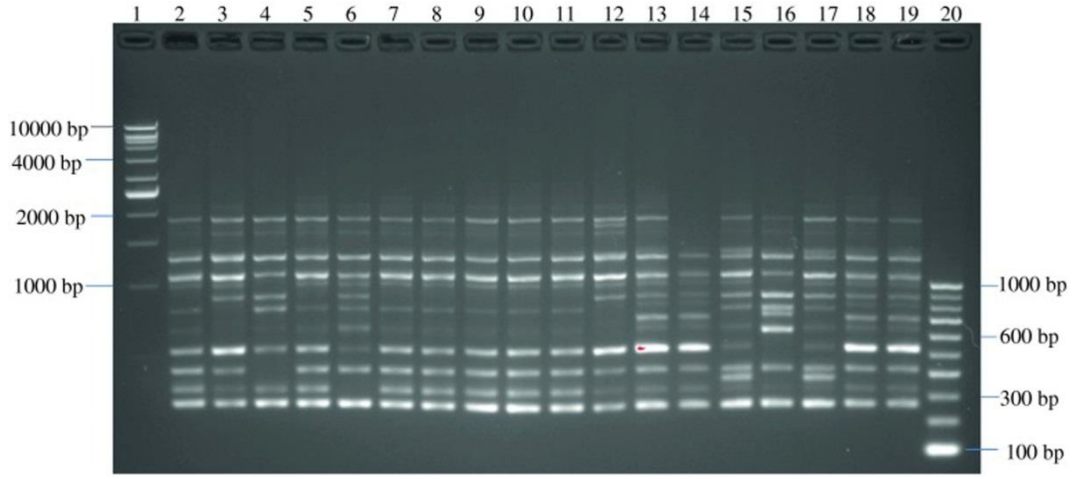

Fig. 1. An example of the polymorphisms detected among some test samples using RAPD primer (P5)

To find the potential of RAPD markers, used for our study, to distinguish our samples in a correct way, polymorphic information content (PIC) was calculated by using formula introduced by Roldain-Ruiz et al. (2000) regarding Table 4, all the PICs were high (minimum was 0.44 and maximum was 0.49), this range of PIC showed the efficiency of molecular marker used to detect polymorphism within the tea plant.

For all Jaccard's coefficient in three methods of clustering (UPGMA, complete and single), the correlation coefficient (r), based on Mantel Z-statistics (Mantel, 1967), was calculated. Mantel Z-statistics showed that Jaccard coefficient had the highest correlation coefficient with UPGMA (0.81). The amount of similarity between the similarity matrix and the cluster were shown with this coefficient.

Pairwise Jaccard similarity coefficient between 20 samples was calculated and ranged from 0.37 to 0.68. The highest and lowest similarities were observed between G14-G5 and G3-G20, with an average of 0.59 based on RAPD data. With a comparison of similarities in our study and other studies, it was found the same results were reported.

Lai et al. (2001) reported maximum and minimum similarities1.00, 0.238, respectively, this wide range of similarity found in wild genotypes of tea, could confirm our results, because Iranian tea germplasm was result of three jat (Rajghur, Betjan and Dhonjan) that used in origin for cultivation and for produce crop. Therefore, it can be stated that Iranian germplasm is more restricted than wild germplasm. On the other hand, comparing our results by papers reported genetic distances (Chen et al., 2005; Mewan et al., 2005), it was found that

our result was correct either. These similar results confirm that RAPD marker in Tea could be applied with high power in distinguishing genetic relationships.

Based on clustering analysis, the 20 samples could be classified into three major groups at 46% similarity. In the first group (A) separated from other groups at 0.39 of similarity just had one member, sample from west of Mazandaran (Tonekabon, Garmaposhteh village) (G14). This separation, according to the geographical distance, was acceptable because all other samples were collected from Guilan province. The second group (B) was separated from other samples at 43% similarity. This group had one member like the first group; this member (G4) belonged to Shahid Eslami Tea Research Station in Lahijan, Guilan province, Iran. Due to, cross-pollination and propagation method of tea in the past (sexual propagation; seed was used in propagating tea at first in Iran) separation of G4 from other samples was not strange.
In the third group (C), the biggest group with 18 members, all samples belongs to Guilan province. The placement of samples from one area showed a close genetic relationship between them. This group can be divided into three sub-groups at 0.51 similarities. The first subgroup showed high level of differentiation from the other subgroups (C2 and C3) and divided from them at % 47.5 similarities.

The first divided sub-group (C1) covered four samples (G2, G12, G15 and G16). Three of the covered samples belong to Kobijar village (G12, G15 and G16) and other one was from Shahid Eslami Tea Research Station (G2). The situation of three samples from Kobijar village near each other with high level of similarity (these samples were not separated from each other until 62% similarity coefficient) could be the result of germplasm conservation in this area. In another hand, placement of G2 (sample of Shahid EslamiTea Research Station) next to the samples of Kobijar village are acceptable because plants in this station were selected in the past from other areas that tea had been cultivated so it was possible that sample G2 was selected from Kobijar village in the past and cultivated in this station. However, it should be noted that G2 was taken in clustering at 0.53 similarities (approximately). This amount of similarity did not validate nor reject this theory.

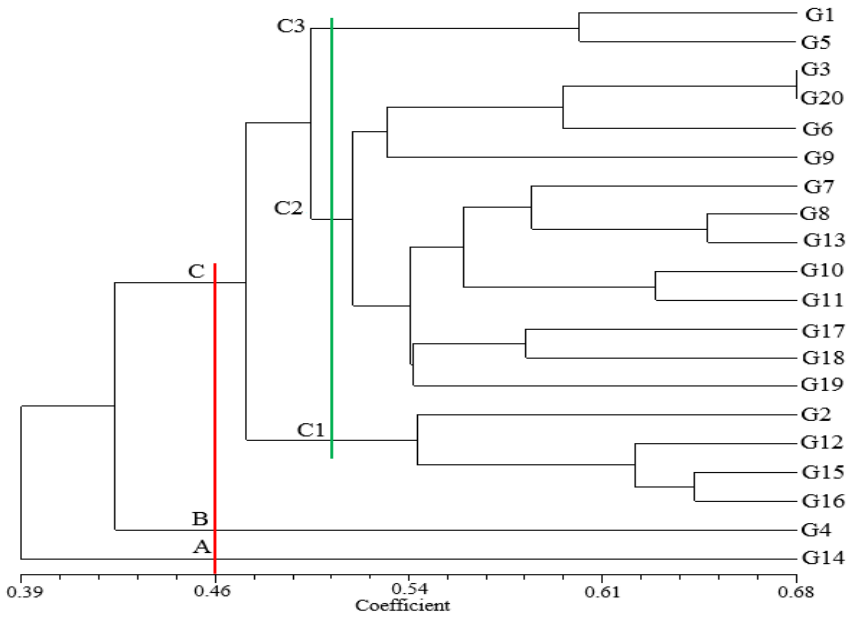

Fig. 2. The phylogenetic dendrogram of 20 tea genotypes (*Camellia sinensis*) constructed from RAPD data using Jaccard similarity coefficient and UPGMA algorithm

Subgroup C2 consists of samples from Shahid Eslami Tea Research Station and Sheykhzahed region (G3, G6, G7, G8, G9, G10, G11, G13, G17, G18, G19 and G20). This sub-group was the biggest subgroup with 12 members. It is noteworthy that in this sub-group, all samples from Sheykhzahed region were separated from Shahid Eslami Tea Research Station samples in %51 similarities (approximately). The maximum similarity was observed between two samples of Sheykhzahed region (G3 and G20).

Moreover, the last sub-group just had two members (G1 and G5). One of these members was from Shahid Eslami Tea Research Station and the other one from Sheykhzahed region.

In this study, samples from Shahid Eslami Tea Research Station showed a high level of conservation and made the special group; however, it should be noted that some samples from this place have been located in others groups (G4: group B, G2: sub-groupC1, G1: sub-group C3). Considering the history of Shahid Eslami Tea Research Station, it is well-known that the planted tea shrubs were collected from different area in the past. Therefore, this deployment in different groups while clustering was acceptable. Lai et al. (2001) also found that samples from different places revealed different grouping in cluster analyses. Mewan et al. (2005) reported the same result by studying tea in Sri Lanka Using RAPD Markers and their clustering divided tea plants from different areas into different groups with some disorder.

ISSR analyses

From 20 ISSR primers tested on four samples, not included in this research, eight primers that showed best amplification and reproducibility were selected and used for ISSR analyses of 20 tea genotypes from north of Iran. Fragments amplified by the usage of ISSR markers were arranged from 100bp to 3000bp, but scorable and acceptable fragments for scoring were between 250bp and 2500bp. The same size range was reported by Beris et al. (2016). Figure 3 showed some samples that amplified by ISSR primer.

Applying ISSR primers have been amplified 72 bands totally which only 49 fragments showed the polymorphism amplified fragment in each primer included 7 for primer I1 ((AG)8G) until 12 bands for primer number 2 (I2 (GA)8T) averages of amplified bands in all of primers were calculated as 9 bands for each used primer for ISSR analyses of 20 tea genotypes from north of Iran. Polymorphic fragments numbers in ISSR primers were from five fragments for primer I1: ((AG)8G), I4 ((AG)8C) and I8 ((ATC)6T) until eight fragments for primers I2 ((GA)8T) and I6 (BDB(TCC)5). The average of polymorphic fragments for all primers was 6.1. According to all amplified fragments and those showed polymorphic pattern, percentages of polymorphism was calculated. The range of this item was from 62.50% for I4 ((AG)8C) and I8 ((ATC)6T) to 80% for I6 (BDB(TCC)5). Previous studies (Chen et al., 2005; Roy & Chakraborty, 2009) reported the same result. Roy and Chakraborty (2009) used ISSR marker for clarifying the genetic diversity and relationships among tea cultivars and reported 88.54% for the average polymorphic percentage. Ji et al. (2011) studied ISSR diversity and genetic differentiation of ancient tea (*Camellia sinensis* var. *assamica*) plantations from China and reported 62.5% as minimum and 89.9% as maximum percent of polymorphic bands Thomas et al. (2006) used 15 ISSR markers for investigating genetic integrity of somaclonal variants in tea (*Camellia sinensis* (L.) O Kuntze) and proclaimed low percentage of polymorphism (min=30.5 and max=78.0) the acquired present of polymorphism was acceptable according to their research and thus, this vindicates our result that percentage of obtained polymorphism was not low for fingerprinting and genetic diversity studies.

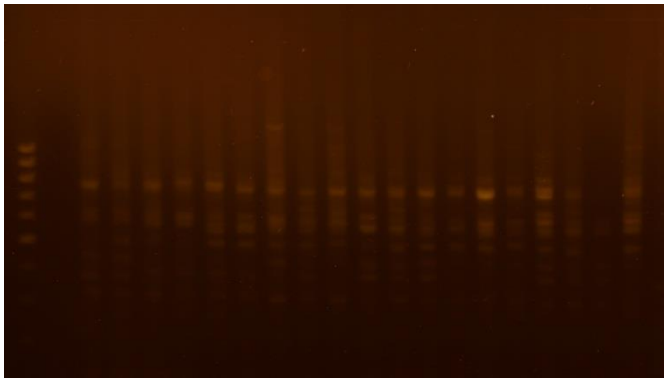

Fig. 3. An example of the polymorphisms detected among some test samples using ISSR primer (I5)

Table 5. Details of amplified bands generated in 20 tea genotypes based on ten ISSR primers

No.	Primer	Sequences	Total no. of amplified bands	No. of polymorphic bands	% of polymorphism	PIC value
1	I1	AGAGAGAGAGAGAGAGG	7	5	71.43	0.35
2	I2	GAGAGAGAGAGAGAGAT	12	8	66.67	0.34
3	I3	CGAGAGAGAGAGAGAGA	9	6	66.67	0.46
4	I4	AGAGAGAGAGAGAGAGC	8	5	62.50	0.50
5	I5	GAGAGAGAGAGAGAGAC	9	6	66.67	0.46
6	I6	BDBTCCTCCTCCTCCTCC	10	8	80.00	0.50
7	I7	ACACACACACACACACC	9	6	66.67	0.39
8	I8	ATCATCATCATCATCATCT	8	5	62.50	0.43
Total	-	-	72	49		
Average	-	-	9	6.1	68.06	0.45

Regarding Table 5, all the PICs were high, and it was ranged from 0.35 (minimum) to 0.50 (maximum), (the range of PIC in dominant markers was zero to 0.50, and if it was high, showed the marker's power in distinguishing polymorphism, genetic diversity, and differences between samples. The calculated range of PIC, showed the efficiency of molecular marker, used to detect polymorphism within the *Camellia* genus and especially in tea genotypes.

Based on ISSR data, pairwise DICE coefficient between 20 samples was calculated and ranged from 0.59 to 0.96. The highest and lowest similarities were observed between G14-G17 and G12-G13, with an average of 0.73 (data not shown). Comparing similarities in our study with others represented the same results. Lai et al. (2001) announced maximum and minimum similarity via assessing genetic relationships in cultivated tea clones and native wild tea in Taiwan using ISSR markers as 0.270 and 0.923, respectively. Yao et al. (2008) analyzed genetic diversity among tea cultivars from three different countries (China, Japan and Kenya) and estimated similarity was 0.538 (maximum) and 0.162 (minimum). In other research, Ben-Ying et al. (2010) in Yunnan used ISSR markers to detect diversity of and relationship of Tea germplasm, the estimates of similarity among tested germplasms passed from 0.445 to 0.819 with an average of 0.512 These similar results confirm that ISSR marker in *Camellia* and related genera could be applied with high power in order to distinguish genetic relationships. In a study conducted in Turkey, which the history of tea cultivation, is not old, like Iran, (it was established in 1940s.), Kafkas et al. (2009) and Beris et al. (2016) reported narrow range of genetic similarity (0.68 to 0.92 by average of 0.76 by kafKas et al. (2009) and 0.456 to 0.743 by Beris et al. (2016).

Based on these polymorphic bands, the similarity coefficient was evaluated and similarity dendrogram (Fig. 4) constructed using UPGMA cluster analysis. Based on clustering analysis, the 20 samples could be classified into five major groups at 74% similarity (approximately).

Comparing the similarity range achieved by Kafkas et al. (2009) and results obtained by the research team of this paper, two cases should be considered: (1) potency and accuracy of used markers in both study, and (2) the way of tea introduction in these countries. To initiate tea plantation in turkey in 1938, the required seed was provided from Batumi (Georgia) (Beris et al., 2005); whereas it was introduced directly from India (one of the sources of tea) to Iran without any mediator, so these differences between similarities ranges were acceptable.

Cluster analysis for 20 tea genotypes was done based on the calculated DICE similarity coefficient and UPGMA algorithm. Five major groups were generated at 46% similarity. The first two groups (groups A and B), divided from other groups at 0.66 similarity coefficient, were separated from each other in 68%, and also generated the special group. However, the other three groups have a special way with each other until 0.72 level of similarity that third group (C) was separate from fourth and fifth groups (D and E). These last two groups (D and E), also were divided from each other at 0.74 similarity coefficient. As it is known, these separations and groupings are carried out in a narrow range, which indicates the acceptable genetic linkage of tea plants, because all tea plants in Iran were generated from a small population introduced in the past.

Group (A) contain just one genotype from Shahid Eslami Tea Research Station (G17). According to geographic distance, this separation was not acceptable due to existing geographic distance, though regarding the fact that self-incommutability and other pollination in tea plants is likely to occur, fragmentation of traits and heterozygosis is extremely high and given that tea propagation was done by seed in the past (sexual way), this division is acceptable. Beris et al. (2016) also, reported that the variations among tea clones exist in Turkey due to their sexual reproduction.

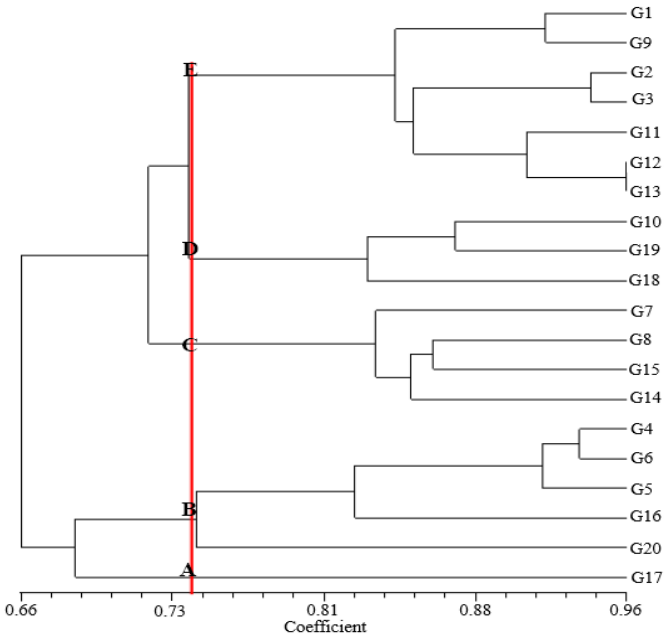

Fig. 4. The phylogenetic dendrogram of 20 tea genotypes (*Camellia sinensis*) constructed from ISSR data using Dice similarity coefficient and UPGMA algorithm

Three genotypes from Sheykhzahed region (G5, G6 and G20); one genotype from Shahid Eslami Tea Research Station (G4) and one genotype from Kobijar village were generated as the second group (B). The important point in this group is the high mixing of samples from three sampling areas of the north of Iran, which is acceptable due to the propagation method (using seed for propagation) in the past. Of course, it is necessary to point out that the sample of the Kobijar village (G20), which has geographical distance from the two other sampling regions, was divided faster from other four samples.

Third group (C) have four members, two members from Shahid Eslami Tea Research Station (G7 and G8), one members from Kobijar village (G15) and one member from Mazandaran province, Tonekabon, Garmaposhteh village (G14), locating locating samples with a large geographical distance in one group can be justified due to seed propagation and free pollination. On the other hand, all tea plants cultivated in different regions originated from first tea plants that Kashef alsaltaneh brought from India and cultivated in Lahijan, so the genetic similarities among them are natural. This sample (G14, genotype from Tonekabon, Garmaposhteh village) was inserted in this study for in Iran's tea trees have been cultivated on the long narrow region of the southern margins of Caspian Sea. Therefore, we intended to investigate whether there are any genetic relationships between plants grown in eastern and middle parts of tea cultivation regions (which includes larger areas of plantations). The fourth group (D), consist of three samples from Shahid Eslami Tea Research Station (G10, G18 and G19) were separated from each other at 87 percent of similarity.

The larger group generated by cluster analyses was fifth group (E) which was included seven genotypes (G1, G2, G3, G9, G11, G12 and G13). This group covered 30 percent of all studied samples. Furthermore, in this group, mixing of this three studied area as (Shahid Eslami Tea Research Station, Sheykhzahed region and Kobijar village) was observed.

CONCLUSIONS

RAPD and ISSR analyses among 20 cultivated genotypes of tea (*camellia*) from three regions of Iran were successfully employed to compute genetic variability and to calculate genetic relationships. According to our data, it was confirmed that RAPD and ISSR markers in tea were successfully utilized for estimating genetic diversity and relationship, which were in agreement as the result of Chen et al. (2005); Thomas et al. (2006); Roy and Chakraborty, (2009); Ji et al. (2011) and Ben-Ying et al. (2010).

RAPD and ISSR data generated from 20 genotypes with then and eight primers, respectively, were sufficient to provide inferences on genetic differentiation and relationships among them. PIC values were also recorded high in both markers (RAPD=0.48 and ISSR=0.45), showing the efficiency of the molecular marker used to detect polymorphism within the tea group. In both markers (RAPD and ISSR), studied samples were slightly different in similar groupings. All 20teagenotypes were classified into three and five main groups by the usage of RAPD and ISSR (respectively) with some differences.

This study represented the first attempt to use two molecular markers, RAPD and ISSR, to study the genetic diversity of 20 Iranian tea genotypes from three different regions of Iran that their relationships were somewhat clarified. The results from this study also open a door to tackle the long-standing problem of tea classification and identification in this country. However, we suppose that this kind of researchers needs to be continued since Iran has a very large and various tea germplasm because of the propagation method, it is being propagated by seed, which gives researchers a chance to find new genotypes, which should be classified, investigated and introduced as a new cultivar.

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Conflict of Interest
The authors declare that they have no conflict of interest.

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Effects of ethanol and chitosan treatments on the quality and storage life of minimally processed pumpkin (*Cucurbita moschata* Duch)

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ABSTRACT

Purpose: Pretreatments of ethanol and chitosan immersion were examined for their potential to maintain physiochemical attributes of fresh cut pumpkin. **Research method:** Fresh cut pumpkin cubes were dipped into different ethanol solutions (20%, 30%, 40%, 50%) or chitosan concentrations (0.5%, 1%, 1.5%). All samples were stored for 15 days at 10°C. **Main findings:** Among four concentrations being applied, the 30% ethanol sample (ET 30) sustained the highest sensory quality until the final day and effectively retained fruit firmness, total soluble solids, total phenolic content compared to the 20% ethanol treatment (ET 20) stored at the same condition. Chitosan application retained better content of carotenoid, phenolic compounds, firmness, and reduced weight loss compared to non – chitosan treatment but there was no significant difference among concentrations. As a result, overall quality index of the coated samples surpassed control ones, especially 1% chitosan. The coating did not affect total soluble solids and antioxidant capacity. **Limitations:** The investigations of antioxidant and cell wall degrading enzymes were absent to support for the study's results. **Originality/Value:** The combination of 30% ethanol and 1% chitosan suggested a possible application in practical context as it outperformed in maintaining the quality and prolonging storage time of the product up to 15 days at 10^{0}C.

INTRODUCTION

Pumpkin (*Cucurbita moschata* Duch) has become a popular crop as the vegetable is able to adapt to various types of soil and microclimate. As the consumption of ready–to–eat foods increases rapidly in crowded cities, pumpkin mostly comes in minimally processed forms. According to USDA, minimally processed products are the ones that undergo processing without being fundamentally altered.

The challenges of minimal processing, as it exposes internal tissues to surrounding, are facilitated water evaporation, enzymatic browning by polyphenol oxidase and microbial spoilage (Garcia & Barrett, 2002). The effects of starch – based coatings have been widely studied on pumpkin. However, polysaccharides–based coatings owe their effectiveness to the gas barrier properties. Nutritional depletion, therefore, cannot be hindered sufficiently. Furthermore, a thick coating layer can provide the commodity a micro–anaerobic condition, initiating fermentation and lead to rapid deterioration. On the other hand, low temperature and modified atmosphere packaging possibly leaves pumpkin chilling injuries and damaged texture.

Ethanol treatment is a classic method that has been used to solve common problems of fresh cut produce such as enzymatic browning, microbial spoilage (Gao et al., 2018). Effects of ethanol on endogenous enzymes such as cell wall degrading enzymes, hydrolases were expected in minimally processed pumpkin (MPP) as observed in sweet cherries (Bai et al., 2011). Ethanol was also used in extending the storage life of fresh-cut Chinese yam (Gao et al., 2018), indicated by least changes for O_2, CO_2 in headspace package, and reducing physiological metabolism and preserving the surface of fresh-cut eggplant (Hu et al., 2010).
Chitosan, derived from the deactylation of chitin, one of the most abundant polysaccharides in nature, can be an ideal coating material because of its ability to form film layer, hydrophilic nature and antimicrobial properties (Li & Yu, 2001). In the study of Suwannarak et al. (2015), chitosan 0.25% or 0.5% exhibited the most effective result for quality improvement and shelf life extension of the carved pumpkin, cantaloupe, and carrot. The addition of chitosan coating on minimally processed pumpkin was concluded to be efficient in minimizing water vapor, carotenoid degradation and microbial growth, hence, maintaining high quality of the vegetable for a longer period of time (Suwannarak et al., 2015).

The objective of this study was to assess the effects of different ethanol and chitosan concentrations on retarding weight loss, maintaining firmness, visual attributes, and preserving total soluble solids, total phenolic compounds, antioxidant capacity and carotenoid content of fresh cut pumpkin stored at 10°C.

MATERIALS AND METHODS

Materials

Fresh pumpkin fruits (*Cucurbita moschata* Duch) were collected from a farm in TienGiang province at intermediate level of maturity with no skin defects, uniform color, size and shape. Fruits were washed under running tap and peeled by sanitized knife. Seeds and sponge parts inside were removed completely and fruit flesh was cut into dice of 2 cm × 2 cm.

Experimental design

Experiment 1

Ethanol treatment procedure followed the experiment of Gao et al. (2018). Pumpkin cubes would be immersed in ethanol solutions (Merck Chemicals Ltd., Darmstadt, Germany) of

20%, 30%, 40% and 50% for 2 minutes and subsequently soaked in 0.5% chitosan solution for 8 minutes. Control sample was submerged in distilled water instead of ethanol solution.

Experiment 2

Chitosan solutions were prepared by dissolving chitosan powder (Sigma-Aldrich, St. Louis, MO, USA) in 0.5% acetic acid solution (Merck Chemicals Ltd., Darmstadt, Germany) at ratio 2:1 (Soares et al., 2018). Pumpkin was soaked into 30% ethanol determined from previous experiment before an 8 minute chitosan immersion. Control sample was immersed in 0.5% acetic acid. There was a group of no ethanol and chitosan dipping to intimidate supermarket condition. After coating steps, dry treated cubes in room condition and pack in styrofoam tray of 100 g portion, cover with PVC film and store at 10°C. Samples were analyzed at three day interval.

Analytical methods

Determination of overall quality index

Different deterioration stages were assessed visually in scale of 5 with detail descriptions corresponding to subjective scores (Fig. 1) by trained personnel. The rating scale was developed based on verbal description of Cantwell and Suslow (2014).

Fig. 1. The overall rating chart of fresh cut pumpkin with photographs of pumpkin at different stages of visual deterioration corresponding to subjective scores and description.

Determination of weight loss (%)

Weight loss in %, was calculated as the ratio of the weight of the pumpkin portion at day of analysis to the initial weight of the coated portion and mathematically expressed as following formula (1):

$$\% \text{ Weight loss} = \frac{Wi - Wf}{Wi} \times 100\% \quad (1)$$

Where Wi is the initial weight of coated sample and Wf is the weight of sample on analyzing day, determined using a top loading balance (TXB- 622L, Shimadzu Co, LTD., Japan) (Santos et al., 2016).

Determination of total soluble solids (%)

Total soluble solids (TSS) was determined using refractometer (RX- 5000, Atago Co., LTD., Japan) at 25°C and the results were expressed as % Sucrose. Pumpkin flesh was homogenized with distilled water at the ratio of 1:5 (w/v). After centrifugation at 4000 rpm for 10 minutes, the supernatant was used for TSS analysis (Hernández-Muñoz et al., 2006).

Determination of firmness (N)

Fruit firmness was determined by digital fruit firmness tester according to Hernández-Muñoz et al. (2006). The result obtained from Digital Fruit Hardness Tester (FR- 5120, Lutron electronic enterprise Co., LTD., Taiwan) using 2mm tip was expressed in N unit.

Extract preparation

Extraction procedure followed the procedure of Nawirska-Olszańska et al. (2011). Specifically, 5 ml of sample was mixed with 25 ml of 80% methanol (Merck Chemicals Ltd., Darmstadt, Germany) (v/v) and sonicated for 30 minutes at room temperature. The extract was applied with centrifugation (UNIVERSAL 320R, Andreas Hettich GmbH & Co. KG, Germany) at 4000 rpm for 5 minutes at 4°C. The supernatant was then used for measurement of total phenolic compounds and antioxidant capacity.

Determination of total phenolic content (µg GAE g^{-1})

Total phenolic content (TPC) was determined by Folin-Ciocalteau assay as described by Singleton and Rossi (1965). The absorbance was recorded at 760 nm with a UV-visible spectrophotometer (GENESYS 10 UV-Vis, Thermo Fisher Scientific, Inc., USA). Gallic acid was used to construct a calibration curve and results were expressed as µg of gallic acid equivalents per g pumpkin (µg GAE g^{-1}).

Determination of antioxidant capacity (%)

DPPH assay was modified from a method of Lim et al. (2007). The absorbance was measured against a blank at 520 nm with a UV-Visible spectrophotometer. The percentage of free radical scavenging effect was calculated as (2):

$$\text{DPPH scavenging effect } (\%) = (1 - \frac{A}{Ao}) \times 100 \quad (2)$$

Where, A_0 is the absorbance of the control solution and A is the absorbance of the DPPH solution containing sample extract at 520nm.

Determination of total carotenoid content (µg β-carotene g^{-1})

Total carotenoid content was quantified using spectrophotometric analysis, as described by Rodriguez-Amaya (2001). Carotenoid was extracted using hexane (Merck Chemicals Ltd., Darmstadt, Germany) as the only solvent. First, 0.5 g sample was incubated in 15 minutes with 10 ml hexane, then, centrifuge the mixture at 4000 rpm for 15 minutes at 4°C. The absorbance was measured using a UV spectrometer at 450 nm. Carotenoid concentration was expressed as µg β-carotene g^{-1}.

Statistical analysis

All analyses were conducted in triplicate; the data were expressed as mean ± standard deviation. To determine differences among treatments in each experiment, one way ANOVA and the least significant difference (Fisher's LSD) were used. Statistical analysis was carried out using Minitab software package (Version 18.0, Minitab Pty Ltd., Australia) with 95% level of confidence.

RESULTS AND DISCUSSION

Effects of ethanol concentrations on the postharvest quality of fresh cut pumpkin

Overall quality index

Main indices for quality declination include translucent appearance, referred to as white blush, surface shriveling, decaying mold and off odor. High ethanol concentration was better at releasing carotenoid from cell components, thus enhance visual appeal of minimally processed fruits and vegetables (Homaida et al., 2017). However, such treatment also causes off odor as well as degradation of plasma membrane of vegetable tissue (Bai et al., 2011), leading to decreased sensory quality score. Those positive and negative effects occurred at the same time in different replicates, resulting in inconsistent scores of 40% ethanol treatment (ET 40) and 50% ethanol treatment (ET 50) (Table 1). Alcoholic off odor was noticed in 30% ethanol treated fresh cut lotus root slice (Gao et al., 2017) but was absent in 30% ethanol (ET 30) treated pumpkin. The result indicates that ET 30 was effective in preserving market appeal of fresh cut pumpkin.

Weight loss

The physiological weight loss of MPP found in this experiment is remarkably higher than the reported values from previous studies which were less than 5% (Cortez-Vega et al., 2014). The difference may source from packaging materials, PVC film used in this study possesses lower moisture retarding activity than previously used PE package (Kjeldsen, 1993). The fact that no significant difference observed in weight loss percentage ($p = 0.43$) among treatments (Table 1) suggests that ethanol soaking generally had no effects on water loss of fresh cut pumpkin. Respiration rate, which is mainly responsible for the transpiration of fruits after harvesting, was also found not altered by ethanol treatment in tomato and fresh cut banana (Ritenour et al., 1997).

Firmness

High concentration ethanol immersion caused accelerated water loss, leading to increasing penetration force at first, then plant tissue senescence decreased the force (Table 1) (Cortez-Vega et al., 2014). On the fifteenth day, firmness of ET 30 samples was significantly higher than that of ET 20 and CO samples, proving the ability of ethanol to keep membrane rigid during storage time. Ethanol treatment was reported to effectively maintain firmness by Pesis

(2005) due to its effect on endogenous plant cell wall degrading enzymes such as pectinase, cellulase, polygalacturonase which dwell in cell lysosome. With a hydrophobic tail, ethanol can pass through the phospholipid bilayer of cell membrane without causing damage and impact on cell components, including lysosome, thereby limit the activity of cell degrading enzymes (Pesis, 2005).

Total soluble solids

The sugar conversion in MPP which was induced by enzymes such as pectinase, invertase were delayed by ethanol until after day 6, later than reported in previous studies of MPP not treated with ethanol (Santos et al., 2016). This effect of ethanol treatment contradicted the conclusions of previous studies that ethanol treatments had no impact on total soluble solids (Plotto et al., 2006). However, this phenomenon can be explained by the ability of ethanol to disrupt linkages between solutes and plant matrices (Şahin & Şamlı, 2013), directly increases soluble solid content of sample.

Total phenolic content

Generally, phenolic content of MPP increased significantly after fifteen days (Fig. 2b), attributable to the activity of phenylalanine ammonia lyase (PAL) enzyme and the extractability ethanol. The growing phase before day 12 was induced by cut damage, with PAL catalyzing the production of phenolic compounds through phenylpropanoid pathway (Halpin, 2004). The activity of polyphenol oxidase (PPO) enzyme was responsible for the phenolic metabolism, causing tissue browning or off color (Garcia & Barrett, 2002) in MPP during the final period. The ethanol pretreatment of MPP suggested a positive effect on phenolic accumulation as ET 30 retained the most TPC. The compatible polarity between phenolic compounds and ethanol accounted for this difference (Şahin & Şamlı, 2013).

Table 1. Overall quality score, weight loss percentage and firmness over storage time of minimally processed pumpkin of different ethanol treatments stored at 10°C

	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Overall quality index						
ET 50	5.00 ± 0.00^{aA}	4.33 ± 0.58^{aB}	3.33 ± 0.58^{aB}	2.67 ± 0.58^{bB}	ES	ES
ET 40	5.00 ± 0.00^{aA}	4.33 ± 0.58^{aB}	3.33 ± 0.58^{aB}	3.00 ± 0.00^{bB}	2.67 ± 0.58^{aB}	ES
ET 30	5.00 ± 0.00^{aA}	4.33 ± 0.58^{aAB}	3.67 ± 0.58^{aBC}	3.67 ± 0.58^{aBC}	3.33 ± 0.58^{aC}	3.00 ± 0.00^{aC}
ET 20	5.00 ± 0.00^{aA}	4.00 ± 1.00^{aAB}	4.33 ± 0.58^{aAB}	4.00 ± 0.00^{aAB}	3.33 ± 0.58^{aBC}	2.67 ± 0.58^{abC}
CO	5.00 ± 0.00^{aA}	4.33 ± 0.58^{aAB}	4.33 ± 0.58^{aAB}	4.00 ± 0.00^{aB}	3.00 ± 0.00^{aC}	ES
Weight loss (%)						
ET 50	0.00 ± 0.00^{aB}	1.98 ± 3.42^{bB}	10.44 ± 0.63^{abA}	13.54 ± 0.96^{aA}	ES	ES
ET 40	0.00 ± 0.00^{aD}	6.20 ± 0.34^{aC}	11.18 ± 1.68^{aB}	16.11 ± 0.61^{aA}	18.72 ± 0.61^{aA}	ES
ET 30	0.00 ± 0.00^{aD}	6.65 ± 0.86^{aC}	9.53 ± 1.42^{bC}	11.35 ± 2.85^{aBC}	15.81 ± 1.16^{aB}	22.77 ± 1.96^{aA}
ET 20	0.00 ± 0.00^{aE}	6.55 ± 0.35^{aD}	11.94 ± 0.90^{aCD}	19.16 ± 0.65^{aAB}	16.97 ± 7.14^{aBC}	24.88 ± 3.79^{aA}
CO	0.00 ± 0.00^{aC}	4.68 ± 0.84^{abC}	11.48 ± 0.90^{aB}	16.63 ± 6.76^{aAB}	21.35 ± 0.57^{aA}	ES
Firmness (N)						
ET 50	22.37 ± 2.71^{aC}	27.28 ± 3.42^{bAB}	31.59 ± 3.07^{abA}	23.91 ± 1.52^{bBC}	ES	ES
ET 40	22.37 ± 2.71^{aC}	31.62 ± 0.34^{aA}	27.90 ± 1.33^{bcB}	24.96 ± 0.20^{bC}	24.34 ± 1.69^{aC}	ES
ET 30	22.37 ± 2.71^{aC}	24.86 ± 0.86^{cBC}	32.24 ± 2.60^{aA}	26.98 ± 0.41^{aB}	21.99 ± 1.62^{abC}	27.38 ± 1.23^{aB}
ET 20	22.37 ± 2.71^{aABC}	24.33 ± 0.35^{cA}	22.90 ± 0.28^{dABC}	21.69 ± 0.41^{cBC}	20.55 ± 0.59^{bC}	23.75 ± 1.72^{bAB}
CO	22.37 ± 2.71^{aB}	26.07 ± 0.84^{bcA}	26.13 ± 1.90^{cdA}	24.17 ± 0.32^{bAB}	23.65 ± 2.24^{aAB}	ES

Data was expressed as mean ± SD. Means in same column with different lowercase letters are not statically different at 5% significance. Equal capital letters in a row do not differ statically at 5% significance by Fisher's test.
ES: end of storage. ET 50: ethanol 50%; ET 40: ethanol 40%; ET 30: ethanol 30%; ET 20: ethanol 20%; CO: control samples.

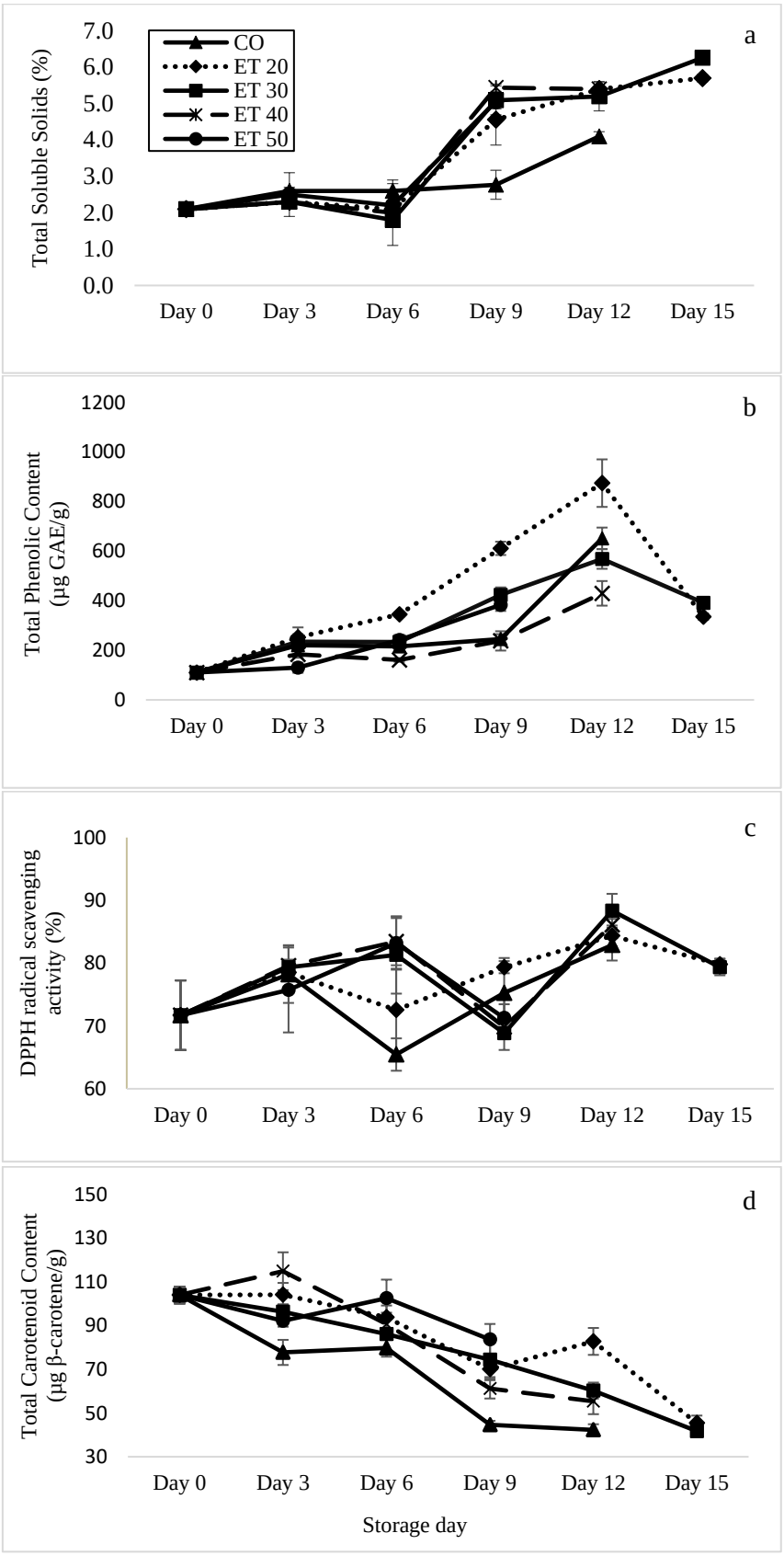

Fig. 2. The changes in (a) total soluble solids; (b) total phenolic content; (c) antioxidant capacity; (d) total carotenoid content of different ethanol treatments stored at 10°C during 15 days.

Antioxidant capacity

After 15 days, antioxidant capacity of samples from all treatments increased by 11% with no significant variation among treatment groups (Fig. 2c). The mechanism of plant coping with reactive oxygen species in response to postharvest stresses includes enzymatic and non – enzymatic detoxification (Toivonen, 2004). Enzymatic antioxidants consist of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), etc. located mostly in cell membrane, peroxisome and mitochondrion (Toivonen, 2004). However, the high dose application of ethanol on MPP could cause the rupture of plasma membrane and initiate phytotoxic effects on mitochondria (Li et al., 2018). Such effects hindered activities of antioxidant enzymes. Despite that, ethanol soaking delayed the drop of antioxidant capacity.

Carotenoid content

Despite the 54% reduction in average value, carotenoid content in treated samples were remarkably higher than in the control throughout storage time (Fig. 2d). The destructive effect of ethanol on chromoplasts (Kulczynski & Gramza-Michalowska, 2019) caused the leakage of more color pigments, thus strongly enhanced visual appeal of displayed fresh cut pumpkin. Contradicting to this advantage, ethanol treatment may cause the leakage other cell components such as sugars, phospholipids due to its polarity feature and lead to lowered carotenoid selectivity (Takahashi et al., 2006). This fact explains the phenomenon of high ethanol treatments (ET 40, ET 50) not causing high retention of carotenoid comparing to low dose treatments.

Table 2. Overall quality score, weight loss percentage, firmness over storage time of minimally processed pumpkin of different chitosan treatments stored at 10°C

	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15
Overall quality index						
CH 0.5	5.00 ± 0.00^{aA}	4.67 ± 0.58^{aA}	4.33 ± 0.58^{abA}	4.33 ± 0.58^{aA}	3.33 ± 0.58^{aB}	3.00 ± 0.00^{aB}
CH 1	5.00 ± 0.00^{aA}	4.33 ± 0.58^{abB}	4.00 ± 0.00^{abBC}	4.00 ± 0.00^{abBC}	3.67 ± 0.58^{aC}	4.00 ± 0.00^{bBC}
CH 1.5	5.00 ± 0.00^{aA}	3.33 ± 0.58^{bBC}	3.67 ± 0.58^{bB}	3.33 ± 0.58^{bBC}	2.67 ± 0.58^{aC}	ES
ND	5.00 ± 0.00^{aA}	4.67 ± 0.58^{aAB}	4.67 ± 0.58^{aAB}	4.33 ± 0.58^{aAB}	3.33 ± 1.16^{aBC}	ES
AA	5.00 ± 0.00^{aA}	3.33 ± 0.58^{bB}	2.67 ± 0.58^{cB}	ES	ES	ES
Weight loss (%)						
CH 0.5	0.00 ± 0.00^{aE}	5.66 ± 1.02^{aD}	9.76 ± 1.40^{abC}	11.29 ± 0.29^{bC}	14.14 ± 0.11^{bB}	20.41 ± 0.53^{aA}
CH 1	0.00 ± 0.00^{aF}	6.33 ± 1.32^{aE}	9.84 ± 1.30^{abD}	12.36 ± 1.52^{abC}	15.91 ± 1.68^{bB}	18.85 ± 1.04^{aA}
CH 1.5	0.00 ± 0.00^{aE}	5.29 ± 0.48^{aD}	9.11 ± 0.78^{bC}	11.69 ± 0.93^{bB}	15.17 ± 1.10^{bA}	ES
ND	0.00 ± 0.00^{aE}	6.17 ± 0.08^{aD}	10.97 ± 0.79^{aC}	13.79 ± 0.70^{aB}	19.01 ± 0.95^{aA}	ES
AA	0.00 ± 0.00^{aC}	6.46 ± 1.28^{aB}	10.42 ± 0.41^{abA}	ES	ES	ES
Firmness (N)						
CH 0.5	26.98 ± 1.02^{aA}	21.88 ± 0.44^{cCD}	24.11 ± 0.55^{aB}	21.10 ± 0.40^{aCD}	20.39 ± 0.28^{aD}	22.02 ± 1.28^{aC}
CH 1	26.98 ± 1.02^{aA}	26.33 ± 1.31^{aA}	24.99 ± 1.48^{aAB}	24.11 ± 0.55^{bB}	23.81 ± 1.15^{bB}	24.96 ± 1.25^{bAB}
CH 1.5	26.98 ± 1.02^{aAB}	23.62 ± 2.35^{bcC}	26.23 ± 2.16^{aABC}	28.03 ± 0.93^{bA}	24.73 ± 1.03^{bBC}	ES
ND	26.98 ± 1.02^{aA}	25.12 ± 1.77^{abA}	20.65 ± 1.09^{bB}	17.84 ± 0.45^{dC}	20.09 ± 0.59^{aB}	ES
AA	26.98 ± 1.02^{aA}	21.62 ± 0.20^{cB}	12.54 ± 0.69^{cC}	ES	ES	ES

Data was expressed as mean ± SD. Means in same column with different lowercase letters are not statically different at 5% significance. Equal capital letters in a row do not differ statically at 5% significance by Fisher's test.
ES: end of storage; CH 0.5: chitosan 0.5%; CH 1: chitosan 1%; CH 1.5: chitosan 1.5%; ND: no dipping, AA: acetic acid.

Effects of chitosan coating on the postharvest quality of fresh cut pumpkin

Overall quality index

Samples soaked in acetic acid were the soonest to be discarded due to watery appearance, sour odor after six days of storage. It is reported that acetic acid in contact with plant could cause rapid desiccation and facilitate food deterioration (Roos & Drusch, 2015). On the contrary, chitosan coating significantly delayed the decrease in visual sensory score (Table 2). The main trait that dropped the quality score of chitosan treatments was surface white blush, also concerned in minimally processed carrot (Bolin & Huxsoll, 1991).The removal of epidermal outer layer of mature plants initiates the formation of another protective layer, causing the milky white appearance (Bolin & Huxsoll, 1991). With the increase of chitosan concentration, the level of whitening was heightened due to polysaccharides nature of chitosan, leaving white color once dried (Arnon-Rips et al., 2019). No dipping treatment (ND) retained good color throughout storage time but sour smell was detected from day 12, threatened its commercial acceptance. In the view of market acceptability, the chitosan 1% (CH 1) treatment was the most favored.

Weight loss

By forming a semi permeable barrier on the surface of minimally processed fruits and vegetables, chitosan retards transpiration rate, slowers water loss and texture degradation (Li & Yu, 2001). However, no statistical disparity was recorded when increasing chitosan concentration (Table 2**)**. A research by Soares et al. (2018) showed increased coating incorporation in higher chitosan concentrations, but no difference in water content was observed after a 16 day storage. The fact that water vapor barrier properties of hydrophilic chitosan film decreased remarkably with time (Arnon-Rips et al., 2019) can explain these observations. Control sample treated with acetic acid (0.5% v/v) behaved similarly to ND sample suggested that the addition of acetic acid in solubilizing chitosan did not have any unusual effects on the fresh cut pumpkin.

Firmness

It can be inferred from Table 2 that firmness retention in MPP was obtained by sufficient chitosan coatings of 1% and 0.5%. The coating matrix lowered Lipoxygenases reactivity, which catalyzes the oxidation of plant plasma membrane by limiting the presence of oxygen in cell, thereby preserving the membrane integrity (Tian et al., 2004). On the other hand, the acidic environment created by acetic acid facilitated the deoxygenation of polyunsaturated fatty acids, subsequently damage cell membrane (Harwood & Moore Jr, 1989), made AA samples unmarketable from day 6. The ND samples, despite being acceptable until day 12, required so low penetration force that may imply an irreversible damage of cell outer layer by senescence (Simon, 1974). Eventually, CH 1 proved to be the best treatment to preserve MPP firmness.

Total soluble solids

The content of TSS increased significantly after 15 days of storage for all treatments with the highest value belonged to ND group (Fig. 3a). The extractability of ethanol observed in the previous experiment was overshadowed by cell wall disassembly in ND which led to the leakage of cell components, in agreement with previous studies conducted on mango, banana and strawberry (Kittur et al., 2001; Petriccione et al., 2015). The amount of soluble solids in MPP remarkably inclined during storage period, consistent with result of Santos et al. (2016).

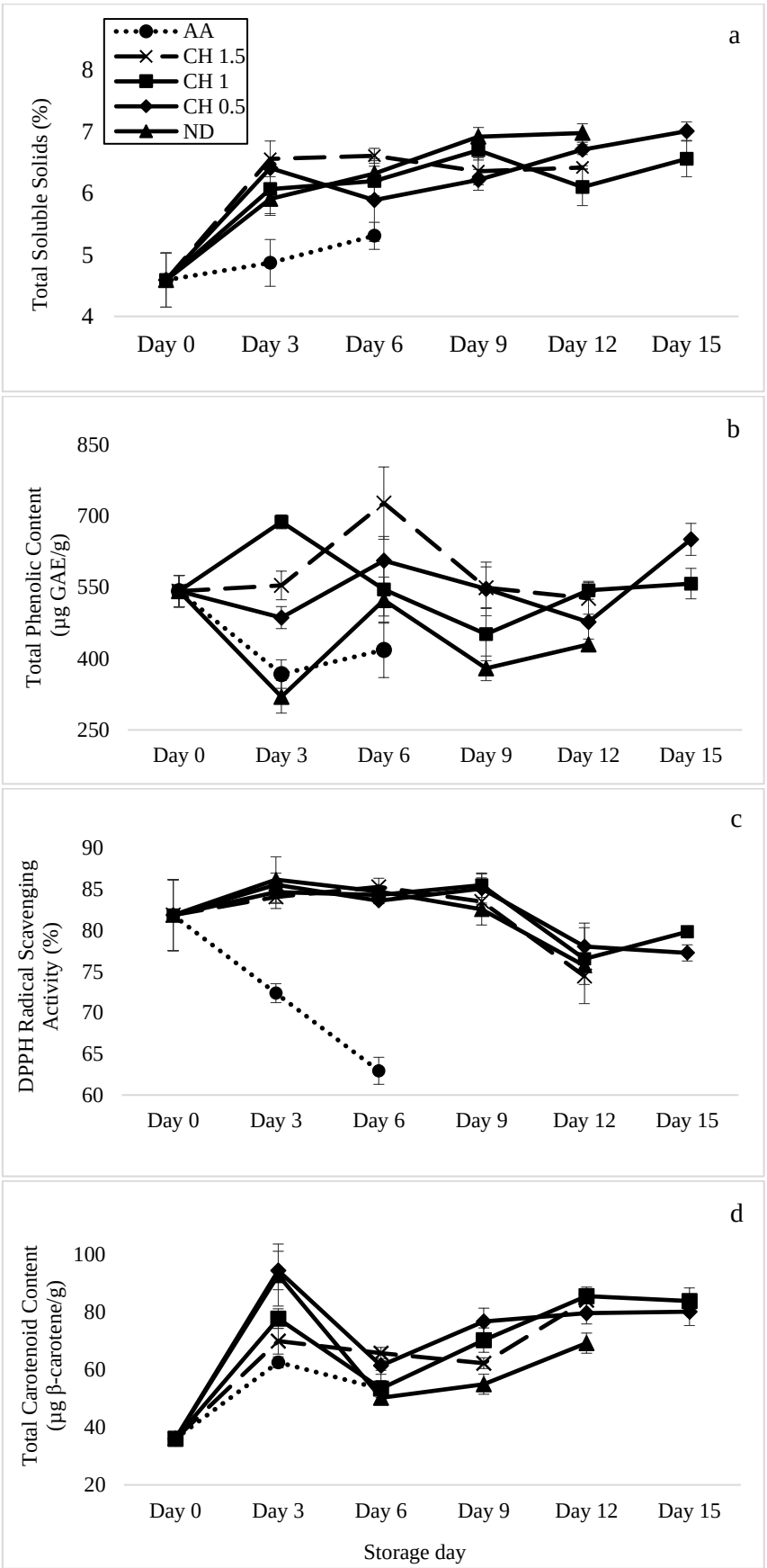

Fig.3. The changes in (a) total soluble solids; (b) total phenolic content; (c) antioxidant capacity; (d) total carotenoid content of different chitosan treatments stored at 10°C during 15 days.

Total phenolic content

The total phenolic content of chitosan – treated groups experienced various fluctuations and ended up statistically the same with initial value (Fig. 3b). High oxygen in the surrounding environment promoted phenolic compounds depletion, especially oxidation by PPO in uncoated samples (Pareek, 2016). Chitosan was proved to trigger defense response in vegetative tissue by activating PAL enzyme, the key enzyme in phenol synthesis pathway (Romanazzi et al., 2017). Such chitosan – induced effect successfully preserved phenolic content in fresh cut pumpkin cubes. The high phenolic content of MPP at the end of storage time suggested that the commodities remained in high quality, cell breakdown due to senescence during storage did not occur yet.

Antioxidant capacity

There was no significant difference in antioxidant capacity among treatment groups except for AA treatment, until the final day of storage (Fig. 3c). The samples applied with acetic acid soaking immediately decreased in the free radical scavenging ability after day 0 as the result of improved lipoxygenase (LOX) activity in acidic environment, causing cell rupture and increased membrane free radical (Engwa, 2018). The antioxidant capacity of MPP in this study was not correlated with total phenolic content suggesting that antioxidant activity of MPP depending much on antioxidant enzymes (superoxide dismutase, peroxidase, and catalase) and non – phenolic compounds (ascorbic acid, glutathione) (Mittler, 2002). The application of chitosan coating was reported to partially inhibit the activity of POD enzyme in fresh cut litchi by Zhang and Quantick (1997), hence limited the radical scavenging capacity of chitosan coated MPP.

Carotenoid content

The protection of chitosan against carotenoid depletion could be due to the selective permeability of chitosan – acetic complex. The coating layer formed by chitosan dissolved in acetic solution gives high permeability to oxygen but sufficiently low absorption and release activity to carbon dioxide (Tian et al., 2004). Such properties limited the contact of carotenoids with oxygen, a potent oxidizing agent, hence retained significantly higher carotenoid content in coated samples (Fig. 3d) (Kulczynski & Gramza-Michalowska, 2019). The result of this study is consistent with previous researches of chitosan application on MPP (Soares et al., 2018), sliced mango (Plotto et al., 2006).

CONCLUSIONS

The results of this study show a promising application of ethanol and chitosan on fresh cut produce, especially pumpkin. The treatment of 30% ethanol maintained higher overall quality index as well as preserved the most of physiochemical attributes of MPP, including firmness, total soluble solids and total phenolic content, despite its side effects in interrupting cell membrane. The coating of 1% chitosan pretreated with 30% ethanol not only acted as a physical barrier but also an interactive outer skin that helped to protect plant cell against aging and retain more nutrients.

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Conflict of Interest

The authors have no conflict of interest to report.

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Assessment and ranking of new gladiolus hybrids in Iran

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ABSTRACT

Purpose: The aims of this research were to collect reliable information about economic traits, introducing superior genotypes for production, exportation and breeding programs in new hybrids of gladiolus. **Research Method**: 48 promising genotypes with 4 parents of gladiolus evaluated on the basis of a randomized complete block design in three replications. The Research was conducted in Ornamental Plants Research Center in Mahallat from 2017-2018. **Main findings**: The results showed that the highest number of florets was observed in OPRC16 (19.0 florets) and the lowest in OPRC412, OPRC413, and OPRC99 (8.00 florets). The highest floret width of 12.00 cm was related to OPRC16 and the lowest of 7.00 cm to OPRC712. The longest spike was 71.00 cm observed in OPRC16 and the shortest was 33.00 cm observed in OPRC411. OPRC311 and OPRC61 exhibited the highest and lowest plant height of 210 and 132 cm, respectively. It was found that the flowers of the new hybrids were almost whitish in color. It was also indicated that the diversity in the traits was mostly related to the genetic factors and the environment was less influential on them. According to the North American Gladiolus Council, the length of the cut branch (spike and branch length) of all hybrids (48 hybrids) and the parents P1, P2, and P3 were categorized in the fantasy group. The hybrids that were placed in the fantasy group in terms of the spike length were more marketable. **Research limitations**: No limitations were founded. **Originality/Value**: The results revealed high diversity among the hybrids and parents in traits. Therefore, hybrids that are superior (fantasy group) in these commercial traits can be introduced as new cultivars.

INTRODUCTION

Gladiolus (*Gladiolus grandiflorus*) is a cross-pollinating and diploid (2n = 2x = 30) plant species from the family *Iridaceae*, the subfamily Ixioideae, the tribe Ixieae, and the sub-tribe Gladiolines (Ranjan et al., 2010). Its other names are sword silly and corn flag (Poon et al., 2012), which is also known as the queen of bulbous flowers (Randhawa & Mukhopadhyay, 1985). The name sword silly has its roots in the sword-shaped leaves of gladiolus. The genus of Gladiolus contains over 276 species throughout the world, but they are mostly native to the western, southern, and eastern parts of Africa although 12 species have been originated from the Mediterranean regions (Cohat, 1993; Rina & Hiroshi, 2016). The acreage of gladiolus cultivation in Iran was reportedly 350 ha in 2017-2018 (Ministry of Agriculture Jihad, 2017). Gladiolus is ranked the eighth among cut flowers and the first among bulbous flowers in the global trade (Pragya et al., 2010). The leading gladiolus producing countries are the US, the Netherlands, France, Portugal, Italy, Belgium, Brazil, Australia, and India (Pragya et al., 2010). According to Misra and Singh (1989), over 30,000 cultivars of gladiolus are cultivated and every year new cultivars are produced (Singh, 2006). The demand for high-quality and commercially valuable cultivars is also on the rise. Several studies have been conducted on gladiolus: phenology (Schwab et al., 2015); estimation of genetic variability (Rashmi & Kumar, 2014); heritability and genetic advance (Patra & Mohanty, 2014); genotypic and phenotypic variability (Pattanaik et al., 2015, Bhujbal et al., 2013); hybridization (Ohri & Khoshoo, 1983a; 1983b; Suresh, 2015; Azimi, 2018; Hossain et al., 2012). Intervarietal hybridization is another common way for transferring desirable attributes between different cultivars and producing progenies with new characteristics (Azimi et al., 2018; Yang et al., 2015; Zamani et al., 2010).

Most cultivars of gladiolus, which are used as cut flowers, have come into existence by inter-species crosses. Due to the complexity of breeding and multiple inter-species hybridizations, it is not possible to classify the cultivars based on their origins, so they are classified by color, color intensity, and flower size (Wilfret, 1980). Several years of hybridization and selection of superior populations of gladiolus have yielded in the production of new types and the replacement of old wild species with diverse new cultivars. These cultivars have drawn attention owing to their diversity in color, flowering time, and disease resistance (Willery, 2010). Azimi (2018) performed genetic, phenotypic, and heredity assessments on new gladiolus hybrids and classified them in different categories.

Many associations of bulbous flowers have been established to expand ornamental bulbs. These associations publish many periodicals, annals, CDs, and books about bulbous plants. They also have other functions including the identification and classification of native ornamental bulbs, breeding, and production of new cultivars (Fairchild, 1979; Howie, 1984; Koeing & Crowly, 1972; Benschop et al., 2010). They also present new cultivars (www.bulbosociety.org). The North American Gladiolus Council works on gladiolus (http://www.gladworld.org). When it comes to the production, storage, and cultivation of ornamental plants, the consumer demand fuels the continuous development of new genotypes (Teixeira da Silva & Kulus, 2014).

Therefore, the main goal of the production and cultivation of ornamental plants is introducing new cultivars with novel characteristics, such as resistance to diseases, new colors, and changes in the structure and morphology of flowers (Tanaka, 2006). Breeding ornamental plants to achieve diversity in production is imperative. Since a major aim of the present study is introducing new genotypes of gladiolus, the results can contribute to producing gladiolus cut flowers.

MATERIALS AND METHODS

The present study assessed the main flower-related traits of 48 promising genotypes (Fig. 1) with the OPRC codes (All promising genotypes were named based on the name of the institute, i.e. 'OPRCn' where 'n' refers to the numerical identifier for the genotype); as well as four commercial parents of gladiolus including 'Amsterdam' (P1), 'White Prosperity' (P2), 'Advance Red' (P3), and 'Rose Supreme' (P4) in the Ornamental Plants Research Center in 2017-2018 on the basis of a randomized complete block design in three replications (10 samples were recorded in each replication). In a single 110-day growth cycle, all agronomic and horticultural operations required for the optimal growth were performed on the genotypes. The 52 genotypes were planted in the research greenhouse of Ornamental Plants Research Center (Latitude: 33° 53' N., Longitude: 50° 29' E., altitude 1732 m, average temperature 23.4 °C, relative humidity 57.1%).

The corm size (a perimeter of 6-8 cm) and plant depth (5.00 cm) were picked according to what was common in the region. The corms were planted by the plot method at a density of 40 plants m^{-2}. The recorded traits including cut branch length (from crown from the soil surface to the tip of the branch in cm), number of florets per branch, spike length (from the first flower to the tip of the flowering branch in cm), and floret size (the size of the first flower of the spike in cm). The traits of floret size, floret number, spike length, and cut branch length were ranked as per the instruction of the North American Gladiolus Council (http://www.gladworld.org). According to this council, the number of florets, the cut branch length, and the spike length are classified into fantasy, specific, standard, and multipurpose and the size of floret is classified into miniature, small, medium, large, and very large.

Statistical analyses including the calculation of descriptive statistics, analysis of variance (ANOVA), and means comparison by the LSD test at the 5% level ($P<0.05$) were all performed in the SAS 9.1 software package.

RESULTS AND DISCUSSION

Cut flower height

According to the results of means comparison (Table 1), among the parents, the highest cut flower height was observed in P1 (146 cm) and the lowest was observed in P4 (82.2 cm). Among the promising genotypes, the highest was for OPRC311 (210 cm), which was the result of P1 × P4 crossing. The lowest was observed in OPRC61 (P2 × P3) whose cut flower height was 132 cm. Most promising genotypes outperformed their parents in this trait. Since all genotypes and parents were in the same conditions. Cut flower height is an important trait. Promising genotypes, which have higher cut flower height than their parents, can be highly successful as commercial cultivars in flower markets. In this respect, Moradi (2009) who studied gladiolus reported that cv. 'Oscar' had the highest height and cv. 'Sefidmasti' had the lowest among all studied cultivars, showing significant differences with other cultivars. The increase in corm size in gladiolus increases the height and length of flowering spikes. Similar results have been reported by Bijimol and Singh (2001), and Moradi-Ashour (2013) for tuberose. Roy and Sharma (2000) reported a high diversity in height among gladiolus cultivars so that 'Vedio' showed a height of 114 cm and 'White Prosperity' showed a height of 152 cm. According to Rai et al. (2000), the height was 128.5, 123.5, 123.3, 121.4, and 135.8 cm for 'White Prosperity', 'White Goddess', 'Red Beauty', 'White Friendship', and

'First Lady', respectively. Sidhu and Arora (2000) revealed that among different cultivars of gladiolus, 'White Prosperity' had the highest height of 135.8 cm in summer.

Floret number

The results of means comparison (Table 1) revealed that the highest and lowest number of florets among the parents were related to P1 (16.5 florets) and P4 (9.0 florets). Among the promising genotypes, OPRC16 had the highest number of florets (19.0 florets) and OPRC412, OPRC413, and OPRC99 had the lowest one (8.0 florets). In their study on different cultivars of gladiolus, Rai et al. (2000) found that the highest and lowest number of florets were related to 'White Prosperity' and 'Green Wood Pecker' (17 and 12 florets), respectively. Sanjai and Singh (2000), also, reported that 'White Prosperity' had the highest number of florets. This may be associated with the nutrient reserves of big corms and their availability that help early growth and development of the plant and finally influence the number of florets on inflorescences. There are similar reports about the reduction of florets on inflorescences with the decrease in corm size (Uddin Farid et al., 2002; Bhat et al., 2009; Memon et al., 2009; Kareem et al., 2013). The production of spikes that have more florets is related to lower competition of plants over water, minerals, nutrients, and radiation (Mojiri & Arzani, 2003). Padaganur et al. (2005), Khalaj and Edrisi (2013), and Moradi-Ashour (2013) reported similar results for tuberose.

Floret size

According to the results of means comparison (Table 1), floret size in the parents was in the range of 8.39-10.52 cm, the highest being for P4 and the lowest for P2. Among the promising genotypes, OPRC16 had the highest floret size of 12.00 cm and OPRC712 had the lowest one of 7.00 cm. In a similar work, Moradi (2009) showed that 'Rose Supreme' had the highest mean floret diameter and 'White Prosperity' had the lowest one. Also, Sindhu and Verma (1995) found that the largest floret was 11.7 cm in 'Sancera' and the smallest was 6 cm in 'Arc'. Likewise, Sidhu and Arora (2000) observed the biggest florets of 8.92 cm in 'Rose Supreme' and Mishra et al. (1987) reported that among 12 gladiolus cultivars, 'Slamone Queen' had the biggest florets. Floret size is a major economic trait. Promising genotypes whose floretsare larger than their parents' florets can be viably commercialized in flower markets. Obviously, introducing commercial cultivars can enhance diversity in flower markets and help its prosperity. In some flowers like cyclamen, the focus is on flower characteristics so that cyclamens have been developed in different flower shapes and sizes (Anderson, 2007), but no such work has been done on gladiolus.

Spike length

The highest and lowest spike length among the parents was observed in P1 (52.25 cm) and P2 (42.47 cm). Among the promising genotypes, OPRC16 had the longest spikes with a length of 71.00 cm and OPRC411 had the lowest ones with a length of 33.00 cm (Table 1). Spike length is an important trait. Promising genotypes that grow spikes longer than their parents are very likely to succeed in flower markets. In this respect, Sharma and Goupta (2003) found that the increase in planting distance increased the number of florets on the flowering spike. In a study on the planting type and corm spacing of gladiolus, no significant impact was reported on the diameter of new corms, the weight, number and diameter of cormlets, the ratio of floret number to flowering stalk length, leaf number, length and width, and stalk diameter (Daneshvar & Heidari, 2009).

Table 1. Mean (±SD) and status of the crossing of 48 promising genotypes and parents of gladiolus in terms of flower-related traits

Crossing	Code	Cut flower height (cm)	Floret number	Floret size (cm)	Spike length (cm)
P1×P2	OPRC11	161.0±8.1	13.0±0.650	8.00±0.40	56.00±2.80
P1×P2	OPRC15	154.5±5.5	15.2±0.541	8.59±0.31	45.45±1.62
P1×P2	OPRC16	193.0±9.7	19.0±0.950	12.00±0.60	71.00±3.55
P1×P3	OPRC21	159.0±8.0	11.0±1.803	9.00±0.99	44.00±10.56
P1×P3	OPRC24	174.0±8.7	11.7±0.833	8.67±0.51	53.33±8.59
P1×P4	OPRC31	172.0±8.6	13.7±0.907	8.00±0.40	54.67±2.94
P1×P4	OPRC35	139.4±5.0	11.1±3.401	8.92±0.48	41.39±3.41
P1×P4	OPRC39	175.0±8.8	10.0±1.790	9.17±0.75	47.67±8.36
P1×P4	OPRC310	172.0±8.6	10.0±0.500	8.45±0.42	45.00±2.25
P1×P4	OPRC311	210.0±10.5	14.00±0.70	9.50±0.48	56.00±2.80
P1×P4	OPRC312	156.0±7.8	11.00±0.55	8.50±0.43	47.00±2.35
P1-self	OPRC45	150.5±5.4	13.10±1.68	8.92±0.48	44.10±1.70
P1-self	OPRC46	157.0±7.9	13.00±5.22	10.33±1.52	51.67±16.87
P1-self	OPRC48	145.0±7.3	12.00±1.82	8.67±0.51	48.00±12.30
P1-self	OPRC49	176.0±8.8	12.00±0.60	8.17±0.49	47.67±8.36
P1-self	OPRC411	149.0±7.5	11.00±0.55	7.50±0.38	33.00±1.65
P1-self	OPRC412	152.0±7.6	8.00±0.40	8.00±0.40	39.00±1.95
P1-self	OPRC413	130.0±6.5	8.00±0.40	9.00±0.45	35.00±1.75
P1-self	OPRC414	174.0±8.7	11.00±0.55	8.50±0.43	40.00±2.00
P1-self	OPRC415	169.0±8.5	12.00±0.60	8.00±0.40	38.00±1.90
P2×P1	OPRC55	133.3±4.8	12.40±2.25	8.52±0.29	38.01±6.17
P2×P1	OPRC56	158.0±7.9	13.70±4.65	9.33±2.34	49.00±19.15
P2×P1	OPRC57	135.0±6.8	12.70±2.94	8.33±1.49	43.67±13.40
P2×P3	OPRC61	132.0±6.6	12.30±0.83	7.33±0.68	46.00±8.90
P2×P3	OPRC64	140.0±7.0	13.00±1.87	8.00±0.94	38.00±5.48
P3×P1	OPRC72	162.0±8.1	10.70±1.26	9.33±1.50	48.33±3.29
P3×P1	OPRC73	165.0±8.3	13.00±0.65	9.33±0.73	55.67±3.95
P3×P1	OPRC75	153.5±5.5	13.80±1.14	10.28±1.59	50.86±5.41
P3×P1	OPRC76	148.0±7.4	15.00±3.53	10.00±1.79	57.00±12.38
P3×P1	OPRC78	173.0±8.7	14.00±0.70	9.00±0.45	54.67±6.84
P3×P1	OPRC711	156.0±7.8	11.00±0.55	8.00±0.40	41.00±2.05
P3×P1	OPRC712	152.0±7.6	9.00±0.45	7.00±0.35	37.00±1.85
P3×P1	OPRC714	193.0±9.7	14.00±0.70	9.00±0.45	58.00±2.90
P3×P2	OPRC81	194.0±9.7	15.70±2.46	8.00±0.40	69.33±12.16
P3×P2	OPRC83	165.0±8.3	13.00±0.65	8.00±1.77	55.00±4.36
P3×P2	OPRC84	163.0±8.2	11.70±0.83	9.00±0.45	46.67±3.33
P3×P2	OPRC87	162.0±8.1	13.30±2.39	9.33±0.73	43.67±13.40
P3×P2	OPRC89	158.0±7.9	11.30±0.80	8.50±0.43	47.00±8.91
P3-self	OPRC93	154.0±7.7	13.00±0.65	9.00±0.96	51.00±7.32
P3-self	OPRC98	162.0±8.1	13.30±0.87	9.00±0.45	54.00±7.37
P3-self	OPRC99	151.0±7.6	8.00±3.48	8.17±0.49	35.00±19.09
P3-self	OPRC911	143.0±7.2	11.00±0.55	7.50±0.38	36.00±1.80
P3-self	OPRC912	151.0±7.6	9.00±0.45	8.00±0.40	42.00±2.10
P3-self	OPRC913	178.0±8.9	11.00±0.55	8.00±0.40	48.00±2.40
P3-self	OPRC914	188.0±9.4	12.00±0.60	7.50±0.38	51.00±2.55
P3×P4	OPRC101	132.0±6.6	12.30±0.83	8.00±0.40	42.00±12.25
P3×P4	OPRC107	146.0±7.3	12.70±2.94	8.67±1.22	44.33±12.84
P3×P4	OPRC108	142.0±7.1	12.70±1.30	8.00±0.94	49.33±11.18
Parent	P1	143.2±4.4	16.50±0.50	9.43±0.12	52.25±2.38
Parent	P2	128.0±2.9	12.10±0.51	8.39±0.10	42.47±3.71
Parent	P3	130.2±1.0	13.10±0.42	8.44±0.21	51.47±0.24
Parent	P4	82.2±7.0	9.00±1.00	10.52±1.10	46.51±3.09
$LSD_{0.05}$	-	4.61	2.40	1.15	10.71

Ranking of promising genotypes and parents based on the North American gladiolus council

Floret size

Two groups of large-flower and small-flower plants are used for commercial purposes (Halevy, 1998). The small-flower group, known as Orchidola gladiolus, is specifically used to produce cut flowers because of their shorter production time and adaptability with winter conditions (Gonzalez et al., 1998). According to the most reliable association of gladiolus in North America, floret size is classified into miniature, small, medium, large, and very large (Table 2). The results showed that promising genotypes and parents were not in the miniature and large groups. Only OPRC16 was placed in the large group, and other promising genotypes and parents were classified in the small and medium-sized groups (Table 2). Presently, commercial cultivars of gladiolus with diverse floret sizes are in a better position in flower markets.

Cut branch length

According to the North American Gladiolus Council, the cut branch length (spike and stalk length) is classified into fantasy, specific, standard, and multipurpose (Table 3). The results showed that all promising genotypes and the parents P1, P2, and P3 were placed in the fantasy group. The parent P4 was placed in the standard group (Table 3). Flowering stalk height is an important trait underpinning the marketability of cut flowers. It is also a crucial trait from the breeding perspective because it has applications when grading is made by floret size. Flowering branch length and diameter are invaluable characteristics of gladiolus appearance that, in addition to influencing physiological properties, improve flower resistance to farm-to-market transport. As well, the difference in plant height relates to their competition for radiation, moisture, space, nutrient, and aeration (Karavadia & Dhaduk, 2002).

Table 2. Ranking of promising genotypes and parents based on the floret size

Sr. No.	Ranking	Floret size (cm)	Genotypes
1	Miniature	<6.3	-
2	Small	6.3-8.9	11, 15, 24, 310, 312, 48, 49, 411, 412, 414, 415, 55, 57, 61, 64, 711, 712, 81, 83, 89, 99, 911, 912, 913, 914, 101, 107, 108, P3, P2
3	Medium	8.9-11.4	21, 31, 35, 39, 311, 54, 46, 413, 56, 72, 73, 75, 76, 78, 714, 84, 87, 93, 98, P4, P1
4	Large	11.4-14	16
5	Very large	>14	-

Table 3. Ranking of promising genotypes and parents based on the cut branch length

Sr. No.	Ranking	Floret size (cm)	Genotypes
1	Fantasy	>107	All promising genotypes and the parents P1, P2, and P3
2	Specific	107-97	-
3	Standard	96-81	P4
4	Multipurpose	<80	-

Spike length

The North American Gladiolus Council divides gladiolus in terms of spike length into fantasy, specific, standard, and multipurpose (Table 4). The results revealed that most promising genotypes, as well as P1, P2, and P3, were placed in the group with the spike length of >45 cm. The parent P4 and some promising genotypes were placed in the specific group (Table 4). According to the results, the promising genotypes that are in the fantasy group are in a better place for marketability.

Floret number

Floret number is divided by the North American Gladiolus Council into fantasy, specific, standard, and multipurpose (Table 5). The results indicated that most promising genotypes and parents were in the specific group. The fantasy group contained OPRC16 and P1. The genotypes 412, 413, 712, 99, 912, and P4 were categorized in the multipurpose group. The promising genotypes 21, 24, 35, 39, 310, 312, 411, 414, 72, 711, 84, 89, 911, 913, and 914 were categorized in the standard group (Table 5). Presently, the commercial cultivars of gladiolus with more florets are more preferred in flower markets (Fig. 1).

Table 4. Ranking of promising genotypes and parents based on the spike length

Sr. No.	Ranking	Spike length (cm)	Genotypes
1	Fantasy	>45	11, 15, 16, 24, 31, 39, 310, 311, 312, 46, 48, 49, 56, 61, 72, 73, 75, 76, 78, 714, 81, 83, 84, 89, 93, 98, 913, 914, 108, P4, P3, P1
2	Specific	32-45	21, 35, 45, 411, 412, 413, 414, 415, 55, 57, 64, 711, 712, 87, 99, 911, 912, 101, 107, p2
3	Standard	20-32	-
4	Multipurpose	<20	-

Oprc712 Oprc55 Oprc46 Oprc11 Oprc311

Fig.1. Promising genotypes of gladiolus.

Table 5. Ranking of promising genotypes and parents based on the floret number

Sr. No.	Ranking	Spike length (cm)	Genotypes
1	Fantasy	>16	16, P1
2	Specific	15	11, 15, 31, 311, 45, 46, 48, 49, 415, 55, 56, 57, 61, 64, 73, 75, 76, 78, 714, 81, 83, 87, 93, 94, 101, 107, 108, P3, P2
3	Standard	12	21, 24, 35, 39, 310, 312, 411, 414, 72, 711, 84, 89, 911, 913, 914
4	Multipurpose	10	412, 413, 712, 99, 912, P4

CONCLUSION

The traits of floret number and size, spike length, and cut stalk length are of crucial importance in cut flowers. Collecting reliable information about flowers is very important for the use of genotypes in production, exportation, and breeding programs. The results revealed that the promising genotype OPRC16 outperformed the other genotypes and their own parents in most flower-related traits.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Effects of different storage temperature conditions on ripening quality and shelf life of mango (*Mangifera indica*) fruits in Ghana

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ABSTRACT

Purpose: Physiologically matured fruits of Haden, Kent, Palmer, and Keitt mango varieties were used for experiment. The fruits were held at ambient (29-31 ^{o}C) and simulated-transit temperature (10-13 ^{o}C) conditions. Quality which includes fruit firmness, weight, and spoilage, were assessed and used to determine shelf life of stored fruits. **Research Method**: A Completely Randomized Design with four replications was used. For each of the four varieties, five mango trees were sampled at random in each of the four replications of a mango plantation when fruits were physiologically matured. **Findings**: For the ambient and simulated-transit temperature conditions, Kent (4.09 days and 3.85 days, respectively) and Keitt (4.08 days and 3.92 days, respectively) fruits stored longer. Haden fruits ripened significantly earlier (9.50 days and 3.5 days, respectively) than Keitt fruits (11.01 days and 5 days, respectively). Ripening time was statistically not different among Haden, Kent, and Palmer fruits. Softness, colour, and decay were limiting quality factors for all mango fruits stored at both conditions. Higher shriveling rates were observed in Haden and Palmer fruits with a slight preponderance of the former, for both conditions. Average weight loss was highest (6.50 % and 3.31 %, respectively) for Haden and lowest (4.09 % and 2.34 %, respectively) for Keitt, but generally lower in fruits stored under transit conditions. **Research limitations**: No limitations to report. **Originality/Value**: A single quality attribute cannot be used to express loss of quality of mango fruit over the normal physiological range of mango fruit growth and development.

INTRODUCTION

The Ghanaian mango industry relies heavily on the Florida cultivars (Haden, Kent, Palmer, and Keitt) for fresh and processed mangoes traded locally and for export (FAGE/USAID/TIPCEE, 2007; Abu et al., 2011). There are several other cultivars that are also recognized commercially but are mainly consumed locally. Haden, Kent, Palmer, and Keitt mango cultivars all perform well under the climatic conditions in Ghana. Ghana's mangoes are now largely exported by both air and sea freights. Though air-freight is more expensive, it gets fruits to their destination markets faster and therefore more likely to be in good quality. However, to be competitive on the international market considering the high air freight charges (between $ 0.70/kg and $ 1.15/kg; Takyi, 2007), Ghana must consider using sea freight for mango export and thus benefit from cheaper freight rates and the opportunities to move bigger volumes to the market.

Domestic and international trade of fresh mango fruit has also been limited by its highly perishable nature and its susceptibility to post-harvest diseases, extremes of temperature, and physical injury (Litz, 2003). The fruit requires a short period to ripen and this short period seriously limits its commercialization in distant markets (Iqbal, 2001). Mangoes must be consumed soon after harvest. In many producing countries, there is an annual glut followed by scarcity. For all of these reasons, mangoes are still considered as luxurious, expensive items in the markets in many industrialised countries (Litz, 2003).

Most of the postharvest technologies for mango fruits have been developed for controlling diseases and insects and for protection against injury during packaging and transport (Tridjaja & Mahendra, 2002). Kitigawa (1994) earlier on reported that mango fruits have poor storage qualities and storage methods have been characterized by variable results and the occurrence of physiological disorders, but technologies for longer term storage such as controlled or modified atmospheres have not been applied successfully to the fruit. Fruit stored under modified atmospheres often show undesirable characteristics, i.e. poor color and eating quality, and the presence of undesirable flavors. But some studies have shown clear evidence that mango ripening can be delayed satisfactorily (Schouten, 2005). Clearly, other alternatives are, therefore, required to delay ripening and subsequent softening.

Temperature is the most important environmental factor that influences the deterioration rate of harvested fruits and vegetables. According to Kader and Mitcham (2008), for each increase of 10 ^{o}C above optimum, the rate of deterioration increases by two- to three-fold and that exposure to undesirable temperatures results in many physiological disorders. Temperature also influences how ethylene, reduced oxygen, and elevated carbon dioxide as well as spore germination and growth rate of pathogens affect the commodity (Kader & Mitcham, 2008).

All the same, any precautionary measures (highly recommended) toward quality maintenance may be inadequate and even fruitless if the quality factors (in relation to storage life and ripening quality of these fruits at ambient and transit temperatures) that limit mango marketability are unknown. In this regard, the prerequisite of remedial development is to assess the ripening condition and trend of mango fruit storage to enhance knowledge on quality factors that limit mango marketability. Thus, the objective of the study was to determine the sensitivity of Haden, Kent, Palmer, and Keitt mango fruits to ambient and transit temperature storage conditions, and consequently, the development of physiological disorders.

MATERIALS AND METHODS

Experimental site and plant materials

Laboratory studies were conducted to determine the sensitivity of the fruits of four mango varieties (Haden, Kent, Palmer, and Keitt) to ambient and transit temperature storage conditions and the consequent development of physiological disorders involved in quality deterioration. The study was carried out at the bio-chemistry laboratory of Food Research Institute, Legon, Accra. The experimental period consisted of two major (April to July) and two minor (December to February) seasons. The experiment was conducted following completely randomized design with four replications.

Sampling

For each of the four varieties, five mango trees were sampled at random in each of the four replications of a mango plantation (Prudent Export and Import Company Ltd mango plantation at Ayenya, situated in the Somanya-Dodowa mango production zone of the Dangme West District of Greater Accra Region) when fruits were physiologically matured at 112, 126, 133, and 140 days after fruit-set for Haden, Kent, Palmer, and Keitt, respectively (Abu, 2010).

Shelf life at transit temperature conditions

For shelf life tests under transit temperature (10-13 ^{o}C) conditions, corrugated fiber board carton packaged fruits (9 cartons; one carton each of counts 4 to 12) of each of the four varieties were first kept in different climatic chambers for up to 21 days in order to simulate the manner of packaging and the average period that fresh mango fruits usually stay in transit during shipment, i.e, from Ghana to Europe (Abu, 2010). After the simulation period fruits were transferred to the ripening chamber (similar to ambient conditions) for ripening to be effected.

Shelf life at ambient temperature conditions

For shelf life tests under ambient temperature (29-31 ^{o}C) conditions, fruits were randomly picked from each variety and put into open plastic containers on a laboratory bench in different rooms of the laboratory. A sample for each variety consisted of 9 cartons; one carton each of counts 4 to 12 to justify comparison under the two different temperature regimes. Fruits were examined and rotated daily and those found to be damaged after each day's examination were discarded. The number of days to the appearance of any sign of damage on a fruit was recorded as the shelf life and the affected fruit (s) discarded from the lot, up to the last fruit. These was averaged out and recorded as shelf life of the fruits in the particular variety (Abu, 2010).

Ripening, weight loss, and spoilage test

Days taken to ripen (eat-ripeness stage) at ambient condition (29-31 ^{o}C, 90-95% RH), weight loss (%) during ripening, and days taken to spoil after ripening i.e. at sales/fresh market conditions (20-22 ^{o}C, 85-90% RH) regarding fruits due for assessment under ambient conditions were determined. These same parameters were determined for fruits due for assessment under transit conditions, but only after the fruits were simulated for transit temperature (10-13 ^{o}C) conditions for twenty-one (21) days (Abu, 2010).

Days taken to ripen from physiological maturity:

Starch Test: Five fruits per tree each of the four varieties sampled in each replication were randomly picked for starch determination using the iodine test which could serve as a useful indicator of maturity/ripening (Dadzie & Orchard, 1997), on fortnightly basis.

The following procedure was used;

a) Samples were cut transversely from the midpoint of the fruit approximately 2-3 cm thick.
b) One side of the cut surface of the pulp was stained for 5 seconds in potassium iodide/iodine solution.
c) The starch present in the pulp (where possible) reacted with iodine causing a dark blue color change. Where the starch in the pulp changed to sugar (during maturation/ripening), no iodine reaction occurred and the area stained a pale tan color.
d) Assessment of the starch pattern of each fruit was done by comparing the stained cut surface with a mango maturation/ripening chart (Dadzie & Orchard, 1997).

Weight loss (%) when ripe:

This was computed considering difference in weight before and after ripening.

Days taken to spoil/damage factors after ripening:

This was computed as outlined and defined under "Visual determination of fruit quality", below.

Fruit firmness

A computerized texture analyzer (TA-XT2) was used to determine fruit firmness (the plateau of the force which occurs after the bio-yield point which is an indication of the underlying flesh firmness of the fruit) and 'bio-yield point' (when the probe punctures through the skin of the mango fruit causing irreversible damage) of the mangoes by penetration. Fruit firmness and 'bio-yield point' are indicators of mango fruit skin and the underlying flesh firmness of the fruit, respectively.

Here, twenty sound freshly harvested physiologically matured fruits of each of the four varieties were used for the tests. Two readings were taken per fruit and averaged for recording using the computerized texture analyzer. Once tests have been performed, values of parameters for sample analysis were automatically obtained by a MACRO, program of the software of the Texture Analyzer (Rosenthal, 2010). The average measurement of the twenty fruits represented the value of the particular fruit quality attribute parameter of the variety assessed at a time. Tests were carried out at different times for the different varieties depending upon their physiological maturity stages.

Visual determination of fruit quality

A typical picking and shipping schedule for mango fruits consigned by sea and by air to the EU from Ghana (Abu, 2010), was also consulted in relation to sea and air freight. Visual determinations were made in relation to the development of physiological disorders such as fruit softening, changes in fruit colour, development of decay, and shriveling, by regular visual observation/inspection. This was done as follows:

Sound freshly harvested physiologically mature mango fruits of the four varieties were stored at both ambient and transit temperature conditions for the shelf life assessment. Tests were carried out at different times for the different varieties depending upon their physiological maturity stages.

For storage tests under transit temperature (10 ^{o}C) conditions, corrugated fibre board carton packaged fruits (nine cartons, one carton each of counts 4 to 12) of each of the four varieties were kept in different climatic chambers for up to 21 days in order to simulate the manner of packaging and the average period that fresh mango fruit usually stays in transit during shipment.

For storage tests under ambient temperature (29-31 ^{o}C), fruits were randomly picked from each treatment (variety) and put into open plastic containers on a laboratory bench. A sample for each variety consisted of a mixture of three fruits each of count 4, 5, 6, 7, 8, 9, 10, 11, and 12 to justify comparison of the two different temperature regimes. Fruits were examined and rotated daily and those found to be damaged after each day's examination were discarded. The number of days each fruit took to show any sign of damage was recorded as the shelf life and the affected fruit(s) discarded from the lot up to the last fruit. These was averaged out and recorded as shelf life of the fruits in the particular treatment (variety).

Definition of Damage: To determine what damage was, fruits were either defined as slightly damaged, undesirably colored, or sound.

The slightly damaged were further grouped into three, comprising: slightly physiologically damaged (wrinkles, shrinkage, and softening due to wilting and ripening); slightly pathologically damaged (sunken spots, rotting, mycelia growth, and disease symptoms due to bacterial and fungal infections); and slightly mechanically damaged (cuts, punctures, scuffs, and abrasions due to open wounds and, bruises due to impacts, compressions, and vibrations).

Undesirably colored fruits were those with poor/abnormal color.

Sound fruits were those free from any damages.

Statistical analysis

All data were analyzed using the Analysis of Variance (ANOVA) technique (Snedecor & Cochran, 1980) with the GENSTAT statistical program. Least Significant Difference (LSD) at 5% probability was used to determine treatment differences among varieties. Separate analyses were carried out with the data for each of the seasonal trials. The errors for these ANOVAS were tested for homogeneity of variances (Snedecor & Cochran, 1980) and found to be statistically not different at $p>0.05$, so the results for the seasonal experiments were pooled for analysis.

RESULTS

Ripening quality at ambient and transit temperatures

Fruit of Haden, Kent, Palmer, and Keitt ripened (eat-ripeness stage) in 9.5, 10.5, 10.0, and 11.0 days, respectively, under ambient conditions (29 to 31 ^{o}C and 90-95% RH) after removal of field heat i.e. by controlling the fruit temperature at 20-25 ^{o}C before the initiation of the ripening process. Days to ripening was not significantly different among Haden, Kent, and Palmer fruits but was statistically different ($p<0.05$) between Keitt and Haden (Table 1).

After exposure to transit conditions for 21 days, and then ripened under ambient conditions (29 to 31 ^{o}C and 90-95% RH), fruits of Haden, Kent, Palmer, and Keitt ripened (eat-ripeness stage) in 3.5, 4.5, 4.0, and 5.0 days, respectively; indicating the same trend in ripening time as it were for ambient conditions, but much faster.

Shelf life

Generally, under ambient conditions, there was a slow development of unpleasant fruit characteristics (fruit softening, changes in fruit color, development of decay, and shriveling) as compared to transit conditions. Under ambient and transit conditions, spoilage was observed much earlier in Haden (3.15 days for ambient and 2.91 days for transit) and Palmer (3.16 days for ambient and 2.87 days for transit) fruits than in Kent (4.09 days for ambient and 3.85 days for transit) and Keitt (4.08 days for ambient and 3.92 days for transit) fruits as in Table 1. This resulted to limiting the shelf lives of the fruits to 3.15, 4.09, 3.16, and 4.08

days for Haden, Kent, Palmer, and Keitt respectively (Table 1; days to spoilage) under ambient conditions; and to 2.91, 3.85, 2.87, and 3.92 days for Haden, Kent, Palmer, and Keitt respectively (Table 1; days to spoilage) under transit conditions.

Signs of decay in Haden and Palmer fruits became visible after 3 days at 20-22 ^{o}C, and for Kent and Keitt varieties, after 4 days at 20-22 ^{o}C (Table 1). Fruit pre-exposed to transit conditions (10-13 ^{o}C; 85 to 90% RH), ripened (29-31 ^{o}C; 90 to 95% RH), and stored at sales/fresh market conditions (20-22 ^{o}C and 85-90% RH) did not suffer any chilling injury.

Table 1. Days to ripen, weight loss, days to spoilage, and shelf life (days) of Haden, Kent, Palmer, and Keitt Mango fruits

Variety	Variables and temperature conditions						
	Ambient conditions			Transit conditions			
	Days to ripen (eat-ripeness stage; 29-31 ^{o}C, 90–95 % RH) from physiological maturity	Weight loss (%) when ripe	Days to spoilage (under sales/fresh market conditions; 20-22 ^{o}C, 85-90 % RH) after ripening	Days stored under transit conditions (10-13 ^{o}C, 85-90 % RH) at physiological maturity.	Days to ripen (eat-ripeness stage; 29-31 ^{o}C, 90-95 % RH) from physiological maturity	Weight loss (%) when ripe	Days to spoilage (under sales/fresh market conditions; 20-22 ^{o}C, 85-90 % RH) after ripening
Haden	9.50	6.50	3.15	21	3.50	3.31	2.91
Kent	10.52	4.96	4.09	21	4.50	2.50	3.85
Palmer	10.01	6.37	3.16	21	4.00	3.12	2.87
Keitt	11.01	4.09	4.08	21	5.00	2.34	3.92
LSD (0.05)	1.04	1.85	0.85	-	1.03	0.75	0.92

Means of four estimations expressed on fresh weight basis.

Table 2. Fruit bio-yield point and firmness index (N) of Haden, Kent, Palmer, and Keitt mango fruits when physiologically matured

Variety	Bioyield point (N)	Fruit flesh firmness (N)
Haden	93.12	145.30
Kent	104.18	177.98
Palmer	117.81	149.87
Keitt	122.91	194.98
LSD (0.05)	21.45	37.51

Means of four estimations expressed on fresh weight basis.

Table 3. Typical picking and shipping schedule for mango fruits consigned by sea and by air to the European Union from Ghana

Operation	Time (days required)	
	Sea freight	Air freight
Picking and packaging	1	1
Pre-cooling and accumulation of load	1	1
Transport to port, port handling and accumulation of load	1	1
Voyage time	14-21	1/4
Discharge handling	1	1
Transport and distribution	2	1
Total	20-27	5 1/4

Weight loss

Average weight loss was highest (6.50%) for Haden and lowest (4.09%) for Keitt during ripening under ambient conditions. The same occurred during ripening after exposure to transit conditions.

Fruit bio-yield point and fruit firmness

Fruit bio-yield point and fruit flesh firmness index values (N) at physiological maturity are presented in Table 2. Values of fruit flesh firmness were higher than values of bio-yield point in all the varieties (Table 2). Significant difference ($p<0.05$) in fruit flesh firmness occurred only between Keitt (highest - 194.98 N) and Haden (lowest - 145.30 N). Keitt and Kent fruits were significantly not different ($p>0.05$) in their flesh firmness; this was the same for Haden and Palmer fruits. Significant difference ($p<0.05$) in force for the bio-yield point occurred between Keitt (highest - 122.91 N) and Haden (lowest - 93.12 N). Bio-yield point test for Palmer recorded a significantly higher force when compared with that of Haden. However, differences in force for the bio-yield point were not significant ($p>0.05$) between Kent and Haden (Table 2).

Typical picking and shipping schedule

A typical picking and shipping schedule for mango fruits consigned by sea and by air to the European Union from Ghana is presented in Table 3.

DISCUSSIONS

Ripening quality of fruits at ambient and transit temperatures

Days to ripening were significantly different between Keitt and Haden fruits but similar for three varieties (Haden, Kent, and Palmer). The differences in ripening time among the different varieties could be attributable to their gene constitutions (Rathore et al., 2007) whiles the significant difference in ripening time between Keitt and Haden fruits could be due to the comparatively vast difference in their physiological maturity periods (Abu, 2010). An earlier report by Lakshminarayana (1980) indicated that in a climacteric fruit, such as mango, the fruit is not considered to be of desired eating quality at the time it initially becomes mature, but requires a ripening period (typically 8 to 10 days at about 25 ^{o}C) before it achieves the taste and texture desired at the time of consumption.

The trend for ripening under transit conditions was similar to that of ambient temperature conditions but occurred rather earlier. This has been explained in terms of the degenerative changes in the chemical constituents as well as of the skin (Rodeiro et al., 2006), resulting from both respiration and transpiration sources (Rocha Ribeiro et al., 2007) as maturation progressed in transit.

Shelf life

Under ambient temperature conditions fruits took longer time to lose their quality characteristics as compared to fruits under transit conditions. Under both conditions the results suggest that Haden and Palmer were fast to deteriorate than Kent and Keitt. Softening or changes in mango fruit texture during ripening have been previously attributed to the degradation of pectic compounds by pectic enzymes, which activity significantly increases as the fruit ripens (Barreto et al., 2008). Shriveling of the mango fruit skin did not increase above an objectionable rating during storage, regardless of the storage temperature.

Increased softness was the quality factor that determined the maximum shelf life of the fruit after they were transferred from the ripening chamber to sale or fresh market storage

conditions (20-22 ^{o}C, 85-90% RH) (Krishnamurthy, 1988; Reddy & Raju, 1988; Mitra, 1997; Yahia, 1999; Mahayothee et al., 2002; Litz, 2003; Cecilia et al., 2007). Although softness was the first quality factor to reach the limiting quality rate, color changes and decay should not be disregarded as they also contributed to the loss of quality in the fruit stored. For fruit ripened from direct physiological harvest, softening was considered to be the major quality limiting factor for Haden, Kent, Palmer, and Keitt fruits as it reduced their shelf lives to 3.15, 4.09, 3.16, and 4.08 days respectively. Softening of the fruit was likewise the major quality limiting factor for the fruits of the mango varieties stored at the sale or fresh market storage conditions after ripening (prior to simulation at transit conditions) and reduced the shelf lives of the fruits to 2.91, 3.85, 2.87, and 3.92 days, respectively.

Weight loss

At ambient and transit temperatures, weight loss was highest (6.50%) in Haden and lowest (4.09%) in Keitt and the observed values corroborate with the values previously reported by other authors (Krishnamurthy, 1988; Reddy & Raju 1988; Mahayothee et al., 2002; Schouten, 2005). Cecilia et al. (2007) reported 3.9% and 3.7% weight losses in "Tommy Atkins" and "Palmer" respectively, stored at 20 ^{o}C for 5 days. The authors also reported that the maximum weight loss value obtained for "Tommy Atkins" mango stored for 18 days at 12 ^{o}C (7.8%) corresponded to a shriveling rate of only 4.7, which is well below the maximum acceptable rate before the visual quality of the fruit becomes objectionable. Cecilia et al. (2007), however, indicated that the maximum weight loss obtained for "Palmer" mango (6.5%) after 14 days at 12 ^{o}C corresponded to a shriveling rate of 3.5, which was close to the maximum acceptable before the fruit become unacceptable for sale. It was therefore concluded that a weight loss between 9 and 7% may be suggested as a maximum acceptable before "Tommy Atkins" or "Palmer" mangoes become unacceptable for sale (Cecilia et al., 2007). The values of weight loss obtained in this study do not seem to be crucial in terms of development of shriveling in Haden, Kent, Palmer, and Keitt mangoes when compared with the findings of Reddy and Raju (1988), and with the findings and conclusion of Cecilia et al. (2007).

Fresh fruits are living tissues and are subject to continuous change after harvest. Thus the weight losses and other objectionable characteristics the fruits developed during storage is a response to; the loss of stored food reserves in the commodity during respiration which translated to loss of salable dry weight and hastening of senescence as the reserves which provide energy for maintaining the living status of the commodity are exhausted; transpiration or water loss since it results in not only direct quantitative losses (loss of salable weight), but also causes losses in appearance (due to wilting and shriveling), textural quality (softening, juiciness, flaccidity, limpness, and loss of crispness), and nutritional quality; compositional changes (loss of chlorophyll, development of carotenoids, development and changes in anthocyanins, changes in carbohydrates, and changes in organic acids) which may continue after harvest and could be desirable or undesirable; ethylene production or ethylene effects that could be desirable or undesirable; and atmospheric composition that can either delay or accelerate deterioration of fresh horticultural crops (Kader et al., 1985; Kader, 2008; Kader & Mitcham, 2008). Deterioration set in much earlier in the early maturing varieties (Haden and Palmer) most probably because they are longer rapidly respiring commodities (Abu, 2010).

Fruit firmness, picking, and shipping schedule

Dadzie and Orchard (1997) indicated that the texture or firmness of the pulp of fruits is an important postharvest attribute that could be used as a maturity or ripening index which could also facilitate comparison of the state of softening of fruits and vegetables. According to Abu (2010), assessment of firmness is important in the evaluation of fruit's susceptibility to

physical or mechanical damage during postharvest handling. In this study softening has been found to be the major quality limiting factor for Haden, Kent, Palmer, and Keitt mango fruits during storage, as it reduced their shelf lives to 3.15, 4.09, 3.16, and 4.08 days respectively. These analyses show that Kent and Keitt fruits store better than Haden and Palmer fruits under both ambient and transit conditions and are therefore recommendable for sea freight or for longer distances where relatively much time is spent before delivery, while Haden and Palmer fruits are recommendable for air freight. The sea freight operations take much longer time (20-27 days), almost three to four times that of air freight (about 6 days).

CONCLUSION

Generally, ripening and subsequent deterioration occurred earlier in fruits that were simulated for transit conditions. Days to ripening were significantly different between Keitt and Haden fruits but similar for Haden, Kent, and Palmer fruits. The trend for ripening under transit conditions was similar to that of ambient temperature conditions but occurred rather earlier.

Under ambient temperature conditions fruits took longer time to lose their quality characteristics as compared to fruits under transit conditions. Under both conditions, deterioration set in much earlier in the early maturing varieties (Haden and Palmer) than the late maturing varieties (Kent and Keitt); weight loss was highest in Haden and lowest in Keitt; and softening was found to be the major quality limiting factor for all the varieties under study during storage. These analyses show that Kent and Keitt fruits store better than Haden and Palmer fruits under both ambient and transit conditions and are therefore recommendable for sea freight or for longer distances where relatively much time is spent before delivery, while Haden and Palmer fruits are recommendable for air freight. The attributes obtained from the quality evaluations for the different temperature regimes showed that a single quality attribute cannot be used to express loss of quality of mango fruit over the normal physiological range of mango fruit growth and development.

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Conflict of interest
The authors have no conflict of interest to report.

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Assessment of allelopathic potential of *Aphanamixis polystachya* on selected field crops

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ABSTRACT

Purpose: *Aphanamixis polystachya* (Wall.) R.N. Parker, belonging to Meliaceae family is very well known for its medicinal properties. But its allelopathic potentiality not yet has been reported. Hence, aqueous extracts of different parts of *Aphanamixis polystachya* were examined to investigate their allelopathic potentiality. **Research Method**: Different parts of *A. polystachya* extracts at four different concentrations (1:5, 1:10, 1:15 and 1:20 (*w/v*)) along with control (distilled water without extracts) were tested against jute, mungbean, mustard, radish, rice, wheat and tomato. The experiments were conducted following completely randomized design with three replicates. **Findings**: Among the test crop species, shoot growth of mustard was most sensitive (43% average inhibition (*a.i.*)) followed by radish (41% *a.i.*) to the extracts of different parts of *A. polystachya*., whereas shoot growth of tomato (14% *a.i.*) was less sensitive to the extracts followed by rice (25% *a.i.*) and mungbean (29% *a.i.*). Root growth of radish was most sensitive (41% *a.i.*) species followed by mustard (39% *a.i.*) and jute (36% *a.i.*) to the extract of different parts of *A. polystachya*. Root growth of mungbean (13% *a.i.*) was less sensitive to the extracts followed by tomato (18% *a.i.*) and rice (20% *a.i.*). Among the plant parts, leaf showed most phytotoxic activity on the shoot growth (41%) and twig on the root (40%) growth of the test plants. However, stem extract was less sensitive to both shoot and root growth of the test species. These results confirm that *A. polystachya* has allelopathic properties and may possess allelochemicals. **Research limitations**: There was no significant limitation to the report. **Originality/Value**: To the best of our knowledge this is the first report about the allelopathic potential of *Aphanamixis polystachya*.

INTRODUCTION

Allelopathy refers to the inhibitory or stimulatory effect of one plant to their neighboring plants and/or their associated micro and/or macrofauna by the production of allelochemicals (IAS, 2017). The substances that is released by allelopathic plants are commonly known as allelochemicals, which are released into the surrounding environment through volatilization from the leaves, , leaching from the above ground parts by precipitation, decomposition of leaf litter or sloughed root tissues, microbial transformation from the decayed leaf, stem, leaf litter or roots, through root exudates, from pollen of some crop plants or other processes in both natural and agricultural systems (Islam & Kato-Noguchi, 2013c). These substances upon release, may suppress the germination, growth and establishment of neighboring native plants, even the secreting plant itself either directly by affecting their physiological properties (Weir et al., 2004), or indirectly by modifying the rhizosphere soil properties through influencing the microbial biomass carbon and microbial community (Zhou et al., 2013).

In forest ecosystems, trees release allelopathic substances for long periods, which may accumulate in soil to toxic levels with passage of time. The accumulation may also occur due to reduction in microbial decomposition under certain conditions (Reigosa et al., 1996; 1998; Singh et al., 1999). Generally, one or few species dominate the forest system, which could lead to accumulation of allelochemicals of these particular species to the forest soil. The substances released from the allelopathic tree species affect the understory species with/without any effect on the secreting plants or their progeny (Kohli, 1999; Malik, 1999). Moreover, introduction of high yielding exotic tree species in forest plantation may also increase accumulation of allelochemicals in soil because of their very high demand for growth resources *viz.*, moisture and nutrients may cause their deficit in soil, leading to increased production of allelochemicals. In addition, the soil microflora also may not be adapted to such allelochemicals and therefore, the chemicals may accumulate in soil to toxic levels. Even though, the allelochemicals released from the dominated species may not cause any harm to the understory flora of that region where they co-evolved, may suppress the understory species in regions of its new introduction (Reigosa et al., 1998).

Aphanamixis polystachya (Wall.) R.N. Parker, belonging to Meliaceae family, a large evergreen tree found to grow in most of the hotter parts of India, as well as the lowlands and hill forests of Bangladesh, Malay and Ceylon (Chan et al., 2011; Rahman et al., 2017). It is an evergreen timber tree with bunches of rounded sub-globose fruits and glossy deep brown seeds, mainly grows in the tropical areas of Asia. The plant is commonly known as Roina or Pittraj in Bangladesh, and are very well known for its medicinal (Chan et al., 2011; Rahman et al., 2017), insecticidal (Talukder & Howse, 1993) and biodiesel properties (Palash et al., 2015).

For example, boiling of *A. polystachya* root bark in abdominal complaints like enlargement of glands, liver and spleen disorders and corpulence (Apu et al., 2013b). Seeds have refrigerant, laxative, anthelmintic activities; used against the diseases of the blood and scale back muscular pain (Apu et al., 2013b). Oil of the seeds is used to treat rheumatism and conjointly has pesticidal character. Bark and seeds of the plant are useful for ulcer (Hossain et al., 2009). Moreover, *A. polystachya* has been reported to possess analgesic (Hossain et al., 2009), antimicrobial (Chowdhury & Rashid, 2003; Yadav et al., 2010; Apu et al., 2013a), antioxidant (Krishnaraju et al., 2009; Sikder et al., 2010; Apu et al., 2013a), antitumor (Chan et al., 2011), CNS depressant (Hossain et al., 2009), cytotoxic (Sikder et al., 2010; Apu et al., 2013a), hepatoprotective (Gole & Dasgupta, 2002), insecticidal (Talukder & Howse, 1993), laxative (Chowdhury & Rashid, 2003), membrane stabilizing (Sikder et al., 2010), anticancer (Apu et al., 2013b) and thrombolytic (Apu et al., 2013a) properties. The plant has also

antibacterial, mild antifungal (Rahman et al., 2017), antifeedant, repellant properties, and contact toxicity to beatles (Talukder & Howse, 1993). Besides, the pharmacological and/or toxicological properties, not a single research have so far been conducted to explore the phytotoxic properties of *A. polystachya*. Therefore, the current research was an attempt to investigate into the allelopathic potential of *A. polystachya* on the seedling growth of selected field crops.

MATERIALS AND METHODS

Location and site of the experiment

The experiment was conducted at the Agro Innovation Laboratory of the Department of Agronomy, Bangladesh Agricultural University, Bangladesh.

Collection of plant materials

Five different plant parts *viz.* bark, stem, leaf, root and twig of *Aphanamixis polystachya* were used for this study. The fresh plant parts were collected during March and April, 2018 from the nearby village of the experimental site.

Test plant

Jute (*Corchorus olitorius*), Mustard (*Brassica juncea*), Mungbean (*Vigna radiata*), Radish (*Raphanus sativus*), Rice (*Oryza sativa*), Tomato (*Solanum lycopersicum*) and wheat (*Triticum aestivum*) were used as test crop species. Radish was used in this experiment because it is highly sensitive to allelochemicals even at very low concentrations (Tsuzuki et al., 1995).

Extraction and bioassay procedure

The extraction and bioassay were done according to the procedure developed by Islam et al. (2018). The collected parts of *A. polystachya* plant were washed with tap water, then with distilled water. One hundred gram of each part was then chopped and crashed into paste by a mechanical grinder and soaked with 400 mL distilled water and homogenized in a warring blender for 5 minutes at room temperature (25 ºC). The extract was then filtered through one layer of filter paper (No. 2; Double Rings® Hangzhou Xinhla Paper Industry Co. Ltd., China). The filtrate was then put into 500 mL volumetric flask and filled with distilled water up to the mark, and homogenized by manual shaking. The prepared concentration was considered full strength concentration *i.e.* 1:5 (*w/v*), and was stored at 4ºC (normal freezing condition) in a refrigerator until further used. The extraction was done separately for each plant parts of *A. polystachya.*

The prepared full strength concentration of bark, stem, leaf, root or twig aqueous extracts were diluted into another three concentrations *viz.* 1:10, 1:15 and 1:20 (*w/v*), and a control (distilled water without extract) was also maintained. The bioassay experiment was replicated thrice. Twenty seeds of each jute, mustard, mungbean, radish, rice (sprouted seed), tomato and wheat were arranged on the filter paper in Petri dishes. After 48 h of incubation the shoot and root length of selected seven crop species were measured. The inhibitory potential of each extract was then examined against indicator plants following standard laboratory bioassay method. All the laboratory experiments were conducted following completely randomized design (CRD) with three replications.

Calculation of inhibition percentage

The percentage of inhibition was calculated according to the equation described (1) by Islam et al. (2018):

$$Inhibition\ (\%) = 1 - \frac{Length\ in\ aqueous\ extract}{Length\ in\ control} \times 100 \quad (1)$$

Statistical analysis

Data recorded on growth inhibition was compiled and tabulated for statistical analysis. The data were analyzed statistically by using R Statistics Software (Version 3.5.0).

RESULTS

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of jute

The aqueous extracts of different parts of *A. polystachya* plant significantly influenced the shoot and root growth of jute (Table 1). From the result it is clear that the inhibitory activity of the different extracts was concentration dependent. Except stem, all other plant parts of *A. polystachya* showed inhibition at all the concentration used in the study. At 1:5 *(w/v)* concentration, *A. polystachya* twig extracts showed more than 90% shoot growth inhibition of Jute, while at the same concentration both twig and leaf extract showed more than 90% root growth inhibition (Table 1). At 1:5 *(w/v)* concentration, extracts of all other parts showed more than 80% shoot and root growth inhibition. Stem extracts of *A. polystachya* at concentration lower than 1:5 *(w/v)* stimulated the shoot and root growth of jute (Table 1).

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of mungbean

Shoot and root growth inhibition of mungbean were also significantly affected by the aqueous extracts of different parts of *A. polystachya* plant (Table 2). Table 2 showed that the inhibitory activity of the different extracts was concentration dependent. Stem extracts of *A. polystachya* showed stimulatory activity on shoot and root growth of mungbean at concentration 1:10 (*w/v*) or below, whereas bark extract of *A. polystachya* showed stimulatory activity on the root growth of mungbean at the same concentration. In addition, leaf and root extracts of *A. polystachya* showed stimulation on the root growth of Mungbean at the lowest concentration used in this experiment i.e. 1:20 (*w/v*). At concentration 1:5 (*w/v*), leaf and root extracts showed more than 80% shoot and root growth inhibition of mungbean (Table 2).

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of mustard

The aqueous extracts of *A. polystachya* plant parts had also significant influence on shoot and root growth inhibition of mustard where growth inhibition increased significantly with the increase of the aqueous extract concentrations (Table 3). Bark extracts of *A. polystachya* showed stimulatory activity on root growth of mustard at concentration 1:10 (*w/v*) or below. Whereas, root and stem extracts of *A. polystachya* showed stimulation on the root and stem growth of mustard, respectively at the lowest concentration used in this experiment *i.e.* 1:20 (*w/v*). At 1:5 *(w/v)* concentration, *A. polystachya* bark, leaf, root, stem and twig extracts showed 55, 90, 85, 89, 94% shoot growth, and 59, 98, 89, 96 and 99% root growth inhibition of mustard, respectively (Table 3).

Table 1. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Jute

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	13.56 b	18.63 c	21.17 c	87.30 b	7.83 c	27.067 c	23.06 d	87.40 b
Leaf	21.73 a	17.40 c	36.97 b	87.70 b	3.33 d	7.63 d	60.36 b	95.70 a
Root	6.67 c	40.00 a	53.33 a	74.73 d	32.47 a	61.13 a	64.66 a	89.86 b
Stem	-10.97 d	-39.37d	-8.40 d	84.53 c	-41.33 e	-78.70 e	-26.66 e	81.26 c
Twig	19.43 a	28.43 b	36.97 b	91.00 a	22.80 b	45.60 b	55.63 c	94.06 a
Level of sig.	***	***	***	***	***	***	***	***
C.V (%)	2.30	1.22	5.33	1.60	2.20	3.57	2.68	1.93
LSD	4.80	3.48	4.92	2.57	2.48	3.20	1.78	3.26

In column, means followed by different letters are significantly different. *** means at 0.1% level of probability. The positive value indicates inhibition, whereas the negative value indicates stimulation by the extract.

Table 2. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Mungbean

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	32.63 a	21.20 b	23.36 b	64.13 b	-33.23 e	-24.63 e	-41.16 e	50.86 c
Leaf	7.36 c	48.03 a	39.00 a	83.07 a	-11.57 c	31.40 a	20.53 b	89.33 a
Root	23.30 b	22.90 b	23.26 b	82.43 a	-2.83 b	1.86 c	8.60 c	83.96 b
Stem	-5.86 d	-2.30 d	-8.83 c	40.53 d	-20.00 d	-16.43 d	-15.83 d	41.03 d
Twig	7.83 c	12.33 c	17.63 b	48.73 c	2.20 a	17.87 b	25.70 a	54.00 c
Level of sig.	***	***	***	***	***	***	***	***
C.V (%)	1.43	1.85	1.03	1.40	5.68	4.85	3.43	2.68
LSD	4.53	6.86	6.76	1.68	3.18	3.67	2.69	3.22

Other details are same as Table 1.

Table 3. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Mustard

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	23.00 c	14.93 d	17.23 d	55.17 d	-34.27 e	-24.80 d	-41.83 e	58.53 d
Leaf	33.93 b	23.86 b	34.87 b	89.93 b	24.83 b	7.33 c	5.87 d	97.67 ab
Root	18.16 d	40.00 a	18.17 d	84.87 c	-6.63 d	14.90 b	23.50 c	89.43 c
Stem	-1.90 e	19.23 c	26.43 c	88.73 b	6.300 c	15.70 b	39.067 b	95.83 b
Twig	41.96 a	37.83 a	49.43 a	93.63 a	67.27 a	70.133 a	80.53 a	99.00 a
Level of sig.	***	***	***	***	***	***	***	***
C.V (%)	3.90	6.75	3.17	1.91	1.82	6.84	4.66	1.46
LSD	1.69	3.45	1.74	2.97	4.29	2.14	1.88	2.42

Other details are same as Table 1.

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of radish

The aqueous extracts of *A. polystachya* plant parts significantly influenced the growth inhibition of radish (Table 4). This result indicated that the inhibitory activity of the different extracts of *A. polystachya* was concentration dependent. In the present study, root extract stimulated the shoot and root growth of radish at concentration of 1:20 *(w/v)* while leaf extract stimulated the root growth at concentration of 1:20 *(w/v)*. Except these, all other extracts showed inhibitory activity on the shoot and root growth of radish at any concentration used in this study (Table 4). At 1:5 *(w/v)* concentration, *A. polystachya* bark, leaf, root, stem and twig extracts showed 78, 97, 86, 93 and 93% shoot growth inhibition, and 86, 97, 91, 95 and 97% root growth inhibition of radish, respectively (Table 4).

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of tomato

The growth inhibition of tomato was also statistically significant among the aqueous extract of different plant parts of *A. polystachya* at different concentrations (Table 5). Table 5 showed that the inhibitory activity of the different extracts was concentration dependent. All the extracts of *A. polystachya* plant parts except twig for shoot growth, and bark and leaf for root growth stimulated the shoot and root growth at lowest concentrations. At 1:5 *(w/v)* concentration, both leaf and twig extracts showed more than 90% shoot growth of tomato, while at the same concentration leaf, stem and twig extract showed more than 90% root growth inhibition (Table 5).

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of rice

Different parts of *A. polystachya* significantly inhibited the percent shoot and root growth inhibition of rice at different concentrations (Table 6). The growth inhibition of rice increased with the increasing concentrations of the aqueous extracts of any parts of *A. polystachya*. Root and stem extracts of *A. polystachya* stimulated the shoot growth of rice at concentration 1:15 (*w/v*) or below, while this parts stimulated the root growth of rice at concentrations 1:10 (*w/v*) or below. The twig extracts of *A. polystachya* showed the opposite trend i.e. concentration 1:10 (*w/v*) or below stimulated the shoot and 1:15 (*w/v*) or below stimulated the root growth of rice. At 1:5 *(w/v)* concentration, only bark extract of *A. polystachya* showed more than 90% shoot and root growth inhibition of rice, while at the same concentration twig extract showed more than 85% root growth inhibition (Table 6). At 1:5 *(w/v)* concentrations, all other parts extracts of *A. polystachya* showed less than 80% shoot and root growth inhibition.

Effect of aqueous extracts of *A. polystachya* plants parts on growth inhibition of wheat

The growth inhibition of wheat was also significantly influenced by the aqueous extract of *A. polystachya* plant parts at different concentrations (Table 7). Table 7 shows that inhibitory activity of the different extracts was concentration dependent. Except root extract of *A. polystachya* for shoot growth, and stem and twig extracts for root growth, all other plant parts of *A. polystachya* showed inhibition at all the concentrations used in the study. At 1:5 *(w/v)* concentration, *A. polystachya* bark, leaf, root, stem and twig extracts showed 46, 88, 87, 60 and 76% shoot growth inhibition, and 74, 97, 91, 73 and 84% root growth inhibition of wheat, respectively (Table 7).

Table 4. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Radish

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	16.93 c	17.16 d	15.43 d	77.76 d	17.93 b	16.30 c	32.40 d	85.63 d
Leaf	56.77 a	43.90 a	81.76 a	97.30 a	-2.40 d	17.43 b	76.10 a	96.60 a
Root	-19.20 e	23.16 c	28.13 c	85.76 c	-1.33 e	27.90 a	35.76 c	91.10 c
Stem	21.10 b	9.03 e	17.53 d	92.76 b	11.66 c	1.23 d	16.26 e	94.90 b
Twig	4.00 d	26.26 b	35.86 b	92.66 b	20.46 a	29.10 a	57.90 b	96.50 a
Level of sig.	***	***	***	***	***	***	***	***
C.V (%)	4.73	4.11	1.68	1.53	3.71	2.57	5.30	1.62
LSD	3.21	1.85	7.26	2.58	2.99	3.41	6.35	1.10

Other details are same as Table 1.

Table 5. Effect of different plant parts of *A. polystachya* on the shoot and root growth of tomato

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	-7.67 c	3.56 c	13.26 b	58.17 b	27.30 b	7.30 c	50.60 a	77.00 b
Leaf	-9.40 d	-5.66 d	33.50 a	91.20 a	43.53 a	19.76 b	18.80 c	91.13 a
Root	-9.17 d	-8.43 d	12.70 b	62.67 b	-85.60 e	-25.37 d	-5.70 d	43.17 c
Stem	-6.20 b	12.43 b	12.43 b	82.60 a	-9.70 d	25.73 a	33.03 b	91.40 a
Twig	15.60 a	22.63 a	35.60 a	91.70 a	-3.13 c	25.43 a	35.23 b	94.73 a
Level of sig.	**	***	***	***	***	***	***	***
C.V (%)	4.73	1.40	306	7.23	3.94	3.9	-11.93	5.59
LSD	13.16	8.26	9.37	10.52	1.56	2.76	3.42	7.80

Other details are same as Table 1.

Table 6. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Rice

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	9.17 a	21.43 a	26.00 a	90.53 a	3.50 b	22.30 a	37.86 a	92.13 a
Leaf	7.90 a	-1.90 b	2.70 d	63.40 b	26.67 a	22.43 a	10.40 c	58.93 e
Root	-18.77 b	-12.77 d	10.17 b	50.57 d	-30.10 c	-12.70 b	-16.70 d	67.67 d
Stem	-20.86 c	-8.07 c	5.63 c	54.63 cd	-21.33 d	-21.46 c	-23.67 e	76.37 c
Twig	-28.90 d	-14.56 de	-6.73 e	58.73 c	-21.50 d	-20.80 c	12.87 b	88.77 b
Level of sig.	***	***	***	***	***	***	***	***
CV (%)	3.45	-5.06	-7.36	3.62	-3.37	-2.93	3.48	2.18
LSD	5.26	7.77	7.14	4.33	3.47	2.76	3008	3.16

Other details are same as Table 1.

Table 7. Effect of different plant parts of *A. polystachya* on the shoot and root growth of Wheat

Plant parts	% inhibition							
	Shoot growth				Root growth			
	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*	1:20 *(w/v)*	1:15 *(w/v)*	1:10 *(w/v)*	1:5 *(w/v)*
Bark	18.63 b	34.93 a	39.30 a	46.00 d	27.46 a	29.80 a	34.76 a	73.56 d
Leaf	32.37 a	17.33 c	34.96 ab	87.70 a	15.70 c	6.33 d	29.33 b	97.13 a
Root	-10.46 d	17.80 c	20.40 c	87.43 a	23.93 b	23.50 b	4.23 e	91.16 b
Stem	17.30 b	18.96 bc	11.80 d	59.46 c	-0.63 d	18.36 c	14.53 c	73.06 d
Twig	7.06 c	19.70 bc	30.96 b	75.90 b	-11.56 e	-3.66 e	9.53 d	83.50 c
Level of sig.	***	***	***	***	***	***	***	***
CV (%)	5.59	6.94	1.72	4.06	3.97	4.36	2.65	1.49
LSD	5.47	3.91	6.70	5.46	2.21	2.21	3.67	2.35

Other details are same as Table 1.

DISCUSSION

The allelopathic potential of *A. polystachya* plant parts were evaluated at different concentrations for selecting the most influencing part(s) and concentration(s) which will substantiate the elevated inhibition of studied seven field crops by containing higher allelopathic potentiality.

The aqueous extracts of *A. polystachya* plant parts had inhibitory and stimulatory effects on both shoot and root growth of fall the field crops studied (jute, mungbean, mustard, radish, tomato, rice and wheat), which confirm the presence of allelochemicals in all the extracts. In this study, shoot and root growth of test crops showed stimulatory effect with the extract(s) of *A. polystachya* plant parts at concentration lower than 1:5 *(w/v)*. Both inhibitory and stimulatory effects were reported by Islam and Kato-Noguchi (2013a) where they found stimulatory activity on the hypocotyls /coleoptiles growth of lettuce, alfalfa, rapeseed, timothy, crabgrass, barnyard grass and Italian ryegrass, and the root growth of rapeseed, timothy and crabgrass was caused by the *Mentha sylvestris* plant extract at concentrations ≤ 30 mg Dry weight equivalent extract mL^{-1} while inhibitory activity was recorded with higher concentrations of *M. sylvestris* aqueous methanol extract on studied field crops. The growth inhibition at higher concentration and tendency of growth stimulation at lower concentration could be explained by the recent findings of Islam and Kato-Noguchi (2012), Amini et al. (2016), Sutradhar et al. (2018), M'barek et al. (2018); Islam et al. (2018) and Islam et al. (2019a; b). This type of inhibitory activity is known as concentration dependent activity and are very common in allelopathic research *viz.*, Ghnaya et al. (2016), Islam et al. (2018), Suwitchayanon et al. (2017) and Appiah et al. (2017) where they reported that the inhibitory effect was dosage dependent and higher concentration showed strongest inhibitory activity on crops.

In this study the leaf extracts at higher concentration showed elevated growth inhibitory activity (more than 85%) among the most test crop species followed by twig and bark extracts. This might be due to the presence of more amounts of allelochemicals in leaf extract than that of other extracts which ultimately enhanced the inhibitory activity on different test crop species. These results are in agreement with the earlier findings of many researchers working with other plant materials. Amini et al. (2016) evaluated the allelopathic potential of 68 medicinal plants where the leaf of *Atriplex canescens* and the flower of *Achillea millefolium* had the strongest inhibitory effect on growth of lettuce than that of flower and fruits extracts of other medicinal plant. Tanveer et al. (2010), Raoof and Siddiqui (2012), Ravlić et al. (2012) and Abu–

Romman (2016) also confirmed that the leaf extract inhibited more strongly the seedling growth than any other extracts. The results differ from those of BaličEvić and Ravlić (2015) who reported that root extract had the highest inhibitory effect on root and shoot length and fresh weight of test species.

From the present study, it was found that the shoot growth of different field crops showed less inhibition than their roots. The greater sensitivity of root compared to shoot is usual in allelopathic study and this is because roots are the first organs to absorb phytotoxic substances from the extract and the permeability of phytotoxic substances into root tissue is higher than the shoot tissue (Islam & Kato-Noguchi 2013a; b), and according to Franco et al. (2015) allelochemicals can affect genes responsible for the cellular characterization of ground tissues and endoderm, reducing root development. Whereas, Levizou et al. (2002) observed low mitotic division in root apex resulted in higher root inhibition of *Lactuca sativa* when treated with *Dittrichia viscose* leaf extracts.

The study also revealed that all the studied field crops were highly sensitive (more than 90%) except mungbean, where mustard showed the strongest sensitivity with the aqueous extract of *A. polystachya* plant parts which confirms that the *A. polystachya* plant contain potential allelochemicals to inhibit the test species. These findings indicated that the inhibitory activities of *A. polystachya* plant parts on different field crop species are not identical. This imbalanced susceptibility to different extracts of *A. polystachya* plant parts could be due to inherent differences in allelochemicals content in different parts of this plant. So, it is clear that the leaves extract followed by twig and bark extracts of *A. polystachya* plant had strong inhibitory effect on the growth of different field crops at higher concentration. This finding was strongly supported by M'barek et al. (2018) who reported that the seedling growth of radish was more sensitive than lettuce, barley and tomato to the different extracts of *Tetraclinis articulate*.

CONCLUSION

Present study showed that the shoot and root growth inhibition of rice, wheat, jute, tomato, radish, mungbean and mustard by leaf, bark, stem, twig and root extracts of *Aphanamixis polystachya* varies significantly. Compared to the shoot growth, root growth of the test species were inhibited more. The leaf and twig have higher allelopathic potential than any other parts of *A. polystachya*. Since leaf and twig of *A. polystachya* extracts had greater inhibitory activities than other parts, these plant parts could be used for isolation and identification of allelochemicals. The findings of this experiment would be helpful for the researchers to know the inter-specific interactions of these plant species with their neighbor plant species under natural settings.

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Disclosure Statement
All the authors declare that there is no conflict of interest in publishing this manuscript.

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The investigation of genetic diversity based on SCoT markers, morphological, and chemical characters in tea (*Camellia sinensis* L.) clones

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ABSTRACT

Purpose: Tea is one of the earliest caffeinated non-alcoholic drinks produced from the tip of young shoots. Evaluation of genetic diversity of clones that existed in tea germplasm can be a help to improve the tea breeding program. **Research Method**: The genetic diversity of 9 tea clones based on morphological, chemical and molecular markers were evaluated at Shahid Eftekhari Fashalam Experimental Station, Tea Research Center, Shaft, Guilan, Iran. **Findings**: Analysis of variance showed a significant difference between the clones for the studied traits. Descriptive statistics showed that green leaf yield had the highest phenotypic variation with CV of 56.47% and water extract showed the least phenotypic variation (4.40%). Clones 399, 285 and 100 had a significantly higher content of the number of plucking shoots, fresh and dry weight of plucking shoot and green leaf yield than other clones. Regarding the water extract, clones 272 and 100 have a significantly higher value than the other clones. Contents of polyphenols in all of clones were high except clones 276 and 278. The cluster analysis classified tea clones into three groups based on morphological and chemical traits as well as SCoT markers. **Research limitations**: Not using other molecular markers and biochemical traits. **Originality/Value**: Great variation of morphological characters was apparent among the selected clones. Based on the Mantel test, the grouping of clones with molecular data was partially corresponding with morphological and chemical traits.

INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kuntze) belongs to the family Theaceae. It is the oldest non-alcoholic caffeine-containing beverage in the world. Tea is an evergreen, perennial, cross-pollinated plant and grows naturally as tall as 15 m. However, under cultivated condition, the bush height of 60-100 cm is maintained for harvesting the tender leaves to be processed for making the beverages (Mondal, 2014). Tea occupies about 2.7 million ha of cultivable land of the world with an annual production of about 2.2 million t (Mondal, 2014). Southeast Asia is the original home for tea. According to Wight (1959), the primary centre of origin of tea was considered around the point of intersection of latitude 29°N and longitude 98°E near the source of the river, Irrawaddy, the point of confluence where lands of Assam, North Burma, Southwest China and Tibet met. Secondary centers of origin were considered to be located in Southeast China, Indochina, Mizoram and Meghalaya (Kingdom-Ward, 1950). The above areas were, therefore, considered to be the zone of origin and dispersion of the genus *Camellia* as a whole (Sealy, 1958). However, presently tea is grown within the latitudinal range of 45°N to 34°S. Tea cultivation was extended to Japan, Indonesia, Sri Lanka, USSR, Turkey, Europe and African countries (Mondal, 2014). In Iran, tea is cultivated in two provinces (Guilan and Mazandaran) with an area under cultivation of 25000 ha.

Since many tea plants are currently being destroyed for many reasons, having information about tea genetics for designing suitable breeding programs is very helpful in obtaining appropriate plants for specific purposes (M Perez-de-Castro et al., 2012). The first and most relevant program for plant breeding is the study of diversity, which can be used to select suitable varieties. The rise of genetic diversity in a population extends the range of natural and artificial selection. Therefore, recognizing genetic diversity of varieties and wild cultivars of plants is essential to facilitate the production and creation of new lines through genetic hybridization and prevent the genetic erosion (Richards, 2011).

Classical methods of estimating the genetic variation among plants are based on the morphological traits, but these traits are influenced by the environmental factors (Govindaraj et al., 2015). Nowadays, methods of the identification and diagnosis of genetic diversity have become more recent and accurate with the use of molecular markers. Identification of the species in recent decades is carried out with confidence and ease using molecular markers. These markers are used to identify species and cultivars, estimate biodiversity and improve the breeding cultivars (Bandyopadhyay, 2011; Govindaraj et al., 2015). The advent of DNA markers technology has helped specialists and plant breeders to overcome many of these problems (Nybom et al., 2014). Diverse researches to assess genetic diversity have also been carried out implementing diverse methods, such as morphology (Wickramaratne, 1981; Toyao & Takenda, 1999; Chen et al., 2005; Rajkumar et al., 2010; Kim et al., 2012), biochemistry (Takeda, 1994; Magoma et al., 2000), using genetic markers, e.g., $RFLP_s$ (Matsumoto et al., 1994), $RAPD_s$ (Lee et al., 1995; Wachira et al., 1997; Kaundun et al., 2000; Kaundun & Park, 2002), $AFLP_s$ (Paul et al., 1997; Raina et al., 2012), SSR_s (Kaundun & Matsumoto, 2011; Fang et al., 2012; Bali et al., 2013), $ISSR_s$ (Lin et al., 2012; Liu et al., 2012; Wang & Ruan, 2012; Rahimi et al., 2019) and SRAP (Khiavi et al., 2020).

Gaining knowledge about the genetic distance between the individuals or populations and knowing the kinship relationships of the species in the breeding programs allows for the organization of germplasms and effective sampling of genotypes (Govindaraj et al., 2015; Nybom et al., 2014). The first step in improving plant characteristics is the identification of the

genetic features of germplasm specimens. So considering this issue, a systematic sampling of the germplasm is possible for conservation and breeding purposes (Govindaraj et al., 2015; Upadhyaya et al., 2008; Van Hintum et al., 2000). Genetic diversity refers to the fact that variety and variability between organisms can be found at different levels among individuals of a population, species of the same sex, and so on. Therefore, it is a unique resource for genetic improving and breeding of plant traits as well as increasing the variety of these traits (Govindaraj et al., 2015; Van Bueren et al., 2011; Xu et al., 2017).
Genetic diversity based on morphological and chemical traits and molecular marker was investigated in tea clones with the aim of breeding in the tea improvement program.

MATERIALS AND METHODS

Plant material and experimental conditions

During 2001-2006, Genotypes selected based on clonal selection method (Gholami et al., 2019) from different gardens in the west of Guilan province and were propagated by cutting and cultivated in Tea Plant Germplasm Collection situated at the Shahid Eftekhari Fashalam Research Station (latitude 37°15′54″N, longitude 38°45′49″E and height of -10 meters above sea level) in a Randomized Complete Block Design with three replications. The length and width of each plot were 5 and 4 meters, respectively (including four rows and six plants per row). In each row, the distance between the plants was 70 cm and the gap between the two rows was 100 cm. Physical and chemical tests of the soil were carried out and the soil texture was found to be sandy loam. All plants were held using similar agricultural management practices. Nine clones were chosen (Table 1) for this study from the Fashalam Tea Plant Germplasm Collection.

Morphology

Nine morphological traits were measured among the summer shoots during July 2018, including: number of plucking shoots, fresh and dry weight of plucking shoot, length of plucking shoot, 5^{th} leaf length, 5^{th} leaf width, leaf area, internodes distance and green leaf yield (IPGRI, 1997). For measuring the number and fresh weight of plucking shoot per unit area, 25×25 cm frame was randomly located in 3 locations per plot and the number and fresh weight of plucking shoots containing two leaves and a bud calculating and then converted to unit area (IPGRI, 1997), for dry weight of shoots, plucking shoots dried in 103°C (IPGRI, 1997), length of plucking shoot was obtained by measuring the length from beginning of shoot growth to the terminal bud (IPGRI, 1997). To determine the green leaf yield per unit area, tea shoots were harvested in standard form (two leaves and a bud) from the experimental plots and their weight was measured by a precision scale (IPGRI, 1997). As for the leaf's length and width, the longest and widest part of 5^{th} mature leave was determined. The leaf area was assessed using the following formula (1):

$$\text{Leaf area } (c.m^{-2}) = \text{leaf length} \times \text{leaf width} \times K \text{ (Ng'etich \& Wachira, 1992)} \quad (1)$$

Also, we measured internodes distance between 5^{th} and 6^{th} mature leaf. We have ten replicates for those length measurements and three for the weighted data.

Table 1. The name, type and origin of tea clones studied at Fashalam station

Row	Clone	Varietal type	Origin
1	272	Chinese type of local selection	West of Guilan
2	277	Chinese type of local selection	West of Guilan
3	100	Chinese type of local selection	West of Guilan
4	285	Chinese type of local selection	West of Guilan
5	74	Chinese type of local selection	West of Guilan
6	399	Chinese type of local selection	West of Guilan
7	276	Chinese type of local selection	West of Guilan
8	278	Chinese type of local selection	West of Guilan
9	269	Chinese type of local selection	West of Guilan

Chemistry

Fresh plant materials (including two leaves and a bud) were collected during July in 2018; and they were then dried at 103°C. Samples were analyzed in order to determine their total polyphenols, caffeine, water extract, and total ash at Tea Research Center in Iran. In each of the experimental units, approximately 100 g of the fresh shoots (with one bud and two leaves) were plucked. Then, the samples were placed inside the labeled paper bags and dried at 70°C for 24 hours. The dried samples were blended, placed inside the paper bags in dry and dark conditions until laboratory analysis.

The ISO procedure (2005) was used for the analysis of the total polyphenols. Ground shoot samples (0.2 g) were weighed into 10-ml extraction tubes. 5 ml, 70% v/v methanol (hot methanol/water extraction mixture) was added to every extraction tube. Then, a vortex mixer was used to stopper and shake the tubes. The tubes were placed in a water bath for 10 minutes. Then, the tubes were allowed to be cool at room temperature. Thereafter, the extracts were centrifuged (3500 rpm, 10 minutes). The supernatant was poured into 10-ml tubes. Then, a cold ethanol/water mixture was added to reach 10 ml volume. 1 ml of the extract was poured into a 100-ml flask to more dilute, and then water was added to reach the mark. Standard solutions of gallic acid (1 ml) corresponding to 10, 20, 30, 40 and 50 µg of anhydrous gallic acid and a similar quantity of water for the reagent blanks were poured in duplicate into the different tubes. 1 ml of the diluted sample extract was poured into the separate tubes and 5 ml of the reagent of Folin-Ciocalteu phenol were added to each of the tubes and mixed. 4 ml of sodium carbonate solution, about 5 minutes after adding the Folin-Ciocalteu phenol reagent, were added to each of the tubes and allowed to remain for 60 minutes at room temperature. By using a 10-mm cell on a spectrophotometer set, optical densities were calculated at a wavelength of 765 nm. Polyphenol contents in the tested sample were measured by a standard curve made by gallic acid, and defined as the contents of gallic acid equivalent. By using the mass of the standards of anhydrous gallic acid, the graph of best-fit linear calibration was drawn in comparison with the standard optical densities of gallic acid, and the content of the total polyphenol, expressed as a percent by the mass based on the sample dry matter, was measured by the ISO procedure (ISO Standards, 2005).

To determine the water extract content, the soluble matter from 2 g of the ground shoots of tea (one bud and two leaves) was mixed with boiling water under refluxing, filtering, washing, drying, and weighing the insoluble residue in hot water, and calculating water extract (ISO Standards, 1994).

To measure the total ash, the organic matter of 2 g of ground shoots of tea (one bud and two leaves) was destructed and heated at 525 ± 25°C to a constant mass with a furnace (ISO Standards, 1987).

Genetic relationship through SCoT analysis

100 mg of the leaf tissues were ground in liquid nitrogen and DNA extracted according to Dellaporta et al. (1983) method. Extracted DNA was diluted with distilled water to 25 ng.μl^{-1} and stored at -20°C.

Polymerase chain reaction was carried out in 10 µl mixtures of reaction containing 2µl from each of template DNA (50 ng), 1µl PCR buffer, 0.3 µl $MgCl_2$, 0.1µl each of dNTP, 0.6 µl of each primer, and 0.2 µl of *Taq* DNA polymerase (Sinaclon Co, Iran) and adjusted value by adding double-distilled water. PCR was performed in a Biometra PCR thermal as mentioned: initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 40 sec, 51-57°C (depending on the primers) for 40 sec, and 72°C for 2 min, and a final 5 min extension at 72°C. The 1.5% agarose (1×TAE buffer) gel was used to observe the band's pattern, and detection was done using UV transilluminator, stained in Ethidium bromide. Eventually, 13 primers (Table 6) producing clear and proliferous fragments patterns were selected for our final analysis.

To score polymorphism for every clone, the absence and presence of a band were scored as 0 and 1, respectively. The binary data of the SCoT marker were scored as: the presence (1) or absence (0) of a band, providing a genetic dissimilarity matrix to use in genetic diversity. Genetic diversity was examined by several indices such as: the number of observed alleles (Na), the number of effective alleles (Ne) (Kimura & Crow, 1964), Neis gene diversity (Nei, 1972), Shannon diversity index (Shannon, 2001) and the polymorphism information content (PIC) (Anderson et al., 1993).

The screening of the entire set of samples was performed three times to assess the repeatability of the SCoT profiles and identical SCoT patterns were obtained.

Statistical analysis

After performing the data normality test (Kolmogorov-Smirnov), the analysis of variance (ANOVA) was performed to test for the differences of clones on the various morphological and chemical parameters measured. Duncan Multiple Range Test was used to compare means at a significance of $P \leq 0.05$. The data analysis was carried out using the statistical package SAS 9.4 (SAS Institute, 1985). Relationships between clones based on morphological, chemical and molecular data were studied using cluster analysis. The PAST software (Hammer et al., 2001) was used for the cluster analysis.

RESULTS AND DISCUSSION

Morphological and chemical traits in selected tea clones

Morphological traits

Variation of morphological and chemical traits in all clones was presented (Table 2). Six of nine morphological traits exhibited a coefficient of variation >20% across clones, which were identified as follows: the number of plucking shoot, fresh weight of plucking shoot, dry weight of plucking shoot, leaf area, internode distance and green leaf yield. Among the above-mentioned traits, green leaf yield showed the highest coefficient of variation among the studied traits and thus had the highest diversity in comparison with other traits. The lowest diversity among the studied clones related to water extract was 4.40%. The trait including number of plucking shoots in tea clones also had a high variation of 40.40%, so there is also a choice between clones for this trait, and suitable clones can be selected. Due to the high diversity of

these traits, these traits can be of interest to the breeders, and selection of clones based on these traits leads to the improvement of these traits. Still, other traits with a lower coefficient of variation have less chance of selection (Table 2).

The results of the analysis of variance (ANOVA) for all the studied traits except the length of plucking shoot showed significant differences between the tea clones (Table 3). The coefficient of variation of the randomized complete block design was between 2.66% for the water extract to 26.29% for the fresh weight of plucking shoot, which indicated the appropriate accuracy of the test. A significant difference between studied traits indicating the difference between the tea clones and the acceptable genetic variation for most studied traits. The diversity between genotypes can improve the traits, and in particular, the amount of genetic diversity is effective in determining the usefulness of selection (Balasaravanan et al., 2003).

The number of plucking shoots, fresh and dry weight of the plucking shoot of clones 399 and 285 were significantly higher than the other clones (Table 4). The 5th leaf width and leaf area of clone 278 measured were measured to be 3.48 cm and 29.71 c.m^{-2}, respectively, resulting in the largest value. Clone 285 showed greater value for green leaf yield, followed by clones 100 and 399 (Table 4).

Chemistry

Different clones of tea may have various chemical contents and these are important factors that contribute to tea quality (Wright & Gilchrist, 1961). Comparing the four chemical traits, all of them showed the low variation with a coefficient of variations (CV) of 4.40 to 8.96 (Table 2), but based on variance analyses, there are significant differences among clones for four chemical traits (Table 3). Regarding the content of water extract, clones 272 and 100 have a significantly higher content of water extract value than the other clones. Clones of 272 and 100 showed a high content of polyphenols compared to other clones (Table 4).

To select the best parents in each cross and achieve maximum heterosis, researchers select genotypes which are genetically heterogeneous. This can be achieved by examining genetic distance among genotypes based on phenotypic traits using the clustering method. While using morphological and chemical traits, clones sorted together in distant groups are used as parents in crossings to acquire greater variety in hybridization and breeding programs.

Different cluster analysis methods were used to determine the variation among the tea clones based on morphological and chemical traits. The results showed that the Wards' method had the highest amount of cophenetic correlation coefficient (0.90). Therefore, cluster analysis was done with this method and tea clones were divided into three groups (Fig. 1). The first group consisted of three clones (100, 285 and 399), the second group contains two clones (272, 276) and the third group consisted of clones of 277, 269, 74 and 278. As stated, clones due to different genetic bases or other environmental factors are placed in completely separate groups. They can justify the ability of morphological and chemical traits to determine this distinction. The results of cluster analysis showed the differences between clones of each group with other groups and the similarity of clones within each group. The reason for differences in clones of groups can be due to differences in the genetic structure or the effect of other environmental factors on the traits.

Table 2. Variation of morphological and chemical traits of nine selected *Camellia sinensis* clones

Row	Traits	Number	Range	Mean±SD	%Phenotypic C.V
1	Number of plucking shoots	9	134.66	143.85±58.12	40.40
2	Fresh weight of plucking shoots	9	70.61	79.14±25.30	31.97
3	Dry weight of plucking shoots	9	12.15	15.35±4.14	26.96
4	Length of plucking shoots	9	1.93	7.62±0.60	7.96
5	5^{th} leaf length	9	3.75	7.50±1.16	15.51
6	5^{th} leaf width	9	1.30	3±0.46	15.41
7	Leaf area	9	18.37	23.14±6.20	26.82
8	Internode distance	9	2.96	3.86±0.96	24.96
9	Green leaf yield	9	561.62	379.29±214.18	56.47
10	Water extract	9	5.19	38.43±1.69	4.40
11	Polyphenol	9	2.03	13.50±0.64	4.74
12	Total ash	9	1.30	6.39±0.42	6.58
13	Dry matter	9	4.87	19.77±1.77	8.96

Table 3. Analysis of variance of the traits in tea clones

S.O.V	df	Mean of squares						
		N.S ($n.m^{-2}$)	F.W ($gr.m^{-2}$)	D.W ($gr.m^{-2}$)	L.S (cm)	L.L(cm)	L.W (cm)	L.A (cm^2)
Block	2	173.03^{ns}	301.90^{ns}	10.52^{ns}	0.06^{ns}	0.95^{ns}	0.09^{ns}	23.66^{ns}
Clones	8	10135.25^{**}	1921.07^{**}	51.46^{*}	1.11^{ns}	4.06^{**}	0.64^{*}	115.59^{**}
Error	16	597.70	433.13	13.53	0.71	0.64	0.17	27.31
CV(%)		16.99	26.29	23.95	11.10	10.66	14.01	22.58
S.O.V	df	Mean of squares						
		I.D (cm)	L.Y ($gr.m^{-2}$)	W.E (%)	P (%)	T.A (%)	D.M (%)	
Block	2	0.16^{ns}	13.43^{ns}	4.21^{ns}	0.26^{ns}	0.08^{ns}	0.17^{ns}	
Clones	8	2.78^{**}	137633.30^{**}	8.56^{**}	1.23^{**}	0.53^{**}	9.42^{**}	
Error	16	0.55	85.80	1.05	0.15	0.03	2.06	
CV(%)		19.15	12.44	2.66	2.90	2.88	7.25	

ns,*, and **: non-significant, significant at 5 and 1% probability levels, respectively.
N.S: number of plucking shoot, F.W: fresh weight of plucking shoot, D.W: dry weight of plucking shoot, L.S: length of plucking shoot, L.L: 5^{th} leaf length, L.W: 5^{th} leaf width, L.A: leaf area, I.D: internodes' distance, L.Y: green leaf yield, W.E: water extract, P: polyphenol, T.A: total of ash and D.M: dry matter.

Table 4. Comparison of means of nine selected clones based on morphological and chemical characters

Clones	N.S ($n.m^{-2}$)	F.W ($gr.m^{-2}$)	D.W	L.S	L.L	L.W
272	86.67^{c}	49.41^{cd}	9.49^{c}	8.37^{a}	8.39^{a}	3.12^{a-c}
277	164^{b}	88.05^{a-c}	15.62^{a-c}	7.72^{ab}	7.83^{ab}	3.01^{a-c}
100	102.67^{c}	72.25^{b-d}	14.20^{bc}	8.16^{a}	7.28^{ab}	3.37^{ab}
285	214.67^{a}	111.55^{a}	21.64^{a}	8.27^{a}	5.02^{c}	2.18^{d}
74	122.67^{bc}	67.37^{b-d}	14.66^{a-c}	7.64^{ab}	6.47^{b}	2.62^{b-d}
399	221.33^{a}	114.04^{a}	20.25^{ab}	7.19^{ab}	8.77^{a}	3.36^{ab}
276	86.67^{c}	43.43^{d}	9.78^{c}	7.30^{ab}	7.41^{ab}	2.52^{cd}
278	89.33^{c}	68.99^{b-d}	14.73^{a-c}	6.44^{b}	8.44^{a}	3.48^{a}
269	206.67^{a}	97.23^{ab}	17.82^{ab}	7.55^{ab}	7.92^{ab}	3.39^{ab}

Table 4 (continued). Comparison of means of nine selected clones based on morphological and chemical characters

Clones	L.A ($c.m^2$)	I.D (c.m)	L.Y (gr/m^2)	W.E (%)	P (%)	T.A (%)	D.M (%)
272	26.21^{ab}	2.98^{cd}	157^{h}	41.60^{a}	13.74^{a}	6.02^{cd}	18.79^{cd}
277	24.05^{ab}	2.30^{d}	218.80^{g}	37.73^{bc}	14.19^{a}	6.75^{b}	17.86^{d}
100	24.81^{ab}	3.71^{bc}	647.73^{b}	40.50^{a}	13.95^{a}	6.29^{c}	19.66^{b-d}
285	11.34^{c}	4.23^{a-c}	705.33^{a}	37.80^{bc}	13.89^{a}	6.30^{c}	19.59^{b-d}
74	17^{bc}	4.39^{a-c}	297.42^{e}	38.58^{b}	13.71^{a}	6.20^{cd}	21.82^{ab}
399	29.62^{a}	4.89^{ab}	583.33^{c}	38.53^{b}	13.65^{a}	6.78^{b}	17.94^{d}
276	18.69^{bc}	3^{cd}	143.71^{h}	36.58^{c}	12.78^{b}	5.88^{d}	22.74^{a}
278	29.71^{a}	4.04^{a-c}	379.25^{d}	36.42^{c}	12.15^{b}	7.18^{a}	21.21^{a-c}
269	26.80^{ab}	5.2^{a}	281.40^{f}	38.11^{bc}	13.46^{a}	6.14^{cd}	18.35^{d}

Means with a common letter do not differ from other means ($p \leq 0.05$).
N.S: number of plucking shoot, F.W: fresh weight of plucking shoot, D.W: dry weight of plucking shoot, L.S: length of plucking shoot, L.L: 5^{th} leaf length, L.W: 5^{th} leaf width, L.A: leaf area, I.D: internodes' distance, L.Y: green leaf yield, W.E: water extract, P: polyphenol, T.A: total of ash and D.M: dry matter.

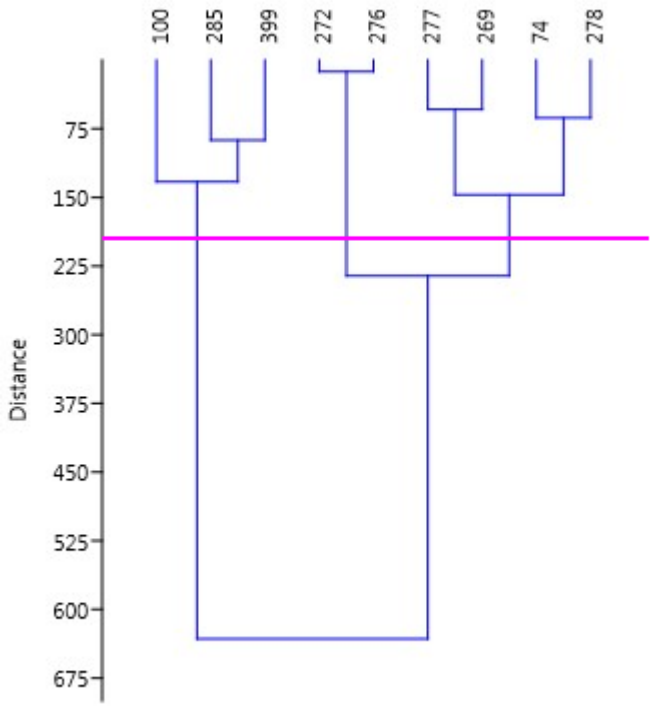

Fig. 1. Cluster analysis based on morphological and chemical traits with ward's method in tea clones.

To show the value of each cluster from 13 measured attributes, the percentage of deviation from the mean of clusters was calculated from the total mean (Table 5). Since clones in each cluster had a greater genetic relationship with clones in the other clusters, the first cluster was composed of three clones and the value of number of plucking shoot, fresh and dry weight of plucking shoot, length of plucking shoot, internodes' distance, green leaf yield and polyphenols showed a higher value than the total average value. The second group consisted of two clones and the average value of the number of plucking shoot, fresh and dry weight of plucking shoot, leaf width, leaf area, internodes' distance, green leaf yield, polyphenol and total ash was less than the total average value. Traits likes' leaf width, leaf area and total ash in clones of the third group showed a higher value than the total average. With regard to the cases mentioned above, crossing among clones sorted in first and second clusters will result offsprings with high yield and quality.

It is emphasized that the tendency toward using similar parents, lack of recognition and using new cultivars in breeding programs leads to a reduction in genetic diversity (Yan et al., 2016). However, farther cultivars with more polymorphism show more genetically distinction, and in terms of hybridization, cultivars with more differences will have the potential for more heterosis or transferring rare traits to the database. To the best of our knowledge, this is the first report that shows promising results of applying morphology and biochemistry for investigating genetic diversity in Iranian selected tea clones.

Yu and Xu (1999) used morphological characters to evaluated diversity in tea germplasm resources of China. In another research, morphology, biochemistry and allozyme studies has been used to present genetic diversity and segregation of *C. sinensis* (cultivated tea) and its wild relatives in Yunnan province of China (Chen et al., 2005). According to Kim et al. (2012), the relationship between catechin-rich and poor lines of tea bushes was mainly analyzed using plants' morphological characteristics and DNA. Genetic diversity of 51 accessions of tea landraces was studied based on agronomic and quality characteristics by Jinang et al. (2013). In a study of 15 tea accessions in Vietnam based on 21 morphological traits, the accessions were clustered by UPGMA cluster and Euclidean distance in two main groups, in the first group 12 accessions of Asami and in the second group three accessions of Chinese type (Phong et al., 2016).

Genetic relationship in selected tea clones

DNA was extracted from leaves of the nine selected tea clones and then analyzed by SCoT analysis using 13 random primers (Table 6). The various sizes of DNA bands were produced in the 13 primers (Table 7).

Amplification of genomic DNA procreated a total of 165 bands with an average of 12.69 bands per primer and generated 122 polymorphic bands patterns with an average of 9.38 bands per primer. The maximum number of polymorphic bands was scored with primer SCoT15 (12 bands, 85.71%). However, a minimum polymorphic band was created by primer SCoT9 and SCoT37 (7, 63.64%). Wachira et al. (2001) announced that 72% of variation inhabited within populations of *C. sinensis* and its wild *Camellia* relatives based on RAPD and AFLP markers. Kaundun and Park (2002) stated that 16% of the total diversity of the RAPD-PCR marker was observed among populations of Korean tea (*C. sinensis*). SCoT is polymorph marker that was used in this study to investigate the genetic diversity of tea for the first time. The results showed that the SCoT marker is capable of detecting polymorphs well. The SCoT marker was used to study the genetic diversity of 8 Iranian modified wheat cultivars. Molecular evaluation results showed that SCoT marker had a high ability to evaluate diversity and differentiability of wheat cultivars (Hamidi et al., 2014).

Table 5. Mean and percentage average deviation of any group from total average for different traits of tea clones derived from cluster analysis

Traits	TA	AFG	PADF	ASG	PADS	ATG	PADT
N.S	143.85	179.56	35.70	86.67	-57.19	145.67	1.81
F.W	79.15	99.28	20.13	46.42	-32.73	80.41	1.26
D.W	15.36	18.70	3.34	9.64	-5.72	15.71	0.35
L.S	7.63	7.88	0.25	7.84	0.21	7.34	-0.29
L.L	7.50	7.02	-0.48	7.90	0.40	7.67	0.16
L.W	3.01	2.97	-0.04	2.82	-0.19	3.13	0.12
L.A	23.14	21.93	-1.21	22.46	-0.69	24.40	1.25
I.D	3.87	4.28	0.41	2.99	-0.88	4	0.13
L.Y	379.29	645.47	266.17	150.36	-228.94	294.13	-85.16
W.E	38.43	38.95	0.52	39.10	0.67	37.71	-0.72
P	13.51	13.84	0.33	13.26	-0.24	13.38	-0.12
T.A	6.40	6.46	0.06	5.95	-0.44	6.57	0.17
D.M	19.78	19.07	-0.71	20.77	0.99	19.81	0.04

TA: Total average, AFG: Average of first group, PADF: Percentage average deviation of first group from total average, ASG: Average of second group, PADS: Percentage average deviation of second group from total average, ATG: Average of third group, PADT: Percentage average deviation of third group from total average, N.S: number of plucking shoot, F.W: fresh weight of plucking shoot, D.W: dry weight of plucking shoot, L.S: length of plucking shoot, L.L: 5^{th} leaf length, L.W: 5^{th} leaf width, L.A: leaf area, I.D: internode distance, L.Y: green leaf yield, W.E: water extract, P: polyphenol, T.A: total of ash and D.M: dry matter.

Table 6. Characteristics of SCoT primers studied

Row	Primers	Nucleotide sequence (5′ to 3′)	Annealing temperature	%GC content
1	SCoT4	CAACAATGGCTACCACCT	48	50
2	SCoT5	CAACAATGGCTACCACGA	48	50
3	SCoT9	CAACAATGGCTACCAGCA	48	50
4	SCoT10	CAACAATGGCTACCAGCC	50	56
5	SCoT11	AAGCAATGGCTACCACCA	48	50
6	SCoT13	ACGACATGGCGACCATCG	52	61
7	SCoT14	ACGACATGGCGACCACGC	54	67
8	SCoT15	ACGACATGGCGACCGCGA	54	67
9	SCoT16	ACCATGGCTACCACCGAC	52	61
10	SCoT18	ACCATGGCTACCACCGCC	54	67
11	SCoT21	ACGACATGGCGACCCACA	52	61
12	SCoT28	CCATGGCTACCACCGCCA	55	67
13	SCoT37	CAATGGCTACCACTAGCC	50	56

Table 7. The percent of polymorphism, polymorphism information content (PIC), generated of molecular data in studied tea clones

Row	Primers	Polymorphic bands	Total bands	%Polymorphism	PIC	MI	Shannon	Nei
1	SCoT4	10	14	71.43	0.37	2.76	0.51	0.33
2	SCoT5	9	13	69.23	0.44	2.92	0.48	0.31
3	SCoT9	7	11	63.64	0.47	2.46	0.60	0.42
4	SCoT10	8	10	80.00	0.48	2.85	0.60	0.41
5	SCoT11	7	9	77.78	0.35	1.81	0.61	0.43
6	SCoT13	11	15	73.33	0.36	2.95	0.50	0.32
7	SCoT14	10	13	76.92	0.41	3.04	0.54	0.35
8	SCoT15	12	14	85.71	0.32	2.88	0.47	0.30
9	SCoT16	11	15	73.33	0.30	2.48	0.62	0.43
10	SCoT18	10	14	71.43	0.35	2.56	0.58	0.40
11	SCoT21	9	12	75.00	0.35	2.30	0.65	0.46
12	SCoT28	11	14	78.57	0.35	2.81	0.58	0.39
13	SCoT37	7	11	63.64	0.40	2.10	0.63	0.44

Molecular genetic diversity between selected tea clones was designated through SCoT-PCR analysis. Genetic similarity across selected tea clones was further analyzed thanks to the results of SCoT analysis. Genetic similarity amongst nine selected clones showed a range between 0.16 and 0.86. The lowest similarity amongst selected tea clones (0.16) was observed in clones 272, 276 and 278. Intercrossing of clones with the lowest similarity (maximum spacing) will give the best result in order to achieve hybrids or attain maximum separation in the next generation. The UPGMA cluster analysis with Jaccard coefficient based on SCoT marker showed the highest amount of cophenetic correlation coefficient (0.91) and placed the tea clones in three distinct groups (Fig. 2). The first group included of clones 272 and 276. The second one consisted of clones 100 and 399, and the third group contained clones 269, 277, 278, 285 and 74 (Fig. 2). Possibility to achieve optimal results can be achieved by crossing between distant clones selected from spaced clusters. It is expected that these results could be used in breeding programs of highly valuable tea clones.

Correlation between the similarity coefficient matrix of molecular markers and matrix of morphological and chemical data with the mantel test was significant (0.44). This indicates that there is a correlation between the pattern of variation represented by the markers and the morphological and chemical data. The grouping of two methods (based on morphological, chemical and molecular marker) was partially identical, and one clone was in different group in two methods. This result may imply that the two systems have different estimates of genetic relationships between clones. The main reason for the discrepancy between grouping clones based on morphological and chemical traits can be that the most quantitative traits are controlled by a large number of gene and are strongly influenced by the environment. In addition, SCoT markers are randomly distributed throughout the genome (Rahimi et al., 2019). Other researchers also examined genetic variation of various accessions and varieties of tea with different markers, showing the diversity between them and placing the accessions in different groups (Mondal, 2002; Balasaravanan et al., 2003; Matsumoto et al., 2004; Hu et al., 2014; Beris et al., 2016).

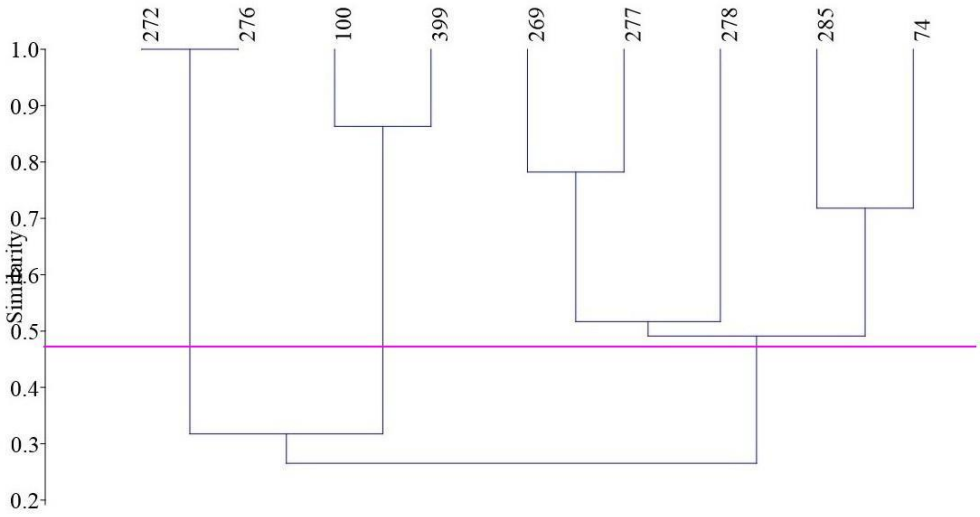

Fig. 2. Cluster analysis based on SCoT markers with UPGMA method and Jaccard's genetic similarity in tea clones.

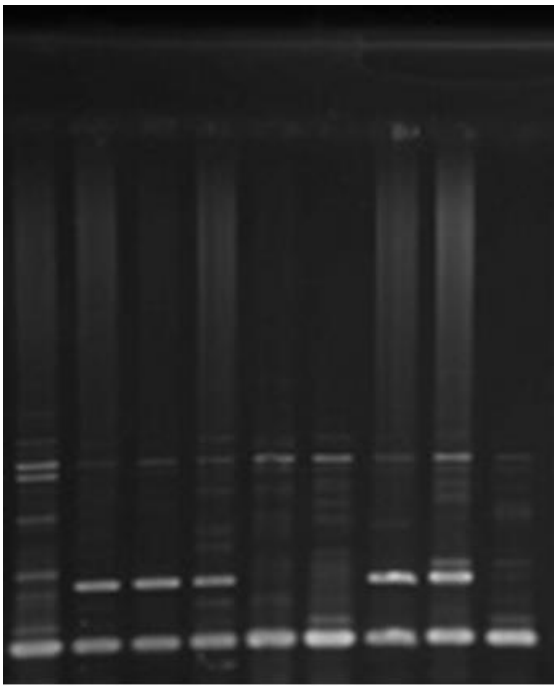

Fig. 3. Amplified fragment for primer SCoT 13.

CONCLUSIONS

The results showed that there was a high genetic variation among the tea clones in terms of morphological and chemical traits as well as the SCoT markers. The grouping of two methods (based on morphological, chemical and molecular marker) was partially identical. Having information about genetic diversity of tea germplasm for designing suitable breeding programs is very helpful to obtain suitable plants for specific purposes.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Impact of Ultraviolet-B radiation based on altitude on photosynthetic efficiency, growth performance and crop yield: a review

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ABSTRACT

Purpose: Ultraviolet-B radiation was inducing enormous stress at highland and coldest area since it increases more than 40% at highland when we compare with lowland. Therefore, this review aims to assess and depict impacts of Ultraviolet-B radiation on photosynthetic efficiency, growth performance, and yield of crops based on altitude. **Findings:** Indicate that ultraviolet-b radiation has a severe effect on photosynthesis, especially the coldest time. It reduces photosynthetic efficiency in such an area, but it depends on the type of the crop and cultivar difference. On the other hand, it reduces growth performance and biomass accumulation based on altitude. There is a contrasting view on a net-assimilation rate on different studies condition. The effect of UV-B on crop yield was more contrasting in some studies says no effect on other studies it says it affect, but this contradictory result was mainly due to the difference in study conditions, still current studies on Yield revealed that UV-B has a high impact on yield. **Research limitations:** Ultraviolet-B radiation has high effect on the highland area, but there is no much research focuses, but UV-B was profoundly affecting photosynthetic efficiency, growth performance and yield of crops on highland area. **Directions for future research:** UV-B was reducing crop production, and productivity at highland and this review gives more insights on UV-B impact at the highland and allow UV-B adaptive and preventive investigation in the future.

INTRODUCTION

Chlorofluorocarbon and N_20 are causing depletion of the stratospheric ozone layer, which protects plants, animals, and humans from Ultraviolet radiation (Sharma, 2001). Starting from the last eighty years, Ultraviolet-B radiation reaching to the earth increases as a result of stratospheric ozone depletion by chlorofluorocarbon due to industrialization of the world (Sharma, 2001). Ultraviolet radiation was part of the non-ionizing region of the electromagnetic spectrum and account 5 up to 9% of solar radiation (Hollosy, 2002). It generally categorized into three wavelength ranges: Ultraviolet-C (100–280 nm) is particularly detrimental to living things, but not reach to the earth surface due to blockage by stratospheric ozone layers; Ultraviolet-B (280–315 nm) partially absorbed by stratospheric ozone layer and the most harmful that affect living things but Ultraviolet-A (315–400 nm) are the only harmless part of Ultraviolet radiation (Hollosy, 2002). Ultraviolet-B was particularly affecting the productivity of herbaceous dicot crop plants because 18-41% of it penetrates the mesophyll cell of this plant (He et al., 1994). Meanwhile, strength mostly depends on the altitude of the location since based on altitude there is a difference in thickness of the stratospheric ozone layer (Helsper et al., 2003; Bjorn, 1996). According to (Pfeifer et al., 2006), as elevation increase, Ultraviolet-B may increase more than 40% at high elevation area and this difference is mainly due to change in seasonal ozone depletion at highland. The increase or decrease of the Ultraviolet-B effect on plants also depends on the altitude, strength, duration of exposure, time of the day, angle of the sun, and the plant species (McKenzie et al., 2003). Ultraviolet-B has harmful effects on the morphology of crop plants mainly the increase of thicker leaves, reduction of petioles length, increase leaf curling, and change in leaf shape; diminish stem elongation, increased auxiliary branching and altered root: shoot ratio depending on the altitude (Robson et al., 2014). Similarly, if the UV-B dosage exceeds the limits of tolerance, plant anatomy will be changed, and biomass is decreased (Coleman & Day, 2004; Kakani et al., 2003; Zhao et al., 2004). Biomass accumulation, the partition of assimilating, leaf area and plant height significantly reduced when plants are exposed to ambient and enhanced UV-B radiation (Zhao et al., 2004; Gao et al., 2003). On metabolism, UV-B reduces photosynthesis, decreased proteins, impair chloroplast function, and decrease the relative growth rate, and damage DNA (Agrawal, 1992). Correspondingly, leaf area and plant height drastically reduced when plants are exposed to ambient UV-B radiation for a long time (Zhao et al., 2004; Gao et al., 2003). UV-B also enhances protective response in plants such as the biosynthesis of flavonoids and anthocyanin components (Jansen, 2002; Jansen & Bornman, 2012; Robson et al., 2014). These flavonoids and anthocyanin prevent the transmittance of the UV-B to the plant cells, thus the exclusion of UV-B damage to the plant molecules (Jansen, 2002; Jenkins, 2014). Therefore, this review aims to assess, and depict the impact of Ultraviolet-B on photosynthesis, growth performance, and yield of crops on different altitudes.

UV-B radiation difference based on altitude and latitude

The first research on ultraviolet light as a wavelength was carried out in 1800 by Ritter (Berg, 2008). UV radiation on the earth's atmosphere was first well-known in 1881 by Hartley, when he was able to measure ultraviolet energy hitting the land surface and found it different depending on altitude. Now, regions on earth between 40°N and 40°S latitude receive 2-11 kJ m-2 d^{-1} of UV radiation and the potential to increase in the future due to more significant ozone loss (Taalas et al., 2000; Rowland, 2006). As shown in Figure 1, Exposure to UV-B along different lines of latitudes vary due to ozone depletion, increases of ozone depleted by 40% in the Antarctic in contrast negligible increases occurred in the mid-latitudes since the

1970s (McKenzie et al., 2003). Higher altitude were having higher levels of UV-B due to a thinner atmosphere, closeness of the sun to the earth's surface, solar angle, and low albedo (Caldwell et al., 1980). This finding was in agreement with (Pfeifer et al., 2006), which reported that UV-B irradiance could increase more than 40% at a high elevation; this variation is due to change in the level of ozone depletion with altitude change as a result of regional aerosol conditions, solar elevation and also low albedo.

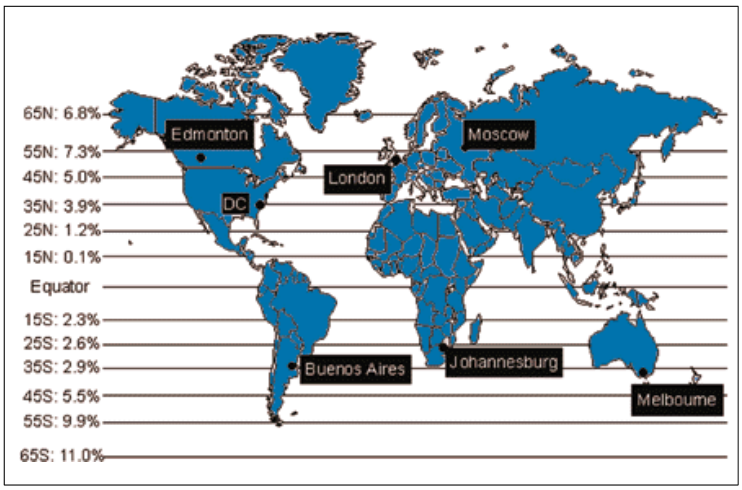

Fig. 1. UV-B trends of average annual increment and its strength based on latitude and altitudes (Lidon et al., 2012).

Ozone layer and Ultraviolet-B Radiation

Without the stratospheric ozone layer, a lot of UV-B radiation from the sun would not be blocked, reaching the earth's surface and causing incalculable damage to most living species. However, it is formed in the stratosphere when UV radiation from the sun strikes molecules of oxygen and causes the two oxygen bit to split apart, Freed atom bumps electrons into another O_2 it joins up forming ozone (O_3) this process is known as photolysis (Morrisette, 1989). Ozone measurement unit is the Dobson Unit, and it measures how thick the layer of the ozone when it is compacted into one layer at 0 degrees Celsius and with a pressure of one atmosphere above it, every 0.01 millimeter thickness of the layer is equal to one Dobson Unit (Margitan, 1991). Ozone concentration in the stratosphere across the globe is 300 DU or (a thickness of only 3mm at 0°C, and 1 atmospheric pressure). The ozone layer in the stratosphere filters UV-B wavelength from the earth's surface selectively (Rowland, 2006; Sarkar, 2011). However, currently, exposure to UV-B was increasing mainly due to depletion of the stratospheric ozone layer because of the high release of chlorofluorocarbon into the atmosphere and the breakage of the (O_3) atoms by chlorine (Rowland, 2006). The incidence of UV radiation varies by season with wintertime it increases as high as 35% per year while there is a 7% increase in summer due to greater ozone loss in colder temperatures catalyzing chlorine depletion of the gas (Kerr & McElroy, 1993). The same finding was reported by (Godin et al., 2001), stated that the stratospheric ozone trends in mid-latitude regions (25˚-60˚) show that ozone abundance over recent years was ≈ 4% below its 1979 values. The winter/spring and summer/autumn losses were of the order of 5.5% and 2.8%, respectively. According to studies at the middle of October, the highest UV-B symptom on plants grown at highland was seen, and this indicates stratospheric ozone depletion, UV-B, and cold temperature may have high correlation on its effect on photosynthesis, growth performance and yield of crops at the highland areas as showed in Figure 2. Similar findings were reported

by (Wuebbles et al., 1998) Ozone concentrations clearly drop in the southern spring (September to October), recovering their normal values in November, although this recovery has been progressively delayed in recent times.

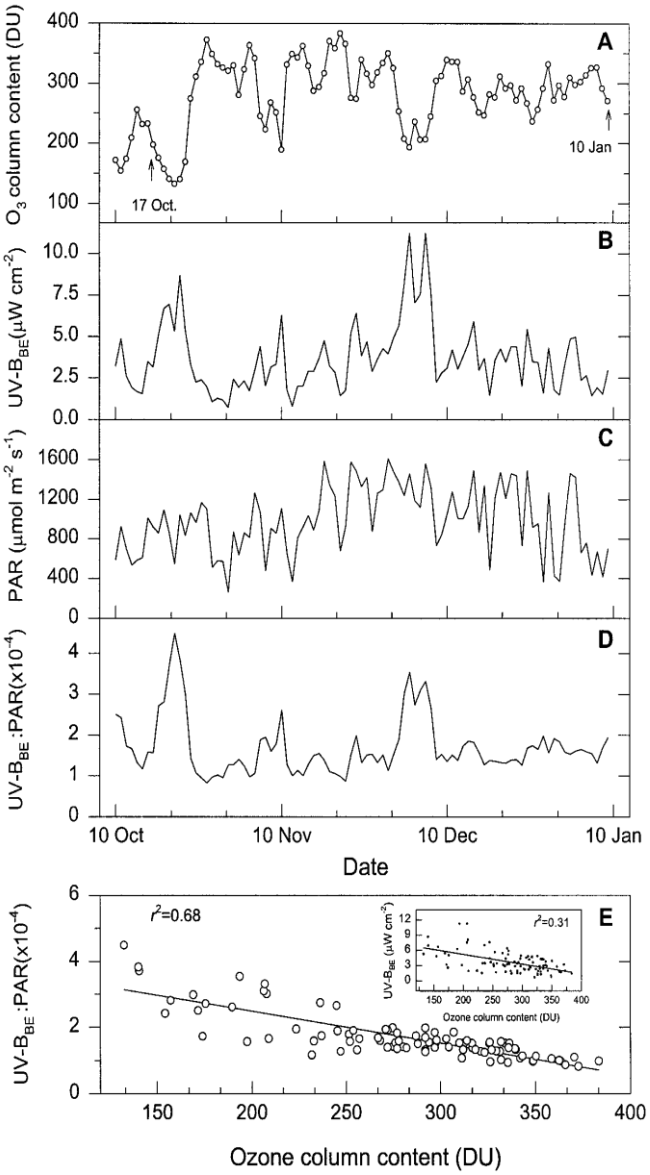

Fig. 2. Trends in total ozone column content (A), mid-day biologically effective UV-B (UV- B_{BE}; (B), mid-day PAR (C), and mid-day ratio of UV-B_{BE}-to PAR (D) at Palmer Station, Antarctica during the experiment. pointes indicate when plants were placed under UV-B treatments (October17,1998) and when they were harvested at the end of the experiment (January10, 1999). Ozone column content was measured with the National Aeronautical and Space Administration Total Ozone Mapping Spectrometer. Mid- day UV-B $_{BE}$ was taken as the mean of five measurements made at 15-min intervals between 12 noon and 1:15 PM by the SUV-100 spectroradiometer at Palmer Station that is part of the U.S. National Science Foundation's Polar UV Monitoring Network. PAR was measured with quantum sensors. The mid-day ratio of UV-B_{BE}-to-PAR was calculated in unit so Radiant flux density (e.g.m Wcm^{22}), after converting PAR data from units of photon flux density to radiant flux density by assuming an average wavelength of 550 nm. There were negative correlations between ozone column content and UV-B_{BE}-to- PAR (E) and UV-B_{BE}. Source: (Xiong & Day, 2001).

Impact of UV-B on photosynthetic efficiency of crops

The reduction of the stratospheric ozone has led to an increase in UV-B radiation in recent decades (Schrope, 2000), and this increasing UV-B radiation reduce photosynthetic efficiency by directly altering photosynthetic process (Reddy et al., 2003), water metabolism (Fuhrer & Booker, 2003), and partitioning the carbon from growth pools to secondary metabolic pathways (Bassman, 2004). However, mostly it can damage plant cell membrane structure (Tanyolac et al., 2007). Yet, UV-B has a severe effect on plant metabolism, and according to the chlorophyll fluorescence test at highland, 0.72 was recorded, and this indicates UV-B reduce photosynthesis efficiency (Nogues et al., 1998), as shown in Figure 3, Table 1 and 2. The increasing levels of UV-B radiation currently shown inhibition of photosynthesis in pea, (Reddy et al., 2003; Zhao et al., 2004) in cotton, and (Allen et al., 1998) in oilseed rape.(Yao et al., 2006) reported that ambient and enhanced UV-B affects photosynthetic pigments that may reduce photosynthesis. Similar findings were reported by (Kataria et al., 2013) decrease in biomass has been associated with reduced rate of photosynthesis due to the impact of ambient UV-B. However, this low photosynthetic efficiency depends on the type of crop, and cultivar differences. According to the same findings reported by (Briscoe & Chittka, 2001; Irani & Grotewold, 2005; Chalker-Scott, 1999; Gould, 2004) anthocyanin are primarily known for their bright red colors and in plants anthocyanin was synthesized in response to excessive UV-B condition. (Jansen, 2002; Jansen & Bornman, 2012; Jenkins, 2014; Robson et al., 2014) reported similar findings that stated UV-B protective response in plants such as the biosynthesis of flavonoids and anthocyanin components that synthesized as a response to UV-B. These flavonoids and anthocyanin prevent the transmittance of the UV-B in to the plant cells, thus the exclusion of UV-B damage to the plant molecules (Jansen, 2002; Jansen & Bornman, 2012; Jenkins, 2014; Robson et al., 2014). (Oren-Shamir & Levi-Nissim, 1997) reported that the increase in anthocyanin content in the leaf in response to the UV-B showed more favorable performance ratings due to color. Plants produce a wide range of flavonoids and related phenolic compounds which tend to accumulate in leaves of higher plants as response to UV radiation (Tevini & Teramura, 1989; Rozema et al., 1997). It has been suggested that plants developed UV-absorbing compounds to protect them from damage to DNA or physiological processes caused by UV radiation (Stapleton, 1992).

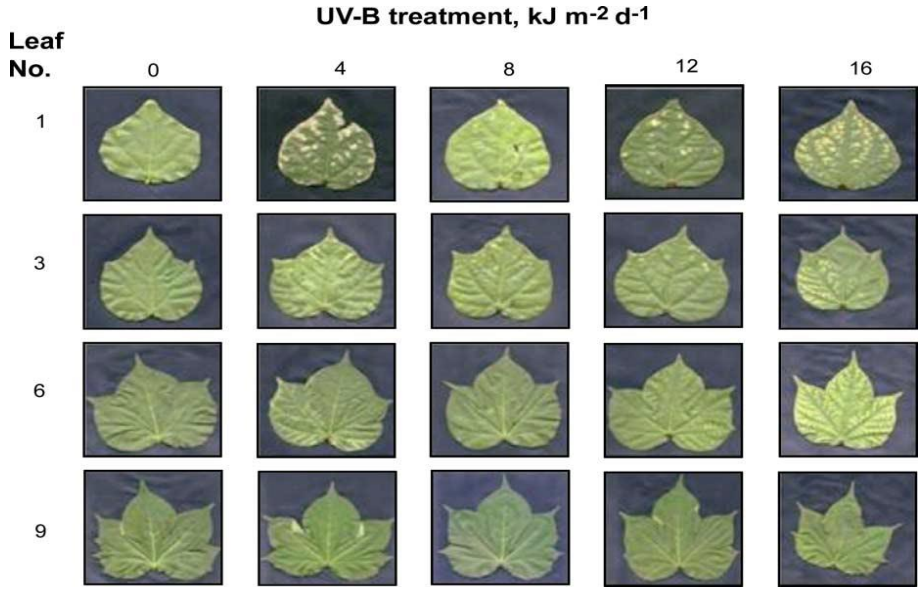

Fig. 3. Symptoms showing the damage caused by UV-B radiation on cotton leaves (Reddy et al., 2003).

These UV-absorbing compounds accumulate in the epidermis; preventing UV radiation from reaching the photosynthetic mesophyll cell (Stapleton, 1992; Braun and Tevini, 1993). (McKenzie et al., 2003; Caldwell et al., 1980) reported that higher elevations having higher levels of UV-B due to a thinner atmosphere, closeness of the sun to the earth surface, solar angle and low reflectivity. (Pfeifer et al., 2006) reported that UV-B irradiance increase more than 40% at highland area; this difference is due to change in the level of ozone depletion with elevation change and at highland area there is high seasonal ozone depletion and UV-B radiation.

Table 1. photosynthetic efficiency of sunflower plants grown without UV-B (-UV-B) and with UV-B (+UV-B) after 12 and 21 days of UV-B exposure

		Leaf 5		Leaf 13	
Sample	Variable	- UV-B	+UV-B	-UV-B	+UV-B
1st	*Ci*	174.29±13.03a[1]	152.43±17.85a	nd[2]	nd
	E	5.30±0.27a	3.81±0.20b	nd	nd
	gs	0.41±0.05a	0.20±0.02b	nd	nd
	A	26.87±0.21a	19.22±0.70b	nd	nd
	Fo	0.272±0.007a	0.292±0.005b	nd	N
	Fv	1.836±0.009a	1.818±0.020a	nd	dn
	Fv/Fm	0.868±0.001a	0.861±0.003a	nd	d
2nd	*CI*	257.83±9.41a	235.83±4.09a	208.50±8.14a	190.83±9.04a
	E	4.15±0.12a	3.68±0.28a	4.69±0.10a	4.25±0.19a
	gs	0.39±0.03a	0.29±0.05a	0.53±0.04a	0.37±0.04b
	A	13.63±1.15a	13.88±1.08a	23.70±0.67a	21.94±0.69a
	Fo	0.280±0.003a	0.282±0.005a	0.271±0.007a	0.263±0.003a
	Fv	1.830±0.017a	1.814±0.023a	1.839±0.016a	1.817±0.023a
	Fv/Fm	0.867±0.014a	0.865±0.024a	0.872±0.002a	0.874±0.001a

Source : (Cechin et al., 2007).

Table 2. Chlorophyll content (mg g^{-1}) of leaves of sunflower plants grown without UV-B (-) and with UV-B (+UV-B) after 12 and 21 days of UV-B exposure

		Leaf 5		Leaf 13	
Sample	Variable	- UV-B	+UV-B	- UV-B	+UV-B
1st	Chl*a*	1.81±0.01a[1]	1.61±0.07b	nd[2]	nd
	Chl*b*	0.58±0.01a	0.56±0.02a	nd	nd
	Chl*a*/*b*	3.09±0.03a	2.88±0.02b	nd	nd
	Chltotal	2.39±0.02a	2.16±0.09a	nd	nd
2nd	Chl*a*	1.75±0.12a	1.64±0.03a	2.27±0.14a	2.25±0.08a
	Chl*b*	0.69±0.05a	0.59±0.02a	0.78±0.03a	0.76±0.03a
	Chl*a*/*b*	2.57±0.18a	2.79±0.04a	2.91±0.10a	2.95±0.04a
	Chltotal	2.43±0.15a	2.23±0.05a	3.05±0.17a	3.01±0.11a

Source: (Cechin et al., 2007).

Impact of UV-B on growth and dry biomass accumulation of crops

The growth rate is a measure of how fast dry matter is stored in standing crops, and relative growth rate is an increase of dry mass per increment in time divided by existing biomass, but net-assimilation rate represents a plant's net photosynthetic effectiveness in capturing light; assimilating Co_2 and storing photoassimilates. According to Liu et al. (2013) report, UV-B radiation decreases the seed growth rate of three soybean cultivars on average by 12.5%, as shown in Table 3. The seed growth rate is shown to be a function of the cotyledon cell number, and the supply of assimilate to the developing cotyledons. (Egli et al., 1989), and (Feng et al., 2001) indicated that UV-B radiation reduces total biomass and yield per plant by 24.2% and 23.3% respectively. The same findings reported by (Kakani et al., 2003) state that exposure to UV-B radiation decrease the growth of leaves and stems in many plant species at both controlled environment and field studies, as shown in Figure 4. Zuk-Golaszewska et al. (2004) reported that high levels of UV-B decrease the relative growth rate by affecting nitrogen productivity, leaf area ratio, leaf area productivity, and leaf nitrogen productivity. However, Avery et al. (2004) reported that UV-B radiation often has an inhibitory effect on plant growth (up to 20%) in herbaceous species and, to a lesser extent, in woody perennials. studies done before state that partition of assimilating (net assimilation rate), and growth rate were reduce when plants exposed to enhanced UV-B radiation and this effect was due to its effect on photosynthesis and stomatal conductance (Zhao et al., 2004; Gao et al., 2003). However, contrasting result on net-assimilation rate was reported, prolonged exposure to UV-B light affect net assimilation, and relative growth rate in some rice cultivars (Dai et al., 1997), but high Ultraviolet-B radiation at highland area has no effect on net-assimilation rate this result was mainly due to light quality difference on different altitude, and the difference in cultivar and variety that alleviate the UV-B effect at ambient conditions. On the other hand, a decrease in biomass has been linked to a reduced rate of photosynthesis in many crop species by supplemental and ambient UV-B (Kataria et al., 2013). Other similar findings were reported which state that exclusion of UV-B from ambient level may lead to an increase in biomass production of various land plants and this indicates UV-B has severe effect on biomass production of various land plants (Mazza et al.,1999; Xiong & Day, 2001), and also there has been reported that the aboveground biomass may be reduced with exposure to UV-B radiation (Ballaré et al., 2001; Phoenix et al., 2002; Robson et al., 2003; Rozema et al., 2005).

Fig. 4. Effect of UV-B radiation on growth performance of Avena fatua (from the left 0, 4, 8 and 12 kJ/m2/d UV-B). Source: (Zuk-Golaszewska et al., 2004).

Table 3. Impact of UV-B on soybean yield and yield components

Cultivars	UV-B treatments	Yield per plant(g)	Pod number per plant	Seed number per plant	Seed number per pod	Seed size (mg)
H339	UV-B	12.3**	23.1**	48**	2.06	256**
	CK	20.2	32.8	70	2.12	289
HN35	UV-B	8.9**	26.5**	50**	1.89	175**
	CK	15.2	38.1	75	1.98	202
KN18	UV-B	7.7**	27.3**	58**	2.11	132**
	CK	15.6	48.3	104	2.16	150

Source: (Liu et al., 2013).

Impact of UV-B on crop yield

Many studies indicate that UV-B affects crop yield depending on the altitude at ambient condition and the types of the crop as well as the response of the cultivars. Reactions of various plants to UV-B radiation at both controlled environment and field studies; almost half of the studies showed that ambient, and enhanced UV-B radiation decrease yield, the other half showed no UV-B effect on yield (Kakani et al., 2003). Previous report indicate that UV-B reduces yield and the main reason for the reduction of crop yield with ultraviolet radiation is damage to organ membranes such as chloroplasts which make other stresses specifically, oxidative stress (Correia et al., 1999). These findings were in agreement with Yao et al. (2006) state that ambient and enhanced UV-B radiation probably affects photosynthesis and reduce economic yield. However, the contrasting result was reported by Hakala et al. (2002) who studied sensitivity of many plant species including barley, wheat, oat, clover, timothy, fescue and potato to UV-B radiation exposure (as if ozone layer decrease below 30%), and found no significant variation on biomass accumulation or yield of crop plants. However, recent experiments (Liu et al., 2009; 2013) indicated that UV-B radiation significantly decreases soybean yield per plant, as shown in Table 3. They showed that on soybean cultivars yield decrement was by 43.7%, and this yield reduction was mainly due to change in pod number per plant. Meantime, UV-B radiation decrease the seed size of three soybean cultivars by 12.3% (Liu et al., 2009; Chen et al., 2004) reported that the seed weight of the 15 soybean cultivars decreases quite significantly.

CONCLUSION

Based on the review, the following are concluded UV-B effect was more at the highland area, which revealed by research finding, physiological and visual observation of the UV-B symptom on plants mainly leaf curling. However, severity was high during at the season of low temperature or coldest time. This could be due to high ozone depletion at this time since at low temperature or coldest time the diffusion of gaseous molecules into the atmosphere was high, which could be mainly due to chlorine that depletes the ozone layer, and result in high UV-B incidence. This high UV-B has a severe effect on metabolism that result reduction of photosynthetic efficiency, biomass, growth performance and yield. Therefore**,** future research physiological, and molecular mechanisms of UV-B effect on plants must be studied**.** Natural and artificial UV-B exclusion mechanisms must be practiced at high altitude areas specifically, for dicot plants since they are the most sensitive, and further research must be needed to take UV-B adaptive and preventive measures.

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Conflict of interest
There is no conflict of interest on the manuscript.

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Enhancing high throughput sequencing unveils changes in bacterial communities during ready-to-eat lettuce spoilage

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ABSTRACT

Purpose: Spoilage flora is critical in vegetal ready to eat (RTE) product shelf-life and selecting efficient spoilage control technologies depends on the microorganisms present. This manuscript describes the evolution of the bacteriome of Batavia RTE lettuce, from fresh lettuce up to completely spoiled (day 14) and correlate these results with the sensorial characteristics. **Research Method:** The microbiome of vegetal RTE were examined using culture-dependent and culture-independent (16S rRNA metabarcoding) methods. Culture-dependent methods were related with the metagenomic results and sensory analysis to describe the evolution during spoilage and shelf-life. **Findings:** Our results demonstrated that the RTE lettuce bacteriome during spoilage is dominated by Gram-negative bacteria, mainly *Flavobacterium* and *Pseudomonas*. A bacterial population of 22 operational taxonomic units (OTUs) represent up to 96% of total bacterial reads and is maintained during the spoilage, representing the bacterial core of RTE lettuce. A high correlation was detected between culture-independent and culture-dependent results, both in general and selective culture media. Sensorial analysis of lettuce demonstrated that "odor" was the key parameter to determine the sensorial spoilage time and is related to total microbial load and to high concentrations of spoilage-related bacterial genera. **Limitations:** Hereby presented results are limited by the lettuce variety and by the storage conditions (MAP, 6°C, up to 14 days). **Originality/Value:** This paper describes an overview of the microbial and sensory evolution during spoilage of Batavia lettuce under MAP. A combination of culture-dependent and independent methods and sensorial analysis were used up to 14 days of storage.

INTRODUCTION

Minimally processed vegetables are appealing to consumers because they represent a good compromise between healthy food and convenience. Consumer acceptability and likeness of ready to eat (RTE) fresh-cut fruit and vegetable products are measured as a combination of sensorial attributes, such as visual appearance, texture and flavor, as well as a combination of nutritional and safety aspects. In this regard, the maintenance of these features for a longer time has a critical impact on producers' economic benefits by reducing costs while ensuring consumer satisfaction. The unavoidable spoilage of vegetables with time is characterized by brown discoloration, necrosis, loss of texture, exudation and/or production of off-flavors (Ponce et al., 2002). Most studies linked sensory rejection of RTE vegetables point to an increased microbial load (Jacxsens et al., 2003; Ragaert et al., 2006).

The bacterial community associated with plant tissues (phyllosphere), is a complex ecosystem, were human pathogens and no pathogens can survive and/or grow. The microbiological studies on RTE vegetable products have focused on food security, although no pathogens contribute greatly to sensory impairment. However, the so-called specific spoilage organisms (SSO) are responsible for generating off-odors and flavors, shortening the life of these products. These SSO have been less studied, despite the knowledge of who they are and how they behave is crucial in order to develop efficient conservation and packing strategies.

The number and type of microorganisms found on fresh products are highly variable and dependent on product type, agronomic practices, weather conditions as well as the harvest, transportation and further processing and handling (Ahvenainen, 1996; Olaimat & Holley, 2012). Gram-negative bacteria dominate the microflora associated with most vegetables (Tournas, 2005). The microbiota of vegetables and fruits is made up largely of *Pseudomonas spp*., *Erwinia herbicola*, *Flavobacterium*, *Xanthomonas*, and *Enterobacter*. Lactic acid bacteria, such as *Leuconostoc mesenteroides* and *Lactobacillus spp*., are also commonly found. Finally, yeasts such as *Torulopsis*, *Saccharomyces* and *Candida* as well as various molds (like *Alternaria*, *Penicillium*, *Fusarium* and *Aspergillus*) are part of the dominant microorganisms, mostly in fruits due to their high sugar content (Caponigro et al., 2010; De Azeredo et al., 2011).

The application of molecular techniques in the microbial ecology of food has changed the way of studying the microbial diversity in complex food ecosystems, including vegetables (Abriouel et al., 2008). As a result, molecular approaches have been successfully applied to describe the bacterial ecosystem in (RTE) vegetables, or even to detect viruses in lettuce leaves (Aw et al., 2016). Most of these studies have monitored the microbial communities in lettuce, focusing on the initial bacteriome or on the differences between cultivation season, cultivation characteristics of lettuce variety (Allende et al., 2004; Caponigro et al., 2010; Oliveira et al., 2010; Salgado et al., 2014). New molecular identification techniques in microbial ecology have revealed new dominant species in plant products, such as *Oxalobacter* or *Flavobacterium* in RTE salads, while new microbial species have been described in RTE spinach (Lopez-Velasco et al., 2011). Comparing the results obtained by different research groups, lettuce variety seems to be a critical factor in bacterial composition. For example, Rudi et al. (2002) compared the dominant microorganisms in Norwegian and Spanish RTE lettuces and concluded that *Pseudomonas* was the dominant group in Norwegian lettuce and *Enterobacteriaceae* was dominant in the Spanish ones.

Batavia lettuce (*Lactuca sativa*, L), also known as summer crisp lettuce, is the most common lettuce variety in the north area of Spain, and it is widely used for RTE salads. The aim of this work was to monitor the changes in RTE Batavia lettuce quality and to

characterize the microbial ecosystem during refrigerated storage with a combination of complementary methods, sensory, conventional microbiology and molecular analysis, in order to better understand the spoilage process in this important commercial product. A deep characterization of bacteriome is the base of further works trending to increase the product's shelf-life.

MATERIAL AND METHODS

Product characterization

Batavia lettuce was produced and packaged (400 ± 15 g) under a modified atmosphere (MAP 100% N_2) by a local manufacturer (Spain). Samples were stored at 4 ± 0.5 ºC for 24 hours and transported to the laboratory under refrigerated conditions (day 1) and subsequently incubated at 6 ± 0.5 ºC in a climatic chamber up to 14 days. Commercial shelf-life was eight days according to the manufacturer.

At each sampling time (1, 2, 4, 7, 9, 11, 14 days), 10 g of lettuce were aseptically transferred to stomacher bags, 90 mL of peptone water with added NaCl (APT, 0.5% NaCl w/v, Pronadisa, Spain) and it was homogenized for 1 min in a Stomacher (Seward Stomacher Lab-Blender 400, Seward Medical, London, UK). Appropriate decimal dilutions from the homogenized were prepared in APT and plated to enumerate the following microorganisms: (i) total viable bacteria (TVC) were enumerated in plate count agar (PCA, Pronadisa, Spain) incubated for 7 days at 10ºC; (ii) *Enterobacteriaceae* were enumerated in violet red bile glucose agar (VRBGA, Pronadisa, Spain) incubated aerobically at 37ºC for 1 day; (iii) Lactic acid bacteria were enumerated on MRS (Pronadisa, Spain) for 2-3 days at 30ºC under anaerobic conditions; (iv) *Pseudomonas* were counted in *Pseudomonas* agar (CM0559B with SR103 supplement, both from Oxoid, UK) and incubated for 1-2 days at 25ºC; (v) Yeast were enumerated in Saboureau agar (Pronadisa, Spain) supplemented with chloramphenicol (0.1g.l^{-1}) after 48 hours at 25ºC. At each sampling time, 3 independent lettuce-bags were used as independent replicates. All analyses were done in duplicate for each independent replicate. The bags were disposed after each sampling day.

The CO_2 and O_2 concentrations in the headspace of RTE lettuce bag were determined in duplicate at each sampling time using a PBI Dansensor Checkmate 9900 gas analyzer (Ringsted, Denmark). Additionally, lettuce cleaning water (inlet) was aseptically sampled in the production facilities at the packaging day. One hundred (100) ml of this sample was filtered (Microplus 21 STL system, 0.45 µm pore, Whatman) and filter was used for the same microbiological analysis described for lettuce.

DNA extraction, and amplicon library generation and sequencing

In each sampling day, 15 ml of the samples prepared as abovementioned (1/10 in APT) were spin down at 3600 g for 10 minutes at 4 ºC, the supernatant was removed and the pellet stored at -20 ºC up to DNA extraction for no more than 15 days). The DNA was extracted using Wizard® SV Genomic DNA Purification System (Promega, USA) with minor modifications. Briefly, bacterial pellet and 0.5 g of glass-beads were diluted in 600 µl of lysis solution and beadbeated (6 x 20 sec on and 10 sec off; Disruptor genie, Scientific industries inc., USA). Bacterial debris were spin down (30 sec, 3000 g), the pellet was discarded and the purification continued as proposed by the manufacturer. Finally, DNA was diluted in 50 µl of sterile MilliQ water and DNA concentration was quantified using Nanodrop spectrophotometer (Nanodrop Technologies, USA).

Amplicon libraries were prepared using the primers Gray28F (5'-TTGATCNTGGCTCAG-3') and Rev338 (5'-TGCCTGCCTCCCGTGGAGT-3'), which

target the V1-V2 region of the small subunit ribosomal RNA (16S rRNA) gene (Huse et al., 2008). These primers are appropriate for 454 sequencing following manufacturers specifications for Lib-L preparation. For each reaction, 20-40 ng of DNA was mixed with 25 µl of 2X DNA polymerase master mix red (Ampliqon, USA), 1µM of each primer solution and MilliQ water up to 50 µl. The DNA was subsequently amplified under the following conditions: 10 min at 98 ºC, 30 cycles of 10 s at 98 ºC, 30 s at 53 ºC and 30 sec at 72 ºC, and a final step of 10 min at 72 ºC. The PCR product length was verified using gel electrophoresis with 0.8% agarose in TAE buffer at 70 v (Biorad, Madrid, Spain) and visualised in a Biodoc-it imaging system (UVP, Upland, USA). PCR products were purified using Illustra GFX PCR DNA and Gel Band Purification Kits (GE Healthcare, Spain) following the recommended protocol. DNA concentration was quantified using a nanodrop spectrophotometer (Nanodrop Technologies, USA) and equimolar amounts of each PCR product were mixed in a pool. DNA was amplified by Emulsion-PCR before sequencing on 2 lanes of a 4-lane PicoTiterPlate (PTP). Pyrosequencing was performed with the Genome Sequencer (GS) FLX (Roche-454 Life Science) according to GS FLX Titanium (454-Roche Life Science) method manuals provided by Roche/using the massively parallel pyrosequencing protocol (Margulies et al., 2005) in the CITIUS Biology Service (University of Sevilla, Sevilla, Spain).

Sequence analysis

Amplicon sequence analyses were performed using the Mothur 454 SOP (Schloss et al., 2009; 2011). Briefly, sequences were demultiplexed allowing 1 and 2 mismatches for the index and primer respectively and those shorter than 200 nucleotides were removed. Finally, the reads were aligned against the SILVA v128 (Quast et al., 2013) database with subsequent chimera and chloroplast and mitochondrial sequences removal. Only samples with > 1000 reads were retained for taxonomic analysis.

Sensory analysis

Sensory analysis was performed by six to eight previously trained panellists from the staff at the Food Research Division, AZTI, to evaluate the freshness of the lettuce. The panellists undertook five training sessions, each lasting 1 h, using a predefined glossary of attributes and three review sessions were performed before the start of the experiment. RTE lettuce was evaluated as described by ISO standard 4121:198721 using a five-point descriptive scale, where 1 is absolutely fresh, 3 is the rejection limit and 5 is completely spoiled. The following attributes of RTE lettuce were assessed: Texture, Flavour, Bright, Odour and Colour homogeneity. Lettuce was defined as unacceptable when the score of any sensory attribute was equal to or more than 3.

Statistical analysis

The software PSPP V0.6.2 (Free Software Foundation, Inc.) was used in order to perform the analysis of variance (ANOVA) and the least significant difference (LSD) statistical procedures for sensory and culture-dependent analysis. A confidence interval of 95% ($p \leq 0.05$) was used.

RESULTS AND DISCUSSION

Sensorial analysis and atmosphere evolution

Sensory analysis of the samples determined a shelf life of the product between 7 and 9 days, mainly due to sensorial defects in odour (Table 1), while the texture was the parameter with the lowest change during the storage time. These results agree with previously published

studies. Allende et al. (2004) studied "Lolo rosso " RTE lettuce and reported that aroma was the parameter with the lowest score at the end of shelf life, while the study of Ioannidis et al. (2018) with iceberg lettuce stored in anaerobic MA highlighted the odour as the critical rejection parameter after 6.6 storage days. The apparition of theses off-odours has been related with lettuce metabolism, microbial metabolism or a combination of both parameters (Ioannidis et al., 2018).

Oxygen concentration in the bags was reduced from 2% at day 1 to close to 0% after 4 days, without a significant increase along storage (Fig. 1). In contrast, CO_2 increased during the storage up to 11% in 11 days (Fig. 1). According to the bibliography, CO_2 increase during storage is usual in RTE vegetables (Rudi et al., 2002; Allende et al., 2004; Ares et al., 2008; Ioannidis et al., 2018) as a consequence of vegetal-tissue respiratory metabolism.

Culture dependent results

In the inlet cleaning water, microbiological counts in all considered agars were below the detection limit (10 cfu.ml^{-1}), demonstrating that water was not a contamination source. Cleaning water is intensively chlorate at inlet point to reduce the flora and can represent a selective factor in fresh lettuce.

Table 1. Evolution of sensorial scores along the storage

	Parameters				
Time (Days)	Texture	Flavour	Bright	Odour	Colour
1	8.2	8.1	6.9	7.0	8.2
2	7.0	7.3	9.1	7.1	7.3
4	6.5	6.6	5.1	5.9	6.7
7	6.5	5.7	5.4	5.4	5.6
9	6.0	4.7	4.5	4.5	4.7
11	4.1	4.0	3.3	3.0	3.3
14	2.0	0.6	3.0	0.6	1.6

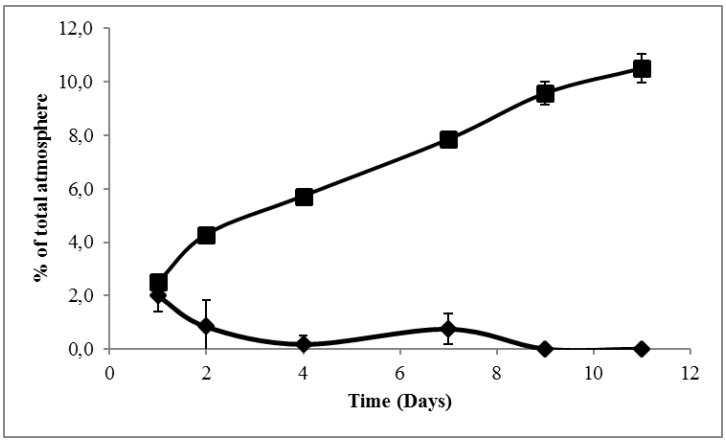

Fig. 1. Gas (O_2 and CO_2) composition (%) during the storage. No represented (up to 100%) was N_2. (■CO_2 concentration; ♦O_2 concentration).

The total bacterial load was around 5.5 Log cfu.g^{-1} at day 1 and increased above 9.8 Log cfu.g^{-1} after 14 days (Table 2). Previous studies indicate a variation in mesophilic bacteria concentration after harvest from 3 to 9 Log cfu.g^{-1} in raw vegetables, depending on the productive and growing conditions (Zagory, 1999; Oliveira et al., 2010; Yu et al., 2018). Bacterial load close to 5.6 Log cfu.g^{-1} has been described in lettuce (Hunter et al., 2010) and in Batavia lettuce variety (Di Carli et al., 2016). An evolution of total bacteria load during storage below 6 ºC from 4 up to 7-8 Log cfu.g^{-1} was observed in fresh lettuce (Allende et al., 2004) and RTE salades (Rudi et al., 2002). In Iceberg lettuce, Bercardino et al. (2018) found an average of 8.5 Log cfu.g^{-1} at middle shelf-life, while Ioannidis et al. (2018) referred 8.3 Log cfu.g^{-1} after 10 days. Data obtained hereby agree with the model proposed by Tsironi et al. (2017).

The selective agars analysis showed that counts in Pseudomonas agar and TVC were similar along the spoilage time (from 10^5 to over 10^9 cfu.g^{-1}, Table 2), suggesting that *Pseudomonas* is one of the mayor genus in lettuce during spoilage. Similar results were obtained in RTE vegetables by other researchers (Rudi et al., 2002; Legnani & Leoni, 2004; Ioannidis et al., 2018).

Initial counts of LAB and *Enterobacteriaceae* were below 4 Log cfu.g^{-1} (Table 2), and both groups would represent below 5 % of total flora in fresh lettuce. Similar initial levels have been described in iceberg lettuce (Ioannidis et al., 2018). While the RTE lettuce became spoiled, counts growth up to 8.6 Log cfu.g^{-1} in VRBG and 6.5 in MRS. Counts on selective media are, usually, less than 10 % to the total counts, but this depends on the considered vegetable. Hereby described results agree with the general metabolic characteristics of both groups (LAB and *Enterobacteriace*ae), those can grow easily even under refrigeration and/or in the absence of oxygen. Along with storage, LAB counts were, approximately, 3 magnitude orders lower than the TVC counts at end of commercial life. A similar situation has been described in the bibliography with RTE lettuce stored in MAP at 5 ºC (Allende et al., 2004; Ioannidis et al., 2018).

Finally, yeast and mould counts started at 4.3 cfu.g^{-1} and increased up to 7.8 cfu.g^{-1} after 11 days (Table 2). These data are lower compared to the ones reported by Rudi et al. (2002) after 10 to 12 days of storage and by Ioannidis et al. (2018) for 10 days.

Bacterial community changes during spoilage time

A total of 286,782 raw reads were obtained, from which 114,277 remained after quality control and chloroplast and mitochondrial sequences removal. The average number of raw reads per sample was 11,950, which corresponds to 4,762 quality filtered bacterial reads (Table 3). Interestingly, although the number of raw reads per sample was not statistically related to sampling time, the proportion of bacterial reads increased with time, as does the number of bacterial OTUs (Fig. 2), suggesting an increased bacterial load and diversity in samples collected later in time. Sample 7A had a considerably lower number of valid reads than the corresponding replicates and was removed from further interpretations.

During the spoilage, *Bacteriodetes* and *Proteobacteria* were the main phyla presented in the RTE lettuce, with more than 90% of the total bacteria identified. *Firmicuttes* is a non-dominant phylum, in contrast with the results described by Yu et al. (2018) in lettuce from North Korea. Differences in origin or in the lettuce variety (not described in their work) would explain these differences. During the spoilage, 709 different OTU (Operational Taxonomic Units) had been identified, and, as observed in Table 3, the number of OTU per sample increased along the spoilage time, up to the day 14. Statistical differences ($p<0.05$) were detected in the number of OTU during spoilage (Fig. 2), indicating higher bacterial diversity in late spoiled samples. Initial OTU richness results seem in agreement with the ones

described before for organic and conventional growth lettuce (Leff & Fierer, 2013) and, on the other hand, the increase in bacterial richness in spoiled salads has been described recently (Di Carli et al., 2016).

In the present work, 56 families and 105 different genera were identified. Our data demonstrate that the bacteriome of RTE Batavia lettuce is dominated by Gram-negative bacteria, mainly *Pseudomonas*, *Flavobacterium*, *Chyseobacterium* and bacteria from the "Enteric_bacteria_cluster" (Table 4). These genera summed around 90% of the total identified sequences in each sample. Sequence analysis showed that all samples from day 4 until day 14 shared 22 OTUs from 8 genera: *Flavobacterium* (10 OTUs), *Chryseobacterium* (4 OTUs), Enteric_bacteria_cluster and *Pseudomonas* (2 OTUs each) and *Janthinobacterium*, *Acidovorax*, *Carnobacterium* and *Duganella* (1 OTU each). These OTUs represented between 75% and 98% of the bacteria detected in each sample when spoilage is detectable (Fig. 3, starting at day 4) and confirmed the stability of the bacterial population present in RTE lettuce duringthe spoilage. The idea of a stable bacterial core in vegetal phyllosphere have been introduced by Rastogi et al. (2012) and seem to be confirmed by the results obtained in the present work since data obtained at day 1 and 2 (fresh lettuce, Table 3) indicated that most of these OTUs are also present at end of storage time.

The class *Flavobacteriales* (including *Flavobacterium* and *Chryseobacterium*) was found the major class present in RTE lettuce during the spoilage (Table 4). *Flavobacterium* has been isolated in different ecosystems, including soil, water and food products. However, their importance during food spoilage is still unclear. *Flavobacterium* genus presented high diversity, with 3 main OTUs but hundreds of OTUs with lower proportional importance. This genus represents up to 64.2±13.1 % of total bacteriome on day 7, although its relative importance decreased after this time. The ability to grow at low temperature and oxygen concentration (Alfaro et al., 2013) would explain the initial proliferation phase. Conversely, other authors have identified *Flavobacterium* in RTE lettuce at lower levels than those described hereby (1% of total bacteria) (Rastogi et al., 2012) or do not describe this genus as important in fresh harvested lettuce (Yu et al., 2018), suggesting that storage conditions would facilitate *Flavobacterium* proliferation.

Table 2. Bacterial counts (Log cfu.g^{-1}) in RTE lettuce along spoilage in different cultivation mediums

Time (Days)	VRBG	*Pseudomonas*	TVC	MRS	Yeast
1	3.8 ± 0.2^{a}	5.2 ± 0.5^{a}	5.5 ± 0.7^{a}	3.6 ± 0.0^{a}	4.3 ± 0.3^{a}
2	4.3 ± 0.5^{ab}	5.4 ± 0.2^{a}	6.0 ± 0.3^{a}	3.7 ± 0.3^{a}	4.8 ± 0.2^{a,b}
4	5.1 ± 0.5^{b}	6.8 ± 0.2^{b}	7.3 ± 0.2^{b}	4.1 ± 0.5^{a}	5.7 ± 0.9^{b}
7	6.7 ± 0.3^{c}	8.1 ± 0.2^{c}	8.3 ± 0.2^{c}	5.4 ± 0.2^{b}	6.8 ± 0.2^{b,c}
9	7.8 ± 0.2^{d}	8.5 ± 0.0^{c}	8.9 ± 0.2^{d}	6.2 ± 0.8^{b,c}	7.1 ± 0.0^{c}
11	8.2 ± 0.6^{d,e}	9.4 ± 0.1^{d}	9.5 ± 0.1^{e}	6.2 ± 0.7^{b,c}	7.8 ± 0.2^{c}
14	8.6 ± 0.1^{e}	9.5 ± 0.3^{d}	9.8 ± 0.2^{e}	6.5 ± 0.2^{c}	7.3 ± 0.4^{c}

Data are average of 3 independent samples.
^{a,b,c,d,e,} different letters in the same columns indicate significant differences ($p<0.05$).

Table 3. Number of reads obtained per sample (raw) and those remaining after quality filtering (QC filter) and after chloroplast and mitochondrial read removal (Bacterial). Number of OTUs identified per sample

Sample ID	Number of reads			Number of OTUs
	Raw	QC filter	Bacterial	
1A	5019	4998	185	27
1B	14949	14934	73	14
1C	18168	18146	70	14
2A	8516	8475	311	24
2B	16068	16035	337	22
2C	13362	13333	187	23
4A	15264	14391	8479	109
4B	19331	19082	1179	52
4C	14683	14467	1777	56
7A	57	56	35	na
7B	3263	3109	1965	31
7C	9426	8965	3502	56
9A	11829	10877	8109	83
9B	16286	15128	11694	78
9C	6674	5345	4347	72
11A	10316	8961	7244	69
11B	16873	15166	12589	93
11C	12536	10685	9555	112
14A	11210	10044	8066	95
14B	16893	14999	10920	83
14C	14072	11653	8706	83
Total	286782	263779	114277	708

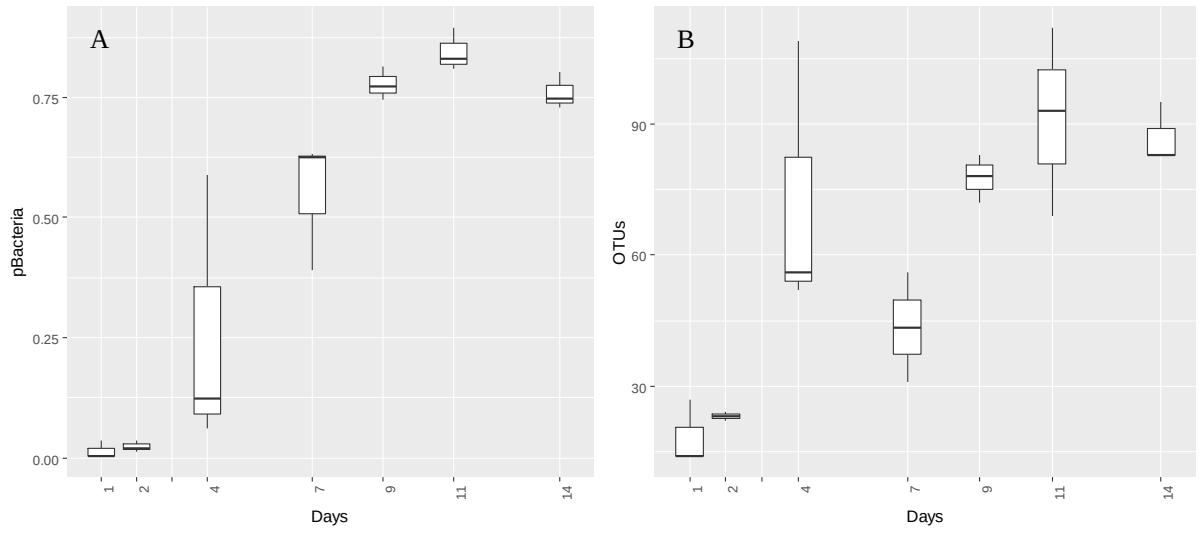

Fig. 2. Proportion of bacterial reads (considering all three replicates) from the total quality filtered reads per sampling time (A) and the total number of OTUs found per sampling time (B).

Table 4. Bacteriome (as % of total bacteria) of RTE lettuce during spoilage up to 14 days

	Storage time (days)						
Genus	1	2	4	7	9	11	14
Flavobacterium	39.1±9.7	42.0±19.4	56.4±17.2	68.3±10.3	64.7±9.9	56.8±10.3	57.2±3.0
Pseudomonas	12.8±2.2	19.4±8.4	24.4±12.3	16.9±3.4	15.9±2.5	18.3±4.8	21.6±5.3
Janthinobacterium	2.5±0.9	2.6±2.3	5.3±0.6	6.3±2.3	9.4±6.3	8.9±2.2	10.4±2.2
Enteric_Bacteria_cluster	1.0±1.6	0.7±0.8	1.7±1.4	2.6±2.3	3.5±1.6	4.9±5.9	2.5±0.4
Chryseobacterium	5.1±3.6	3.7±2.0	4.0±2.9	1.7±0.2	1.3±0.5	2.3±2.2	1.4±0.7
LAB	6.0±7.0	2.3±3.7	1.1±1.0	0.8±0.5	0.8±1.0	1.0±1.3	1.3±0.3
Others	33.6±2.5	29.3±25.7	7.0±1.7	3.3±1.6	4.4±1.7	7.9±6.1	5.7±2.1

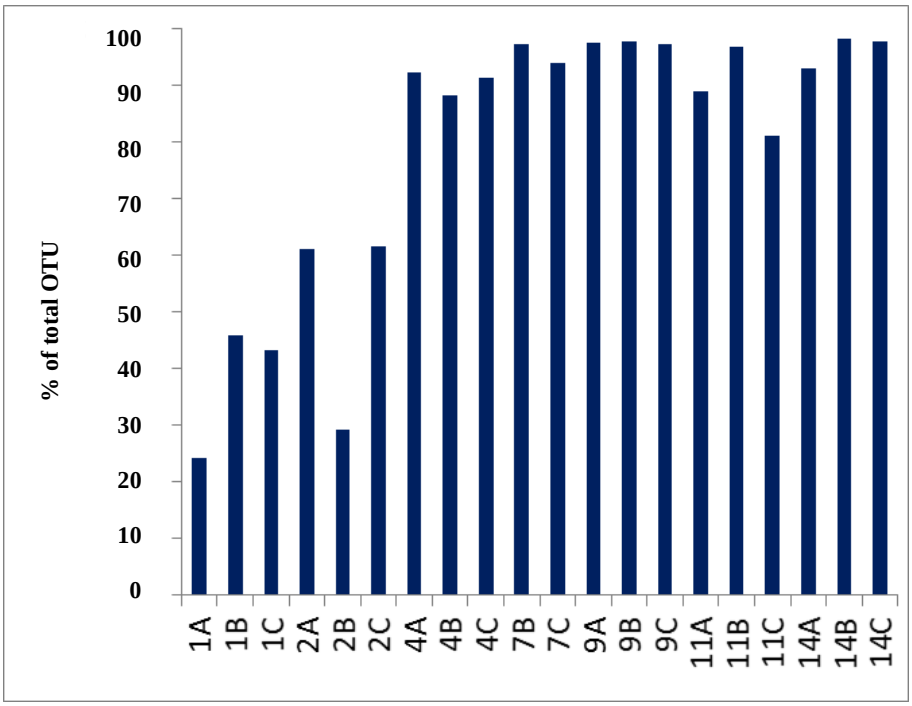

Fig. 3. Bacterial population (as %) that belongs to OUTs present in all samples during spoilage. In X-axis, the number represents spoilage time (in days) and letter the sample alanyzed.

The other major group of bacteria along the spoilage was *Pseudomonas*. Our results indicate that this group represented between 12 and 23% of the total bacterial population (Table 4), mainly corresponding to only one OTU (over 90% of all detected *Pseudomonas* are OTU0003). Some species of the genus *Pseudomonas* has been described before as important genus in lettuce phyllosphera (Rastogi et al., 2012; Jackson et al., 2013) and dominant in RTE lettuce (Rudi et al., 2002) and it may represent up to 90% of the microbial population in vegetables (Zagory, 1999). Our approach is not able to differentiate between *Pseudomonas*, though *P. fluorescens* was identified as mayor representative of this group in oak leaf lettuce (Nübling et al., 2016). The proportion of *Pseudomonas* was stable between 4 and 14 days of storage (Table 4), in between 16-24% of total bacterial load. Similar stability was observed by Ioannidis et al. (2018) in MAP Iceberg lettuce, where *Pseudomonas* reached 25-35% of the total population after 10 days.

Enterobacteriaceae (as "enteric_bacteria_cluster") increased their presence in samples up to 3.4±1.5 % at day 9. Compared with other published data, seems that this bacterial group is less important in Batavia lettuce compared with other varieties. Since, at harvesting time, *Enterobacteriaceae* was deemed the major group in Romaine lettuce (>38% of total bacteria) (Leff & Fierer, 2013), in lettuce produced both in spring and summer (Yu et al., 2018) and in

Iceberg lettuce (Ioannidis et al., 2018). The *Enterobacteriaceae* group includes many different organisms and was not defined up to the genus level in the informatics analysis. In the bibliography, most studies point to the important role of *Erwinia*, *Pantoea*, *Enterobacter*, and *Rahnella* in vegetables (Rudi et al., 2002; Rastogi et al., 2012). Conversely, we had identified *Erwinia* and *Pantoea* in our samples but at very low proportion (less than 0.1% and less than 0.5 % of the population, respectively) while we have not identified Rahnella, despite it have been underlined by Ioannidis et al. (2018) as one of the main bacteria in spoiled iceberg lettuce (up to 15-20% of total bacteria).

Finally, LAB (usually considered as *Lactobacillales*) were present in all samples during the storage, representing usually below 3% of total bacteria, in agreement with the ratio between counts in selective medium (MRS) and general medium (TVC). Main identified genera were *Lactococcus* and *Carnobacterium*, without statistical differences due to time of storage. In Iceberg lettuce, *Lactococcus* and *Leuconostoc* have been described as major LAB genus at spoilage time (Ioannidis et al., 2018). Leff and Feirer (2013) found *Leuconostaceae* as the major LAB family in Romaine lettuce, but we have found very low levels of this family in our samples and, surprisingly, *Lactobacillus* has not been identified in our samples.

Having in mind that OTUs were not identified at the species level, the presence of some pathogen bacteria in RTE lettuce at very low concentrations could not be excluded. In this regards, *Aeromonas* ssp has been identified at late spoilage time (up to 0.1% of the total population at day 14), *Yersinia* ssp represented up to 0.17%, some of the *Pseudomonas* ssp identified could be *P. aeroginosa* and the only OTU identified as *Bacillus ssp* would be a pathogenic species. All these species have been previously described as part of lettuce's bacteriome (Yu et al., 2018). We have not identified other usually pathogens, like *Escherichia*, *Salmonella*, *Listeria*, *Staphylococcus* or *Campilobacter*, probably due to the good sanitation effect of the post-harvest treatment.

The predominant specific spoilage microorganism (SSO) in RTE lettuce would be mainly *Pseudomonas*, *Enterobacteriacea*e and lactic acid bacteria (LAB). Although the proportion of these groups is highly variable, a similar situation can be observed in similar studies (i.e. Rudi et al., 2002). Strains of *Pseudomonas* and *Enteroacteriacieae* have been described as high-spoilers bacteria in RTE vegetables (Ragaert et al., 2006; Kahala et al., 2012; Federico et al., 2015) and, according to Ragaert et al. (2006), *Rahnella* and *Pantoea* (both *Enterobacteriaceae*) are able to produce spoilage-related volatile compounds in lettuce-agar. In the same direction, LAB are described as microorganisms with high spoilage capacity in these products (Pothakos et al., 2014). Within this group, *Leuconostoc* or *Lactobacillus* had been identified as some of the main causes of deterioration in salads (Nguyen-the & Carlin, 1994; Pothakos et al., 2014), but the levels reported hereby are below the, in theory, acceptable initial level (10^4 cfu.g^{-1}) (Jacxsens et al., 2003). *Flavobacterium*, the mayor genus, is described as no-SSO in fish (Parlapani et al., 2013), but some strains can produce aromas related with chicken spoilage (Freeman et al., 1976) and it has been proposed as spoilage bacteria in frozen vegetables (Manani et al., 2006). Further research is required to clarify the role of *Flavobacterium* in the spoilage of RTE salads. In general, detected relevant SSO microorganisms can grow at low temperatures and oxygen concentration, they are able to resist up to 10% CO_2 and are resistant to mild thermal treatments, which hinders the development of strategies to increase the shelf-life of RTE.

Hereby presented results showed a good correlation between the conclusions obtained by culture-dependent and independent methods. Bacterial counts in *Pseuodomonas* agar were in the same magnitude order than counts in TVC, and allowed us to identify *Pseudomonas* as one of the dominant genus identified in the population. Counts in selective agars for LAB (MRS) and *Enterobacteriaceae* (VRBG) and comparison with counts in TVC agree with

metagenomic results, concluding that these genera represent below 5% of total bacteria along the spoilage time. Jackson et al. (2013) also concluded that culture-dependent methods are a good overview of the bacterial populations present in RTE vegetables, while the advantage of culture-independent methods is that these techniques allow evaluating the importance of certain genera (like *Flavobacterium)* without a selective medium.

CONCLUSIONS

Sensorial analysis results indicate a commercial shelf-life of 9 days for Batavia lettuce stored in MAP at 6 ºC, mainly due to the apparition of off-odour after this period. The sensory limit of shelf life overlaps with bacterial counts in TVC and Pseudomonas agar reaching levels higher than 8 Log cfu.g^{-1}. Metagenomic analysis showed that *Flavobacteriales* (mainly *Flavobacterium* and *Chryseobacterium*) is the most abundant bacteria in RTE Batavia lettuce along with storage. *Pseudomonaceae* (especially *Pseudomonas*) is the second family in importance, and *Enterobacteriaceae* and LAB did not represent more than 5% of total obtained sequences. Finally, the microbial population is dominated by a few specific OTUs that were present throughout spoilage. These few OTUs may represent up to 98% of the total bacterial load.

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Conflict of interest
The authors declare no conflict of interest to report.

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Review of the agro-ecology, phytochemistry, postharvest technology and utilization of moringa (*Moringa oleifera* Lam.)

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ABSTRACT

Purpose: This paper provides an in-depth critical review and analysis of current and recent research undertaken on the agro-ecology, photochemistry, postharvest physiology and utilization of *Moringa oleifera* Lam. **Findings:** This treatise provided a comprehensive review of current and relevant research on the horticultural practices, its agro-ecological conditions, and pre- and post-harvest operations and treatments. It showed the alignment of the biochemical production of the various phytochemicals to the ecophysiology of the plant particularly the variety and ecotypes, micro climatic and edaphic conditions, and the crop growth patterns. The detailed aggregation of the different phytochemicals and the sites of synthesis in the plant revealed that the plant produces in excess of 36 phytochemical compounds that manifest its biological efficacy in over 16 different human physiological activity and medicine. Additionally, it revealed the many approaches to the postharvest physiology of the plant parts and the extension of the shelf life and quality for processing opportunities. **Research limitations:** There were no significant limitations to the conducting of this exercise as the literature was available and accesses to communicate with authors were easily facilitated by the internet. **Directions for future research:** This is the only review which collated the findings on the agro-ecology, phytochemistry, postharvest physiology and utilization of *Moringa oleifera* Lam., and how it is linked to the world of ethno-medicine, and pharmacology. Further research is necessary to determine the efficacy of extracts from all parts of the moringa plant as potential and promising eco-friendly alternatives to common chemicals used as novel food preservatives.

INTRODUCTION

Phytochemicals are complex compounds produced by plants and used by humans as herbal medicine or as nutraceuticals for its contribution to flavor, texture, pungency, odor, or color to foods (Bridgemohan et al., 2018). However, they are not required as in the case of essential nutrients, vitamins or minerals (Fahey, 2005). *Moringa oleifera* Lam., or 'drumstick' vegetable is popular in the cultural practice of ethnomedicine due to the accumulation of many functional bioactive compounds including phenols, flavonoids, alkaloids, phytosterols, sugars, glucosinolates, ascorbic acid, minerals, and amino acids, tocopherols, polyunsaturated fatty acids, folate, tocopherols, and organic acids (Amaglo et al., 2010; Saini et al., 2014; Saini et al., 2016; Vinoth et al., 2012; Garcia et al., 2016).

It is a tropical deciduous tree (Fig. 1) that is cultivated for a variety of purposes in both the tropical and sub-tropical regions (Leone et al., 2015). All parts of the plant produce phytochemicals (Table 1), with potential applications in functional food preparations, water purification, and biodiesel production (Saini et al., 2016; Bridgemohan, 2014). Various parts of this plant such as the leaves, roots, seed, bark, fruit, flowers and immature pods act as cardiac and circulatory stimulants; possess antitumor, antipyretic, antiepileptic, anti-inflammatory, antiulcer, antispasmodic, diuretic, antihypertensive, and cholesterol-lowering, antioxidant, including antidiabetic, hepatoprotective, and antibacterial (Kasolo et al., 2010; Anwar et al., 2007; Ijarotimi et al., 2013).

The plant synthesizes these compounds in complex biochemical processes in the leaves and the phytochemical is stored in the different plant parts. However, the agro-ecological conditions under which the plant is cultivated can significantly affect the growth, development, and yield of the plant. Similarly, variations in the availability of essential plant nutrients in the soil in addition to water will influence flowering and seed production. If the plant is cultivated under extreme stress conditions such as, waterlogged, arid, shade or excessive sunlight, or nutrient deficits, then the rate of photosynthesis, partitioning of assimilates, and other metabolic processes that produce the phytochemicals are affected (Bridgemohan, 2011). The processes are undertaken after maturity and can extend and improve the phytochemical content of the plant parts.

Bamishaiye et al. (2011) investigated the proximate and anti-nutrient composition of dried leaves of *Moringa oleifera* harvested at different stages of plant maturity. In their study, leaves were harvested at three-period stages of maturity (10^{th}, 15^{th}, and 20^{th} week) after pruning. The result of the proximate analysis revealed that the 10th week had the highest carbohydrate content (55.14%). The 15th week had the highest moisture (6.3%) while the 20th week had the highest protein content (28.08%), as well as crude fiber (10.11%), ash (9.25%), fat (2.5) and pH (6.27).

The phytochemicals recorded in both aqueous and methanolic extracts of each of the different stages of leaf maturation were the same except for tannin which was present in aqueous extract but was not detected in methanolic extracts. The antinutrients included alkaloids, tannins, phenolics, saponins, flavonoids, steroids but phylobatanin and tripertenes were not obtained. Bamishaiye et al. (2011) recommended that the leaves harvested at the 20th week or late stage of maturity had a higher percentage of embedded nutrients than the two earlier stages of maturity. They also concluded that the presence of some phytochemicals like saponins and flavonoids supported the medicinal action of the plant and associated therapeutic uses and therefore could act as a nutritional and medical alternative for socially neglected populations according to Brilhante et al. (2017) and more recently (Ma et al., 2019; Oguntibeju et al., 2019).

This paper is an in-depth critical review of current and recent research conducted on the agro-ecology, phytochemistry, postharvest physiology and utilization of *Moringa oleifera* Lam., it collates the information using a meaningful and collaborative approach that provides a better and holistic view on the advances made in this field of research, and its contribution to the existing body of knowledge in moringa.

PHYTOCHEMISTRY

Moringa phytochemicals are produced by all of the morphological structures of the plant but are not phyto-nutrients. Phytochemicals are normally referred to as plant compounds that may be beneficial or detrimental, whilst phyto-nutrients may have a positive effect. Gopaul and Bridgemohan (2014) observed that *Moringa oleifera* leaf extracts functioned as a plant growth regulator (PGR) on Pak Choi seedlings.

The proximate analysis of moringa seeds (w/w%) under varying treatments of dehulling or defatted revealed that the crude protein content could reach as high as 45% and the crude fat at 38% (Table 3). This suggested that *Moringa oleifera* is an underexploited source of crop protein for use in animal feed after the oil is extracted (Bridgemohan, 2014). This was supported by Garcia et al. (2016) in a comparative analysis of the potential protein value of some agro-industrial by-products for ruminant animals. Bridgemohan and Knights (2010) in a detailed nutrient analysis of *Moringa oliefera* as a high protein supplement for animals found that the whole seeds had 12 essential amino acids in the recommended proportions for non-ruminants (Table 4). The high-fat content of the dried moringa seeds is an essential source of indigenous bio-energy which has demonstrated potential as bio-fuel and bio-diesel (Bridgemohan, 2010).

The aqueous or ethanolic extracts have demonstrated biological activity in ethno-medicine and veterinary medicine (Bridgemohan et al., 2016). Fahey (2005) reported that *Moringa oleifera* is rich in compounds containing the simple sugar, rhamnose, and unique group of compounds called glucosinolates and isothiocyanates (Table 2). It has hypotensive, anticancer, and antibacterial activity include 4-(4'-O-acetyl-a-L-rhamnopyranosyloxy) benzyl isothiocyanate (Abrams et al., 1993), 4 4-(a-L-rhamnopyranosyloxy) benzyl isothiocyanate (Abuye et al., 1999), niazimicin (Akhtar & Ahmad,1995), pterygospermin (Anderson et al., 1985), benzyl isothiocyanate (Anwar & Bhanger, 2003), and 4-(a-L-rhamnopyranosyloxy) benzyl glucosinolate (Asres,1995).

Fig. 1. *Moringa oleifera* tree.

Fig. 2. *Moringa oleifera* pods.

Table 1. Biological activities of various extracts of *Moringa oleifera* plant

Plant Parts	Extract	Biological activities	References
Foliage	Aqueous	Oxidative DNA damage protective	Chumark et al. (2008)
		Hypolipidemic and antiatherosclerotic	
		Antiproliferation and apoptosis	Sreelatha et al. (2011)
		De-regulation of nuclear factor-kappaB	Berkovich et al. (2013)
		Anti-Herpes Simplex Virus Type1 (HSV-	Kurokawa et al. (2016)
	Hydroalcoholic	Antiperoxidative and cardioprotective	Nandave et al. (2009)
	Ethanol	Antihyperglycemic and hypolipidemic	Irfan et al. (2016)
		Upregulation of TNF-α	Akanni et al. (2014)
		Hypolipidaemic	Atsukwei et al. (2014)
	Methanol	Antioxidant, anti-inflammatory and antinociceptive	Adedapo et al. (2014)
	Methanol / ethanol	Inhibit differentiation of cancer cells	Lea et al. (2012)
	Acetone	Antimicrobial and antioxidant	Ratshilivha et al. (2014)
	Isothiocyanate	Insulin resistance and hepatic gluconeogenesis	Waterman et al. (2014)
Seeds	Ethyl acetate	Antimicrobial	Emmanuel et al. (2014)

Table 2. Proximate analysis of moringa seeds (w/w%) under varying treatments (After Bridgemohan, 2014)

Proximate analysis	Whole grain	Defatted		Partial		Mean	[SD]
		Dehulled	Unhulled	Defatted	Dehulled		
Crude protein	37	45	40	34	32	36	[4.98]
Moisture	5	7	7	6	10	7	[1.30]
Crude fat	38	25	17	22	30	26	[6.90]
Crude fiber	3	3	24	16	18	13	[8.81]
Ash	4	4	4	5	5	5	[0.75]

Table 3. Amino acids [%] profile of moringa seeds under varying treatments (After Bridgemohan, 2014)

Amino acids	Whole grain	Defatted		Partial	
		Dehulled	Unhulled	Defatted	Dehulled
Aspartic acid	1.5	1.8	1.4	1.4	1.6
Threonine	0.8	0.9	0.6	0.6	0.8
Glutamic acid	7	8.5	5.7	4.2	7.4
Proline	1.7	2.1	1.5	1.3	1.8
Glycine	1.8	2.2	1.5	1.2	1.9
Alanine	1.4	1.7	1.1	1.1	1.5
Cysteine	1.5	1.8	1.2	0.9	1.5
Valine	1.3	1.6	1.1	1.1	1.4
Methionine	0.7	0.8	0.5	0.5	0.7
Isoleucine	1.1	1.4	0.9	0.9	1.2
Leucine	1.9	2.4	1.6	1.4	2.0
Lysine	0.5	0.6	0.6	0.5	0.6
Mean	1.8	2.1	1.5	1.3	1.9
[SD]	[1.37]	[0.99]	[1.44]	[1.81]	[1.28]
Total	21	26	18	15	23

Plant ecology

Moringa oleifera can be grown as shrubs for foliage and roots or as trees for pod and seeds. The plant has a wide agro-ecological amplitude (Bridgemohan, 2011). It is a tropical deciduous plant, although it is native of the sub-Himalayan mountains and can be cultivated in both the tropics and sub-tropics (Leone et al., 2015). The plant is propagated by seeds but stem cuttings are also effective at a density of 1600 – 1800 trees/ha. For vegetable foliage and animal feed/fodder production, the shrub architecture is recommended at the higher crop density.

Saini et al. (2013) found crop establishment by seeds is undesirable due to genetic variation through cross-pollination. As a tropical plant, the ideal agro-climatic conditions are warm temperatures (25–35°C), under direct sunlight, at an altitude of 500m, and on slightly acidic to alkaline soil (pH 5.0–9.0). However, it can tolerate high temperatures (up to 48 °C), frost in winter and high altitudes, and a wide variety of soil conditions. The seeds can be planted just after maturity, as the seeds do not undergo dormancy while retaining viability for up to 1 year.

Yang et al. (2006) found that the harvest season and plant leaf stage significantly influenced nutrient contents of moringa leaves. Further, higher protein, vitamin A and glucosinolates contents and AOA were obtained in hot-wet season, compared to the cool-dry season where the iron, vitamin C, and phenolic contents increased significantly.

Bridgemohan (2010) found that during the wet season, the foliage and pod/seed yield was significantly higher than the dry season, but this did not affect the seed oil yield, crude protein content or amino acid profile (Garcia et al., 2016; Bridgemohan & Knights, 2010). The dry season favored the production of mature leaves which are more nutritious than young shoots and could be quickly dried with a minimum nutrient loss under ambient conditions. The major pre-harvest factors that affect the growth and development of the pods were pruning/thinning of the branches (Goordeen, 2018), and irrigation (Yang et al., 2006), especially during the onset of flowering.

Zhang et al. (2014) studied the effects of planting density and cultivation technology on agronomic characters of *Moringa oleifera*. The results showed that the effect of different planting density on the phrenological phase was not obvious, but the increment and the characters of fruits were affected by different planting density. There were differences in the phenological phase, the increment and the characters of fruits at the fertilizing and irrigation conditions, and the growth and fertility of *Moringa oleifera* could be improved and the yield of fruit could be increased. (Bridgemohan, 2014).

Förster et al. (2015) evaluated *Moringa oleifera* ecotype variability for growth and secondary metabolite (glucosinolates, phenolic acids, and flavonoids) profile using 6 different ecotypes grown under similar environmental conditions. They found that the USA and Indian ecotypes had the best growth performance and highest secondary metabolite production. Furthermore, optimal cultivation conditions, exemplarily on sulfur fertilization and water availability for achieving high leaf and secondary metabolite yields were investigated for *Moringa oleifera*. In general, plant biomass and height decreased under water deficiency compared to normal cultivation conditions, whereas the glucosinolate content increased.

Whilst some studies about macromolecular characterization have been made, including a protein with the ability to agglutinate, proteinase inhibitors, lectins, carbohydrates and lipid contents (Olayemi & Alabi, 1994; Santos et al., 2016; Bridgemohan & Knight, 2010), the complete knowledge about what kind of metabolites are present in each organ, and their ecological and biological roles, are poorly elucidated.

Vázquez-León et al. (2017) investigated the effects of the tree age, soil physical and chemical parameters, and climatic factors, on the content of gallic acid, total phenolics, total

carotenoids, and ascorbic acid and on the antiradical activity (DPPH and ABTS assays) of ethanolic extracts obtained from moringa freeze-dried leaves. They found that the bioactive compounds measured as reference and the antiradical activity from moringa leaves correlated with climatic factors (precipitation, humidity, and radiation) and with soil nutrients, principally with the K and P contents. Tree age was positively correlated with the total carotenoids contents and inversely correlated with the ascorbic acid contents. It can be seen that the variations in bioactive compounds and antiradical activity in moringa leaves are influenced by climatic factors, soil, and tree age, and will affect the antioxidant potential present in the plants during different harvest times.

Table 4. Phyto-chemical extracts of *Moringa oleifera* and use in ethno-medicine

Plant parts	Uses	Phytochemicals	References
Foliage	Mineral elements	K, Ca, Fe and Mg	Amaglo et al. (2010); Saini et al. (2014)
	Anti-inflammatory	glucosinolates, 4-O-(a-L-rhamnopyranosyloxy)-benzylglucosinolate (glucomoringin)	Amaglo et al. (2010)
		quercetin and kaempferol	Coppin et al. (2013)
		quercetin, apigenin, and kaempferol	Nouman et al. (2016)
		tetrahydrofolic acid and Formylfolic acid , carotenoids	Saini et al. (2016); Saini et al.(2014)
	Antioxidant	kaempferol, caffeoylquinic acid, zeatin, rutin, chlorogenic acid, beta-sitosterol.	Saini et al. (2014)
	Anticancer,	niazimicin	
	Hypocholesterolemic	isothiocyanate and niaziminin	
		E-luteoxanthin, 13-Z-lutein, all-E-zeaxanthin, and 15-Z-β-carotene -E-zeaxanthin, all-E-β-carotene,	Saini et al. (2014)
	Plant growth regulators	quercetin and kaempferol α-tocopherol	Gopaul & Bridgemohan (2014); Saini et al. (2014)
Roots	Antiurolithiatic	benzyl glucosinolate (glucotropaeolin	Bridgemohan et al. (2016); Amaglo et al. (2010)
Stem / shoot		glucosinolates, 4-O- (a-L-rhamnopyranosyloxy) -benzylglucosinolate (glucomoringin)	(Amaglo et al., 2010)
Pod	Crop protein	amino acids	Bridgemohan, (2008)
	Hepatoprotective	Isothiocyanates, catechin, epicatechin, ferulic acid, and vitamin C	
	Bio-energy	monounsaturated fatty acids	Bridgemohan, (2010); Saini et al. (2014)
	Crude protein	essential Amino acids	Bridgemohan (2010); Garcia et al. (2016); Amaglo et al. (2010)
	Antimicrobial,	omega-3 and omega-6, α-linolenic acid , palmitic acid	Amaglo et al. (2010)
Oil	Ulcerative colitis	oleic palmitoleic (stearic and arachidic acid, and linolenic acid	

POSTHARVEST TECHNOLOGY

Postharvest factors on phytochemical components

The major plant organs of *Moringa oleifera* that produce and store the phytochemicals are the flower seed, foliage, and roots. The edible plant parts of *Moringa oleifera* (leaves, fruits, flowers, and immature pods) form part of the traditional diet in many countries (Odee, 1998). However, the stage of maturity of plants affects the concentration of nutrients of leaves (Yu et al., 2004; Bamishaiye et al., 2011), taste and cooking as well as the amount of anti-nutrients (Sallau et al., 2012). Chodur et al. (2018) conducted studies to determine whether domesticated and wild type *Moringa oleifera* differ in myrosinase or glucosinolate levels and implied that the ultimate impact of these differences on taste affected consumption. Their assessment examined taste and measured levels of protein, glucosinolate, myrosinase content, and direct antioxidant activity of the leaves of 36 *Moringa oleifera* accessions. Taste tests highlighted differences between wild type and domesticated *Moringa oleifera*. Furthermore, there were differences in indirect antioxidant potential, but not in myrosinase activity or protein quantity. While agro-ecological conditions influenced growth and development, and the biochemical synthesis of these compounds, the retention and storage of these phytochemicals proved to be critical in maintaining the integrity, quantity, quality, and perishability of the harvested plant.

The postharvest extraction of phytochemicals (flavonols [myricetin, quercetin, and kaempferol] and phenols) in *Moringa olifera* foliage was conducted using pressurized hot water extraction (PHWE) as a "green" technology (Matshediso et al., 2015). The kaempferol and myricetin concentration decreased at 150°C, compared to quercetin which remained unchanged. Optimum extraction temperature for the flavonols and 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging activity was 100°C, but the total phenolic contents (TPC) increased with temperature (150°C) and then decreased.

Tetteh et al. (2019) evaluated the effects of different harvesting techniques and commonly used traditional-drying methods on the stability of glucosinolate (GS) of harvested *M*oringa *oleifera* leaves. The study revealed that sun- and oven-drying *of* leaves retained significantly higher GS contents compared to solar-and shade-drying. Further, oven-dried leaves had significantly higher levels of GS than leaves freeze-dried. Some of the processing methods like boiling, simmering and blanching affected the anti-nutritional contents of *Moringa oleifera* leaves. Sallau et al. (2012) showed that boiling reduced the cyanide content by 88.1% when compared with simmering and blanching. Similarly, boiling reduced the amounts of oxalate, phytate and trypsin inhibitors.

Harvesting methods

Moringa olifera plant parts are generally harvested manually. In view of the highly perishable nature of harvested flowers, leaves, pods and seeds, rough field handling could induce physical injuries and subsequent secondary infections to compromise postharvest quality and shelf life (Mohammed & Bridgemohan, 2019). Leaves of moringa plants grown in well-drained fertile soils under high-density planting can be harvested after 60-90 days when plants attain 1.5–2.0 m in height (Bridgemohan et al., 2019). Leaves are harvested by cutting leaf stems manually with a sharp knife at 20-45 cm above ground which ultimately results in the proliferation of new shoots. Harvesting operations can be conducted every 35-40 days thereafter. Moringa shoots intended for use as fodder can be harvested at seventy-five (75) days intervals. Under conditions of intercropping, plants can be harvested after two to four months of growth (Nouman et al., 2016).

The initial cutting can be done manually at 20cm up to 1.5m height. Ideally, harvesting procedures should be undertaken at a suitable plant height to avoid the effects of shading by

overhanging companion crops. It is recommended that freshly harvested moringa leaves should not be assembled in heaps or packed tightly since the accumulation of respiratory heat could initiate deterioration, particularly when exposed to high temperatures for extended periods (Mohammed et al., 2019). In situations where moringa leaves are harvested, branches should be pruned, followed by washing in chlorinated water to remove dirt and dust. Drying must be done in the shade, preferably indoors in a clean, well-ventilated space. If leaves are dried in the sun, the vitamin content will be reduced. Leaves should be hung upside down or laid on a drying rack of string mesh to ensure good airflow. It is also important to turn or move leaves as needed to prevent rotting or molding during the drying process (Radovich & Paull, 2008; Mohammed et al., 2019).

Moringa leaves and tender pods should be harvested during the coolest time of the day, early morning or late evening to minimize moisture loss. Moringa pods are harvested depending on the variety as tender immature green (snap stage), semi-firm (fresh-cut), or dry pod state (seeds) (Bridgemohan et al., 2016; Goordeen, 2018). The semi-mature pods are harvested for the soft seeds and are less susceptible to mechanical injuries. During all harvesting and postharvest procedures in the field, and when packed and transported to the packinghouse and more so when pods are prepared for minimal processing (Fig. 2), worker hygiene and sanitation practices must be monitored and implemented consistently (Goordeen, 2019). Thus, careful supervision and proper instructions conveyed to the harvesting crew are essential to the success of the hand-harvesting operation of pods. Careful supervision includes random checks of harvesting bags or pails for trash and poor-quality pods. Packinghouse problems and buyer complaints often result from a poorly instructed and supervised harvesting crew. Pods should be removed from the plants cleanly without tearing them or causing undue damage to the pods or other plant parts. Over handling or rough handling of the pods would result in both visible and latent damages (Mohammed & Bridgemohan 2019; Goordeen, 2018).

Maturation indices

The recommended indicators of maturity and marketable quality of moringa pods include diameter, length, skin and flesh color, snapping strength, firmness and tenderness. Moringa pods with no bulge or only a slight bulge enclosing tender soft immature seeds are considered ideal maturity indices where pods are required for cooking purposes (Goordeen, 2019). However, immature ready to cook pods are susceptible to wilting. Over mature pods with bulging seeds are tough and fibrous. Uniform sizing of pods packed in crates or cartons is critical to acceptance by fresh market buyers. Additionally, pod shapes must be straight, bright green in color and typical for the cultivar.

Pre-cooling

The perishability rate of harvested moringa pods is proportional to the respiration and transpiration rate, respectively, and can be reduced by pre-cooling [14ºC] (Amuthaselvi et al., 2014; Peiris, 1997). The respiration rate increases with temperature 8.31ml/kg.h (14ºC) to 34.61ml/kg.h (28ºC) and is affected by agronomy, cultivar, production environment, and pre-harvest crop management practices. To reduce the impacts of transpiration and further deterioration in moringa pods, it is necessary to control water loss and maximize retention by harvesting at low ambient temperatures during the early mornings or late evenings (Mohammed & Bridgemohan, 2019; Goordeen, 2018).

The high build of field heat in the afternoon in the tropics is undesirable during harvesting as it increases metabolic activities of moringa pods (Bridgemohan et al., 2016; Goordeen, 2019). Accumulation undesirable field heat should be reduced immediately after harvest by

pre-cooling to minimize the effects of microbial proliferation and high respiration, transpiration and ethylene production rates (Mohammed at al., 2019; Kitinoja et al., 2018). All parts of the moringa plant should be pre-cooled in the field by placing plant components in shallow, light-colored, stackable ventilated plastic crates that are either covered with broad leaves or by holding in a covered field shed or under a tree with shade to minimize water loss, respiration, and secondary infections. Pre-cooling moringa pods immersed in chlorinated water cooled at 5-7ºC for 30-40 minutes is an efficient, inexpensive, easy to apply and fast hydro-cooling technique to remove field heat as well as an effective starting point to initiate the cold chain. Limiting the time between harvesting and cooling to no more than 1 or 2 hours will help maximize shelf life. (Mohammed et al., 2019; Goordeen, 2019).

In the absence of refrigeration, alternatives such as shade, harvesting during the coolest part of the day, and drenching the produce with cold chlorinated water should be employed. Field containers packed with moringa plant parts should be cleaned with chlorinated water before packing and transport to the packinghouse and retail market display. Periodic sprinkling or spaying with chlorinated water may also initiate evaporative cooling if sufficient air circulation is present. Once the pods have been packed in cartons, air circulation must continue until the products are properly refrigerated (Kitinoja et al., 2018).

Moringa pods intended for distant fresh markets should be immediately cooled after harvest. The placement of field-warm pods in a refrigerated space, known as room cooling, is recommended only as a last resort. Room cooling may be of some benefit but is slow because it relies only on natural conduction and convection to transfer heat. Palletized and bulk containers of moringa pods may require more than 16-18 hours to cool sufficiently in cooling rooms (Mohammed et al., 2019). To promote cooling and prevent the buildup of respiration heat, the containers should be loosely stacked, leaving space between the pallets for air circulation (Thompson, 2004).

Grading and sorting

Moringa pods are sorted and graded regardless of the stage of maturity based on appearance, freshness, turgidity, and absence of blemishes. Freshness is evidenced by a distinct, audible snap when the pod is broken. The degree of seed development inside the pod indicates processing and marketing quality. Buyers and processors require a well-filled pod but harvesting flat and immature green pods must be out-graded. Dark brown pods indicate dry, over-mature pods that are unacceptable in the fresh market. Pods displaying rusty brown spots or other blemishes indicate disease, injury, or the possibility of deterioration and should be discarded (Mohammed et al., 2019).

Careful supervision of labor is the key to ensuring uniform cleaning, sizing, and packing of hand-harvested moringa pods. Growers may use a grading table or belted conveyor located at the packing shed to remove unmarketable pods. Spreading harvested pods on a conveyor belt or flat surface helps to dissipate field heat before packing, storage, and transportation (Mohammed et al., 2019).

Packaging and transport

Packaging of fresh moringa flowers, pods or leaves is one of the more important steps in the long and complicated journey from grower to consumer. Bags, crates, hampers, baskets, cartons, bulk bins, and palletized containers are convenient containers for handling, transporting, and marketing display of fresh moringa plant components. The recommended container is the shallow light-colored and well-ventilated plastic crates which must enclose the produce in convenient units for handling and distribution (Mohammed et al., 2019). The pods should fit well inside the container, with little wasted space. Packages of moringa pods

commonly handled by hand are usually limited to 22-23kg. The package must protect the produce from mechanical damage and poor environmental conditions during handling and distribution. To produce buyers, torn, dented, or collapsed produce packages usually indicate a lack of care in handling the contents. Produce containers must be sturdy enough to resist damage during packaging, storage, and transportation to the market (Kitinoja et al., 2018).

Because almost all moringa contained packages are palletized, containers should have sufficient stacking strength to resist crushing in a low temperature, high humidity environment. Moringa pods packaged and destined for export markets requires containers to be extra sturdy. The packaged moringa pods must identify and provide useful information about the product such as brand, size, grade, variety, net weight, count, grower, shipper, and country of origin. It is also becoming more common to find included on the package nutritional information, recipes, and other useful information directed specifically at the consumer. In consumer marketing, package appearance has also become an important part of the point of sale displays (Kitinoja et al., 2019).

Recently, (Nayak & Khuntia, 2019) described the use of natural fiber polymer composites materials for packaging applications and advocated that *Moringa oleifera* fruit fiber (MOF) as a reinforcement and promising candidate for packaging applications. In this ongoing research, composites were fabricated by reinforcing treated *Moringa oleifera* pod fibers with polyethylene terephthalate **(**PET**)** thermoplastic polymer in order to investigate the mechanical, thermal and morphological properties. Surface treatments of fibers were conducted to obtain better compatibility with the PET matrix. Nayak and Khuntia (2019) further articulated in this novel study that the mechanical properties increased at the early stage with an increase in treated *Moringa oleifera* fiber content until optimum (20 wt% of fiber) fiber loading thereafter declined. At this fiber loading the mechanical properties obtained were 65.92 MPa of tensile strength, 98.49 MPa of flexural strength, 3.78 GPa of young's modulus and 28.09 kJ/m2 of impact strength. Thermogravimetric analysis (TGA), dynamic mechanical analysis (DMA), and scanning electron microscopy (SEM) were used for analysis. Furthermore, the TGA inferred that the thermal stability of the composites increased as compared to the neat PET matrix. It was found that composites fabricated from 20 wt.% fiber content showed superior mechanical properties as well as thermal properties as compared with other fabricated composites and can be used for packaging applications.

Storage

Fresh moringa pods are generally consumed within 2 days of harvest, more so, since immature *Moringa olifera* pods which possess a high moisture content, are thin-skinned with a cuticle that is protected with very limited amounts of wax and with high susceptibility to physical damages and subsequent secondary infection (Mohammed et al., 2019). This valuable crop has a very short shelf life (1 to 3 days at room temperature). There could also be a loss in nutritional quality due to poor postharvest handling, and different means of food preparation which influence the nutritional and functional qualities of moringa pods.

The need for preservation of moringa pods is essential due to its medicinal and therapeutic properties. Moringa pods treated with an edible coating such as gum of Arabic was considered as a cheaper alternative to cope with the perishable nature of the pods (Viyas & Mahendrakumar, 2018).

Sangeetha et al. (2017) explored the effect of time of harvest, method of harvest and pre-packaging calcium chloride treatments on shelf life and quality of *Moringa oleifera*, cv. PKM 1. Their study indicated that percentage losses in fresh weight increased significantly with increases in the storage period. The time of harvest also resulted in a significant influence on the weight loss of moringa pods. The morning harvested pods showed minimum weight loss

(10.37%) after nine days of storage under ambient conditions probably because the morning harvested pods had minimum water loss when compared to afternoon or evening harvested pods. Similar results were reported by Palada (2003) in amaranthus. Sangeetha et al. (2017) further claimed that the method of harvest also had significant effects on fresh weight losses. Harvesting of moringa pods with pedicles recorded minimum weight loss (11.25%) and decay when compared to pods without pedicles (13.31%). They attributed that the differences in decay could be related to those pods harvested without stalk and stored under ambient conditions, which, perhaps produced more decay loss as the exposed surface of stalk or scar left at the time of harvesting which created avenues for the entry of secondary pathogens. Pathak and Shrivastava (1969) and Singh and Panday (1993) have articulated similar explanations in their study with mango. The effect of postharvest treatments showed that calcium chloride (1%) treated pods recorded minimum weight loss (9.12%) when compared to untreated moringa pods (15.59%). It might be due to $CaCl_2$ that might react with water molecules and it might be acting in a manner to block the amino groups before entering into the enzymatic browning reaction. Similar results were reported by Davoodi et al. (2007) in tomato.

Freshly harvested moringa pods were subjected to three different chemical treatments, NaCl 10%, $CaCl_2$ 1%, and KMS 0.5% concentration (Viyas & Mahendrakumar, 2018). Pods were dipped in 10, 15 and 20% gum of Arabic solution, drained for two minutes followed by overnight room drying and then stored in a refrigerator at 8-10^{0}C and ambient temperature 25-30^{0}C up to 10 days. At the higher temperature, slight fungal growth was noticeable after 6 days but upon prolonged storage, after 10 days the proliferation of fungal growth was extensive making the pods unmarketable. Maximum firmness (5.85 kg/f), chlorophyll (0.14 mg/g), ascorbic acid content (78.94 mg/100g), protein content (2.13 g/100g), carbohydrate content (1.86g/100g), ash content (0.53%) were found for $CaCl_2$ 1% + coating 20% at the end of 30 days of storage under refrigerated conditions. Moringa pods coated with gum of Arabic coating was the best treatment when stored at 8-10°C and 80-85% RH in turn retained ascorbic acid, firmness, protein, crude fiber, chlorophyll, and TSS levels and maintained the lowest amount of moisture loss compared to the other treatments (Viyas & Mahendrakumar, 2018).

Other parts of the moringa plant have been studied to determine optimum quality attributes under different storage regimes. Moringa leaves for example which is also perishable should be stored under cool temperatures and high humidity to avoid excessive wilting and leaflet abscission. Moringa leaves subjected to modified atmosphere packaging (MAP) and supplemented with refrigeration at approximately 10°C (50°F) was reported by Radovitch (2009). Modified atmosphere packaging can create lower oxygen and higher carbon dioxide atmospheres during shipment and storage of horticultural commodities, potentially reducing quality loss caused by high respiration, transpiration, ethylene, and pathogen growth (Kader & Watkins, 2001). Mubvuma et al. (2013) investigated the effect of storage temperatures and the duration of moringa seed to achieve optimum germination percentages. The results of the study indicated that the management of storage temperature and storage duration of seed have the potential to improve the seed quality and germination percentage. Across all treatments, the quality of the seed improved with prolonged storage period up to three months, thereafter the quality of seed decreased with storage time unless stored under low temperatures (10°C). Good germination results were achieved after storing the seeds at a storage temperature of 25°C for a duration of 60 days.

Experiments were conducted by Tesfay and Magwaza (2017) to investigate a novel moringa leaf extract, together with commercially available edible coatings, namely, chitosan and carboxymethyl cellulose (CMC), as postharvest treatments to enhance shelf-life and

improve the quality of 'Fuerte' and 'Hass' avocado fruit. Postharvest treatment included a 2% moringa extract with an emulsifier, two levels of chitosan (0.5, 1%), and CMC (0.5, 1%). Results from the study indicated that moringa extract with emulsifier and moringa containing chitosan and CMC significantly improved pod quality of both cultivars and showed that edible coatings containing moringa leaf have the potential to be commercialized as a new edible coating for future industrial application.

Katayon et al. (2006) also reported on stored *Moringa oleifera* seeds at different conditions and durations using an open container and a closed container at room temperature (28°C) and refrigerator (3°C) for durations of 1, 3 and 5 months. A comparison between turbidity removal efficiency of *Moringa oleifera* kept in refrigerator and room temperature revealed that there was no significant difference between them. *Moringa oleifera* seeds kept in a refrigerator and at room temperature for one month showed higher turbidity removal efficiency, compared to those kept for 3 and 5 months, in both types of containers. The coagulation efficiency of *Moringa oleifera* was found to be dependent on the initial turbidity of water samples. Highest turbidity removals were obtained for water with very high initial turbidity. Coagulation efficiency of *Moringa oleifera* was found independent of storage temperature and container, however, the coagulation efficiency of *Moringa oleifera* decreased as storage duration increased. Besides, *Moringa oleifera* can be used as a potential coagulant, especially for very high turbidity water.

Fotouo et al. (2016) corroborated with several authors (Rashid et al., 2008; Rahaman et al., 2009), in relation to the potential of moringa seed oil or Ben oil for biodiesel production as well as a cooking oil. There was also a consensus among them that oil quality is directly related to the physiological condition of the seeds at the time of extraction. Fotouo et al. (2016) proceeded to investigate the effect of various storage conditions and durations on moringa seed oil quantity and quality as a potential source of biodiesel. They found that moringa seed oil quality did not change significantly after 12 months of storage but after 24 months, the oil content of seed stored at 4°C in paper bags and at 20° and 30°C in aluminum bags were significantly lower than the control. The free fatty acid increased significantly after 12 months at all storage conditions and continued to increase above the recommended value (2%) at 24 months for biodiesel parent oil, except for that of seed oil stored at -19°C in aluminum bags. They deduced that the decrease in oil content and increase in free fatty acids were probably due to hydrolysis and oxidation processes accelerated by the high moisture content in the seed. The density of moringa seed oil remained unchanged throughout storage. The viscosities of oil extracted from seed stored in paper bags at -19°C and that of the oil stored at ambient temperature decreased significantly after 24 months. However, based on available literature the average oil density of less than 2mm2/s will not have a major effect on the final viscosity of the derived biodiesel. Moisture content, temperature, and storage period at which seeds are stored can influence the quality of the derived biodiesel, but seed moisture seems to be the main factor influencing the quality of the extracted oil. Seed can be stored at any of the applied conditions for six months, but if it is stored beyond this period, the use of low temperatures such as -19°C and 4°C and the use of sealed containers such as aluminum bags are recommended. Storage of the extracted oil for more than 12 months is discouraged.

In other studies, Selvi and Varadharaju (2016) examined the shelf life of moringa pods by controlled atmospheric storage. A 'Local variety' and a hybrid 'PKM 1' were selected and given a fungicidal treatment of 1% for 2-3 minutes. Respiration studies of moringa pods were conducted at three different temperatures (14, 21 and 28ºC) with the product to free volume ratios at 1:5, 1:10 and 1:20. Moringa at two different temperatures (14ºC and ambient) was stored in a specially designed PVC chamber with 3, 4 and 5% O_2 concentrations. Loss in firmness for both 'Local' and 'PKM 1' was 12.9, 13.2 and 16.9% for 14ºC with 3, 4 and 5%

O_2 concentration, respectively during 40 days of storage. The ambient stored moringa pods had a higher ascorbic acid loss of 8.2% as compared to 5.5% at 14ºC. The results were comparable with local variety also. 'PKM 1' showed a higher reduction of ascorbic acid of 54.1, 5.3 and 5.9%, respectively for 3, 4 and 5% O_2 concentrations at 14ºC in 40 days. Controlled atmosphere storage at refrigerated conditions revealed that the shelf life of moringa pods could be increased to approximately three to four times compared to ambient conditions. The best treatment for increasing the shelf life of moringa pods up to 40 days at 14°C was 4% O_2 and 5% CO_2.

Tripathi and Variyar (2018) examined the effect of radiation treatment (0.5, 1, 1.5, 2 and 2.5 kGy) and storage (10°C; 15 d) on the quality of ready to cook (RTC) moringa pods and reported an improved shelf life of 12 days for radiation treated samples at 1 kGy with suitable sensory and microbial quality. The radical scavenging activity, phenolic constituents, and isothiocyanates content were better retained in radiation processed products in comparison to control samples at the end of the intended storage duration. Their investigation also established the amenability of gamma radiation processing along with cold storage for improving the shelf life of ready-to-cook moringa pods.

Rikhotso et al. (2019) evaluated the efficacy of chitosan (CH) and carboxymethyl cellulose (CMC) incorporated with moringa leaf extracts (M) on reducing peteca spot (PS) incidence on 'Eureka' lemons. Their results showed that coating treatments and canopy position significantly affected PS incidence. Fruits coated with M + CMC, CMC, CH were less susceptible to PS development both inside and outside canopy compared to the control and M + CH coated fruit. Coating treatments significantly affected phenolic and flavonoid concentration. Moreover, coating treatments significantly reduced mass loss, ascorbic acid loss and delayed a color change of fruit.

UTILIZATION AND DEVELOPMENT OF VALUE-ADDED PRODUCTS

There are several value-added products made from various parts of the moringa tree. Included are moringa pickle, dehydrated pods, moringa flesh mesocarp powder, moringa pulp powder, moringa leaf powder, sauces, juices, dried flowers, oil and cosmetic products (Ponnuswami, 2019). Organoleptic evaluations of raw moringa plant leaves had a slight "bite", reminiscent of watercress or radish, which was more pronounced (Bridgemohan, 2014) in *Moringa oleifera* than in *Moringa stenopetala* (Bridgemohan, 2014). When cooked, the "bite" is eliminated resulting in an after taste similar to "pecany" spinach. Moringa plant flower blossoms and buds are also edible, when cooked. Moringa flowers have laxative properties if more than about 1/4 cup is eaten at one time. Very young Moringa pods are excellent eating when they are about the size of string beans. After that, they get "woody" and "stringy", and will require additional cooking time. Moringa seeds can be fried in a little oil, sometimes they "pop" just like popcorn. Add salt, and eat a few at a time, as they are intensely cleansing. The green drumsticks are used in South Asian cooking, particularly in curries. The leaves are often added in salads and stir-fries and are also cooked as a vegetable dish on their own, with added spices or topped with eggs.

Moringa leaves, flowers, and pods

Moringa leaves are dried and used in a powdered form and the pods are minimally processed and canned in a brine solution and also as pickled condiments (Price, 1985). Leaves and flowers may be dried in the shade or dehydrated and then pounded or ground and used as a food additive to improve the protein content of foods. Leaves and flowers are also used for making tea. An example of a commercial health food drink (Zija™) contains 30 g (1 oz) of moringa leaf, seed, and pod. This is reportedly the first commercially available drink from

moringa. Retailing such a commercial product to the average consumer may be challenging because of high costs. However, local, prepared drink and tea products may offer the value-added opportunity for sale at farmers' formulated markets or health food stores. Young pods are consumed as a vegetable (Fig. 2). Very young pods are fibreless and can be cooked like string beans. Because the weight is low on very young pods, most commercial production involves larger, more fibrous pods that are used in soups, stews, and curries. The nutritious leaves are eaten in many dishes including soups, stews, and stir-fries. Sauteed young leaves and flowers are also eaten. The demand for home consumption of pods and leaves can generally be met by one or two backyard trees. Commercial production of mature seeds for oil occurs in India, Africa, and elsewhere. Recently, Hassan and Fetouh (2019) investigated whether moringa leaf extract (MLE) had the ability to act as postharvest preservative solution to improve the quality and longevity of gladiolus spikes. They subjected gladiolus spikes to various concentrations (0, 1, 2, 3, 4%) of MLE in vase solution and reported that all MLE concentrations significantly extended the vase life of spikes. MLE treated spikes also improved floret opening and reduced post-cutting weight loss. Furthermore, the relative water content (RWC), chlorophyll content, and membrane stability were considerably maintained while the microbial growth was suppressed in vase solution containing MLE treatments. Also, malondialdehyde (MDA) and H_2O_2 production was significantly suppressed by MLE treatments. MLE significantly increased the total phenolics and the activities of antioxidant enzymes (CAT and POX) in the florets. These ameliorative effects of MLE were more pronounced by the concentration 3% but higher level resulted in no improvement in cut gladiolus spike longevity and quality. Hassan and Fetouh (2019) concluded that MLE showed these effects via alleviation of oxidative stress induced in the cut spike, maintenance of photosynthetic pigments and water relations. Based on these findings they recommended that MLE to be applied as a useful and promising eco-friendly alternative to common chemicals used in preservative solutions for cut flowers. MLE could also be commercialized as a novel floral preservative for future floral industry application (Hassan & Fetouh, 2019).

Moringa seeds

Mahmoud (2019) transformed moringa seeds into power as a substitute for wheat flour to make cakes enriched with crude protein (7.6-17.65%), fiber (0.68-1.65%), ash (1.06-1.97), calcium (30 mg/100g) and iron (7.65mg/100g). In Africa, moringa powder is popular and used as a food supplement, whereby, 1–2 tablespoons of dried powder are added to soups and stews daily to enhance the protein content and nutritional value of food. In Africa, 25 g of moringa powder is administered to pregnant women daily to improve prenatal nutrition (Diatta, 2001).

Radovich and Paull (2008) discussed moringa oil derived from the seeds and its potential to add value to a small family farm if extraction can be optimized and if it were marketed to high-end venues as an alternative to imported olive oil. It was predicted that local and internet sales of moringa oil for cosmetic use may also have additional economic benefit based on its extraordinarily long shelf life and ability to capture the scent of added fragrances. Infusions of moringa oil with essential oils such as jasmine and lavender may also be valuable options.

Radovich and Paull (2008) also described in detail the moringa oil extraction method, composition, yield, and organoleptic attributes. The oil contains 60-75% oleic acid and is comparable to olive oil in taste and cooking quality characteristics. The high antioxidant content contributes to excellent oxidative stability and hence lower susceptibility to rancidity. Producing moringa oil on a small scale might be economically feasible if it were marketed to restaurants, hotels, and other high-end venues as a locally produced alternative to imported

olive oil. If the oil is extracted through pressing, costs may be further reduced if press cake is used to replace purchased fertilizer (Radovich, 2009).

Press cake

The press cake leftover after extracting seed oil is utilized as a fertilizer and as a flocculent for water clarification. The seed cake contains positively charged compounds that are effective in settling suspended solids out of the water (flocculation) because most particles have a net negative surface charge while suspended in aqueous solution. There is international interest in using moringa-based flocculants as a locally produced, biodegradable substitute for aluminum sulfate, which is commonly used to clarify water.

Animal feed

The seed cake is normally not used as livestock feed because of the presence of anti-nutritional compounds in the mature seeds. Leaves are readily eaten by cattle, sheep, goats, pigs, chickens, and rabbits and can also be used as food for fish. Several studies demonstrated that significant proportions of traditional fodder can be replaced with moringa leaves. A study in Fiji reported significant weight gain over traditional fodder when 50% of fodder contained moringa leaves (Aregheore, 2002). In Nicaragua, cattle feed consisting of 40–50% moringa leaves is mixed with molasses, sugar cane, and grass. Moringa leaf meal can be used to substitute up to 10% of dietary protein in Nile tilapia without a significant reduction in growth. However, excessive feeding with moringa can reduce weight gain in livestock. Animals given fodder with 80% moringa in the Fijian study cited above showed lower weight gain than animals on 50% moringa fodder. Adverse effects resulting from high rates of moringa in the feed are due to excessive protein levels, and potentially anti-nutritional compounds in the leaves such as nitrate, oxalate, saponin, phytate, and isothiocyanates. Raffinose and stachyose may cause flatulence in monogastric (Foidl & Paull, 2008). Moringa biomass is reportedly low in lignin and may be valuable for ethanol production (Foidl & Paull 2008).

Medicinal uses

Most parts of the plant are used as medicine. The greatest contribution of moringa to health is its high nutritional value with the potential to target health food stores. The plant can be grown organically and this would facilitate the organic certification and greater consumer appeal. The most common direct medical use of the plant is as a poultice of the leaves and bark applied directly to wounds as an anti-microbial and to promote healing (Foidl & Paul, 2008). The anti-fungal and anti-bacterial properties of moringa extracts are well documented and are thought to be derived at least in part from 4-(α-L-rhamnopy-ranosyloxy) benzyl isothiocyanate. This compound is particularly effective against *Helicobacter pylori*, a bacterial pathogen of human beings in medically underserved areas and poor populations worldwide (Fahey, 2005). Isothiocyanates are the source of the mild horseradish smell in moringa roots and bark, which gives the tree one of its common names, “horseradish tree.” Moringa is in the same order as horseradish and other cabbage family members (*Capparales*). Isothiocyanates and related products from the cabbage family have been shown to have anti-tumor and anti-carcinogenic effects. Clinical research at Johns Hopkins University, USA, supported the traditional use of moringa to treat cancer (Fahey, 2005). The strong tradition of medical uses of moringa combined with recent scientific work supporting these tradition beliefs has resulted in increased marketing of supplements and so-called moringa “superfoods” (Radovitch & Paull 2008; Radovitch, 2009).

Utilization of waste products

Rajendran et al. (2016) conducted experiments to determine the uses of deseeded moringa pods (DMP) which is usually wasted after seed removal and subjected to environment dumping and degradation often associated with methane gas production and climate change. The nutrient analysis from their study showed that deseeded moringa pod was rich in fiber, carbohydrate and protein and the bakery products derived from DMP included fortified cookies and soup mix. Physical properties like bulk density, average size of the particle, porosity of the powder, influenced the spread potential and size of the textural characteristics such as hardness and fracturability of cookies. These physical attributes also impacted positively on organoleptic evaluations such as taste, appearance, color, flavor and overall acceptability based on a nine-point hedonic scale which actually turned out to be higher than average for the cookies and soup mix.

In other studies, Soliman et al. (2019), investigated the effectiveness of moringa seed waste as a novel green environmental absorbent for removal of industrial toxic dyes, red 60 (DR60) and Congo red (CR) from aqueous solutions. They elucidated that the absorption rate for both dyes were very high at the initial stages which eventually decreased until equilibrium was achieved. They further added that the absorption rate of the CR dye was not affected by catalyst weight, pH, or solution temperature. Maximum amounts of dyes absorbed were reported to be 170.7 and 196.8 mg/g for both CR and DR60 dyes, respectively, at 25^0C and pH 7. The authors concluded that the overall rate of the absorption process seemed to be controlled by a chemical process mechanism involving valence forces through the exchange or sharing of electrons between dyes and MSW absorbents. Maina et al. (2016) focused on the unexploited property of moringa seed pod (MSP) and morula nutshells (MNS) as a bio-remedial approach for removal of metals (copper, zinc, lead, manganese, cadmium, magnesium, and iron) from wastewater and borehole water. They reported that removal efficiencies were improved after treating sorbents with acids. This method was found to be simple, cheap and environmentally friendly and could be a remedial solution for water scarcity in rural areas where there are no resources to acquire expensive conventional techniques.

A study published by Otunola et al. (2013) showed that the use of moringa leaf waste (MLW) as a dietary fiber source enhanced cookie fiber content by as much as 29.54% relative to a control recipe, and 58% relative to a commercial cookie recipe. All cookies containing MLW were acceptable, scoring above 3.5 on the 7-point hedonic scale. Their investigation also highlighted that the addition of MLW fiber up to 10% in cookies was acceptable, although inclusion at 5% was found to be more acceptable. They concluded that cookies with MLW by-products have the advantage of being a good source of some of the daily requirements of dietary fiber.

CONCLUSIONS

There is an increasing rise in the use of ethno-medicinal and traditional herbs in the treatment of non-communicable, life-style, and pathogenic induced diseases in humans. The popularity of Asian homeopathic and Ayurvedic practices are based on a long successful history in the use of herbal plant treatments. *Moringa oliefera is* one of the many plants so utilized over the centuries in folklore medicine. It has attracted the attention of phytochemists and pharmacologists in the understanding of the mode of action of the active ingredients of the phytochemicals. Agronomists have investigated the crop production under both the wild and cultivated situations, and assayed the changes in the active ingredients, its biological effectiveness, biosynthesis and accumulation within the cell of the plants.

This treatise provides an in-depth assessment of the current and relevant research on the crop agronomy, its agro-ecological conditions, and pre- and post-harvest operations and treatments and utilization of value-added products. It aligned the biochemical production of the various phytochemicals to the ecophysiology of the plants particularly the variety and ecotypes, micro climatic and edaphic conditions, and the crop growth patterns. The detailed aggregation of the different phytochemicals and the sites of synthesis in the plant revealed that the plant produces in excess of 36 phytochemical compounds that manifest it biological efficacy in over 16 different physiological activity related to human health and medicine.

There are many commercial moringa products such as teas, tablets and capsules, and formulae and oils that are used in alternative medicine. However, the agronomic production and post-harvest treatments of the plant parts have been shown to influence the quality of the phytochemicals. As such, this comprehensive review aligns good agricultural practice (GAP) to optimal biosynthesis of moringa phytochemicals for its place in plant-based pharmacology.

Conflict of interest

The authors declare no conflict of interest to report.

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Optimising drying conditions for maximum nutritional quality and bioactivity of *Cucurbita pepo* L var. *fastigata* flesh and seeds

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ABSTRACT

Purpose: Transformation of pumpkin flesh and seeds into dry flours increases their shelf stability and versatility. This study sought to optimise drying conditions for production of flour with high nutritional and nutraceutical value from flesh and seeds of *C. pepo* L var. *fastigata* using Response Surface Methodology, I optimal design. **Research Method**: Pumpkin flesh and seeds were dried following temperature time combinations got using I optimal design. All dry samples were tested for ascorbic acid, total carotenoids, total antioxidant capacity and starch digestibility. Seeds were also tested for *in vitro* protein digestibility, trypsin inhibitor activity, alpha tocopherol, beta tocopherol, and alpha tocotrienol. **Findings**: The predicted optimum drying conditions for production of *C. pepo* L var. *fastigata* flour with maximum nutritional quality and bioactivity were 57°C; 6.9 hours for flesh and 60°C; 3.15 hours for seeds. The most influential model terms were temperature for resistant starch, *in vitro* protein digestibility, trypsin inhibitor activity, total carotenoids, alpha tocopherol, beta tocopherol and alpha tocotrienol; quadratic term of temperature and time for ascorbic acid; and drying time for total antioxidant activity of the flesh and temperature for antioxidant activity for seeds. **Research limitations**: Dryer air flow rate was not included as a variable. **Originality/Value:** Results give, for the first time, an objective basis for choice of the drying conditions *C. pepo* L var. *fastigata* flesh and seeds for maximum nutritional and health benefits.

INTRODUCTION

Pumpkin (*Cucurbita* spp) flesh is rich in carbohydrates and carotenoids while seeds are rich in protein, fat and bioactive compounds such as carotenoids, vitamin E and sitosterol (Kim et al., 2012). Drying of pumpkin flesh increases shelf-life and versatility, enabling use in bakery products, sauces, pasta and instant noodles (Mirhosseini et al., 2015).

Hot air drying is widely used in production of pumpkin powder (Que et al., 2008). Several studies have assessed the effect of drying temperature and methods on properties of dried products. However, there is lack of information on the optimum pumpkin drying temperature-time combinations. Roongruangsri and Bronlund (2016) reported significant effect of drying temperature on the physicochemical attributes of pumpkin powder. Ceclu et al. (2016) found significant differences in rehydration properties of pumpkin slices dried using different methods. Monteiro et al. (2018) reported effect of drying method on drying kinetics, rehydration kinetics and rehydration indices of dehydrated pumpkin slices. The current study sought to determine the effects of hot air drying conditions (including temperature and time) on preserving the nutritional quality and bioactivity in the flesh and seeds of *C. pepo* L var. *fastigata*.

MATERIAL AND METHODS

Mature fruits were cut into 8 pieces using a stainless steel kitchen knife, seeds removed, pieces peeled and sliced to 0.3 cm thick and 2 cm long. Seeds were washed under running tap water for about 5 minutes. Preliminary drying experiments were conducted at 40 to 80°C using an electric dryer (Philip Harris Ltd, Shenstone England) and limits for temperature and time set based on attainment of about 10% moisture content (Ekorong et al., 2015). Samples were picked at intervals of 30 minutes for flesh and 15 minutes for seeds and moisture content determined using the oven method (Bradley, 2003). The selected drying limits were 40-80°C and 3.5-11 hours for flesh and 40-80°C and 1.25-5.25 hours for seeds (Table 1).

Response Surface Methodology I optimal design (Design Expert version 11 Stat-Ease Inc., Minneapolis MN) was used to get 17 independent randomised drying temperature-time combinations (experimental runs) (Table 2) for drying samples. Samples were milled (multi-function mill Model 100, Zhejiang Winki Plastic Industry Co., China) and analysed for different response variables. Numerical optimisation was used to establish the optimum drying temperature-time. Triplicate samples were dried using optimal conditions, milled and resulting flours analysed. The prediction power of the developed models was verified by comparing the theoretical predicted data to the experimental data.

Table 1. The experimental levels of drying conditions used in the I-optimal design

Sample	Factor			Factor range and values		
	Symbol	Name	Units	-1	0	+1
Flesh	A	Temperature	°C	40.00	60	80.00
	B	Time	Hours	3.50	7.5	11.50
Seeds	A	Temperature	°C	40.00	60	80.00
	B	Time	Hours	1.25	3.25	5.25

Table 2. Experimental Conditions to which pumpkin flesh and seeds were subjected

Flesh samples			Seeds samples		
Experimental run	Temperature (°C)	Time (Hours)	Experimental run	Temperature (°C)	Time (Hours)
1	50	9.5	1	50	3.25
2	70	11.5	2	50	4.25
3	70	7.5	3	70	5.25
4	50	11.5	4	70	4.25
5	60	7.5	5	50	4.25
6	50	7.5	6	60	4.25
7	70	5.5	7	60	3.25
8	40	11.5	8	80	1.25
9	70	9.5	9	70	3.25
10	60	9.5	10	70	2.25
11	60	5.5	11	60	5.25
12	60	7.5	12	40	5.25
13	50	9.5	13	50	3.25
14	50	7.5	14	60	3.25
15	60	11.5	15	60	3.25
16	60	7.5	16	50	5.25
17	80	3.5	17	60	2.25

Starch digestibility

Moisture content of the raw samples and flours was determined using the oven method (Bradley, 2003) and resistant starch determined following megazyme resistant starch assay procedure (Megazyme International, 2017). Flour (0.1 g) and raw sample (0.5 g) were each placed in a falcon tube and 4 mL of a solution containing pancreatic α-amylase (10 mg.Ml^{-1}) and 3 enzyme units of amyloglucosidase per milliliter (3 U.mL^{-1}) added. One enzyme unit (U) being the amount of enzyme required to convert one micromole of a substrate under specified conditions of the assay method. The mixture was covered, vortexed, aligned horizontally in a shaking water bath (100 rpm; 37°C), for 16 hours, 4 mL of 99% ethanol added, vortexed and centrifuged at 1,610×g for 10 minutes (225 centrifuge, Fisher Scientific Co. St. Louis, MO). The supernatant was decanted off, 2 mL of 50% ethanol added to the pellet, vortexed, 6 mL of 50% ethanol added, mixed and centrifuged (1500×g; 10 minutes). The supernatant was decanted off, 6 mL of 50% ethanol added, mixed, centrifuged (1500×g; 10 minutes) and decanted. To the pellet, 2 mL of 2 M potassium hydroxide was added, stirred for 20 minutes, 8 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.1 mL concentrated amyloglucosidase solution (3,300 U.mL^{-1}) added, mixed, incubated in a water bath (50°C; 30 minutes) with intermittent vortexing, and the mixture adjusted to 100 mL using distilled water. An aliquot was centrifuged (1500×g; 10 minutes), 0.1 mL supernatant transferred into duplicate test tubes, 3 mL of glucose oxidase peroxidase (GOPOD) added, incubated (50°C; 20 minutes) and the absorbance read at 510 nm against a reagent blank. Quadruplicate glucose standards (0.1 mg.mL^{-1}) were mixed with 3 mL GOPOD, the absorbance determined, and resistant starch calculated as follows (1).

$$\text{Resistant starch (g/100g, dwb)} = \Delta A \times F \times \frac{100\text{ mL}}{0.1\text{ mL}} \times \frac{1}{1000} \times \frac{100}{W\text{ (g)}} \times \frac{162\text{ (g)}}{180\text{ (g)}} \quad (1)$$

Where:

ΔA: absorbance read against the reagent blank
F: conversion factor from absorbance to micrograms of glucose

$$F = \frac{100(\mu g \text{ of standard glucose sloution})}{\text{Absorbance of standard glucose sloution}}$$

100/0.1: volume correction factor since 0.1mL was taken from 100mL
1/1000: conversion from micrograms to milligrams
W: dry weight (g) of the sample analysed

$$W\ (g) = \text{"as is" weight (g)} \times \left[\frac{100 - \text{moisture content (\%)}}{100}\right]$$

$\frac{100}{W\ (g)}$: factor expressing nonresistant starch as a percentage of sample
$\frac{162\ (g)}{180\ (g)}$: conversion factor from free $D-$ glucose, as determined, to anhydro $-D-$ glucose as occurs in starch

In vitro protein digestibility

The multi enzyme method of Hsu et al. (1977) as modified by Park et al. (2010) was used. Protein content of the raw seeds and of the flours was determined using the AOAC 955.04 micro Kjeldahl method (AOAC, 1995). A test solution containing 6.25 mg protein/mL was prepared and its pH adjusted to 8.0 using 0.1 N sodium hydroxide. An enzyme solution of 1.6 mg trypsin (T0303), 3.1 mg chymotrypsin (C4129) and 1.3 mg peptidase (P4762 (all from SIGMA ALDRICH USA) was prepared, its pH adjusted to 8.0 using 0.1 N sodium hydroxide, 1 mL added to a 10 mL sample, mixed, incubated in a shaking water bath (130 rpm; 37 °C) for 10 minutes, pH determined and protein digestibility calculated as follows (2).

$$\text{Protein digestibility (\%)} = 201.464 - 18.103A \quad (2)$$

A: the final pH

Trypsin inhibitor activity

Trypsin inhibitor activity was determined following the bromocresol purple index "BCPI" method as reported by Szmigielski et al. (2010). Bromocresol purple index (quantity of bromocresol purple adsorbed, expressed as per protein mass unit in dry matter) was determined as follows (3).

$$BCPI = \frac{(A_b - A_s) \times C \times V}{A_b \times M} \times \frac{P}{100} \quad (3)$$

A_b: absorbance of the reagent blank
As: absorbance of the sample
C: concentration of bromocresoe dye (mg/mL)
V: volume of bromocresoe dye solution used (50mL)
M: mass of the sample (g)
P: percent protein content of the sample (dry matter basis)
Trypsin inhibitor activity (TIA mg/g protein DMB) was determined as follows (4).

$$TIA\ (mg/g) = -1.3042\ (BCPI) + 27.049 \quad (4)$$

α-tocopherol, β-tocopherol and α-tocotrienol content

α-tocopherol, β-tocopherol and α-tocotrienol were determined as described by Frick and Doyle (2015). To a 0.4 g sample in a 50 mL centrifuge bottle, 10 mL of methanol were added, mixed, 10 mL of HPLC grade mixture of ethyl acetate and hexane (1:1) added, vortexed for

20 minutes, centrifuged (4,251×g; 10 minutes), the organic (upper) layer transferred into a clean tube and the sediment re-extracted with 10 mL of extracting solvent. The filtrates were combined, reconstituted to 100 mL and a 5 µL aliquot analyzed using Agilent Technologies 6420 Triple Quad LCMSMS system (SG16217007, Singapore). The HPLC conditions were a carbon 18 column (Poroshell 120 EC-C18, 2.7 µm, 3.0x50 mm (B16163), USA), two mobile phases composed of a mixture of 0.1% formic acid and 5 mM ammonium formate (A) and 0.1% formic acid (B) flowing at 0.5 mL.minute^{-1}. The separation was performed at 40°C. The Mass spectrometer conditions were gas temperature of 250 °C flowing at 8 L.minute^{-1}.

Total carotenoids

A sample (2 g) was extracted with 50 mL cold acetone in the dark, distilled water (250 mL) slowly added, the aqueous phase discarded and the procedure repeated four times. The extract was filtered through glass wool containing 15 g anhydrous sodium sulphate into a 50 mL volumetric flask, adjusted to volume using petroleum ether and its absorbance measured at 540 nm against petroleum ether as the blank (Rodriguez-Amaya & Kimura, 2004). The total carotenoid content was calculated as follows (5).

$$\text{Total carotenoid}(\mu g/g) = \frac{\text{Absorbance} \times \text{Total volume of extract} \times 10^4}{2592 \times \text{sample weight (g)}} \quad (5)$$

Where;
2592: absorption coefficient of beta carotene

Ascorbic acid content

Ascorbic acid content was determined using the 2, 6 Dichloroindophenol method (AOAC, 1995). Extracts of dark coloured samples were decolorised prior titration by adding a spatulaful of activated carbon to 10 mL followed by centrifugation (906×g; 10 minutes).

Total antioxidant activity

A 1 g ground sample was extracted thrice using 5 mL of 50% methanol while filtering, the filtrate pooled and volume adjusted to 100 mL with 50% methanol (Muanda et al., 2009). Antioxidant activity of the extract was determined by mixing 50 µL extract with 2.9 mL of freshly prepared 50% methanolic solution of 100 µM DPPH, vortexed, allowed to stand in the dark, at room temperature for 30 minutes and the absorbance of the mixture and control (DPPH solution) measured at 515 nm against 50% methanol as blank (Stratil et al., 2006). Antioxidant activity (percent radical scavenging activity) was calculated as follows (6) (Marwah et al., 2007).

$$\text{Radical scavenging activity (\%)} = \left[1 - \left(\frac{\text{Absorbance of the sample}}{\text{Absorbance of the control}}\right)\right] \times 100 \quad (6)$$

Statistical analysis

To determine the optimum drying conditions, all statistics and construction of 3D graphs were generated using the Design-Expert software (version 11, Stat-Ease Inc., Minneapolis MN). I-optimal design of RSM was used to fit polynomial models for the different response variables. The best model suggested by the program (one with highest order polynomial and p-value <0.05, was obtained from the Fit summary. The goodness of fit of the chosen model was determined using the coefficient of determination (R^2 close to 1) and a non-significant p-value for lack of fit (p >0.05). Analysis of variance at 5% level of significance was done in order to determine the significant model terms (P ≤0.05).

RESULTS AND DISCUSSION

The predicted optimum conditions for drying of *C. pepo* L var. *fastigata* flesh and seeds were 57°C; 6.9 hours and 60°C; 3.15 hours respectively. For both the flesh and seeds, all models (equations 7 to 19) were significant (P<0.05) and all experimental values were within the confidence interval of the respective predicted values (Table 3). Furthermore, all models had a non-significant lack of fit implying that the models were valid.

Starch digestibility

High drying temperature and long drying time resulted in increased resistant starch content of the flesh (Figure 1A) and seeds (Fig. 1B). Based on the coefficient of determination (R^2) values, 94.9% (equation 7) and 97% (equation 8) of sample variation for resistant starch of the flesh and seeds, respectively, was attributed to the independent variables namely temperature (A) and drying time (B).

$$\text{Resistant starch (g/100g sample DMB)}_{\text{Flesh}} = 36.12 + 0.40A + 0.10B - 0.12AB - 0.05A^2 - 0.05B^2 \text{ (model P value} < 0.001, R^2 = 0.949, \text{lack of fit P value } 0.563) \quad (7)$$

$$\text{Resistant starch (g/100g sample DMB)}_{\text{Seeds}} = 1.34 + 0.14A + 0.01B + 0.02AB + 0.02A^2 + 0.03B^2 \text{(model P value} < 0.001, R^2 = 0.970, \text{lack of fit P value } 0.287) \quad (8)$$

For the flesh (Table 4) and seeds (Table 5), temperature significantly and positively affected the resistant starch content. Donlao and Ogawa (2017) reported an increase in resistant starch content of rice as drying temperatures increased from 40 to 90°C and a decrease beyond 115°C. The increase in resistant starch of flesh and seeds could be due to drastic loss of moisture at drying temperatures not high enough to cause melting of the starch granules. This is supported by Vamadevan and Bertoft (2015) who report that heating a starchy food in presence of limited water like during drying, shifts the gelatinisation peak to a higher temperature. This inhibits gelatinisation thus reducing starch digestibility. Oh et al. (2018) also reported a negative correlation between dry heating temperature of 110 to 130°C and the *in vitro* starch digestibility of high amylose rice starch.

Table 3. Optimum predicted versus experimental values at the optimised drying

Response	Flesh			Seeds		
	Predicted values		Experimental Value	Predicted values		Experimental value
	Mean	95% CI for Mean		Mean	95% CI for Mean	
RS (g.100g^{-1} sample)	36.04	36.01-36.08	35.99±0.04	1.33	1.31-1.34	1.30±0.02
PD (%)				66.25	65.69-66.81	65.43±0.82
TIA (mg.g^{-1})				5.68	5.55-5.80	5.41±0.28
TAA (%DPPH scavenged)	35.55	33.74-37.33	35.72±0.97	44.75	44.41-45.09	44.63±0.41
AA (mg.100g^{-1})	43.58	42.01-45.15	41.88±0.46	14.42	14.33-14.50	13.89±0.52
TC (mg.100g^{-1})	11.52	11.07-11.97	10.61±0.55	7.00	6.94-7.06	6.56±0.44
α-tocopherol (mg.100g^{-1})				6.51	6.43-6.59	6.47±0.06
β-tocopherol (mg.100g^{-1})				2.92	2.89-2.94	2.83±0.08
α-tocotrienol (mg.100g^{-1})				3.36	3.34- 3.38	3.30±0.05

Data are means of triplicate determinations ± standard deviation.
(Resistant starch: RS; Protein Digestibility: PD; Trypsin inhibitor activity: TIA; Total antioxidant activity: TAA; Ascorbic acid: AA; Total carotenoids: TC)

Table 4. Significance of the nutrient quality and bioactivity parametric model terms for the dried pumpkin flesh

Factor	Total antioxidant activity			Total carotenoids			Ascorbic acid content			Resistant starch		
	CE	SE	P-value	CE	SE	P-value	CE	SE	P-value	CE	SE	P-value
Intercept	35.22	0.69		11.57	0.18		43.46	0.65		36.12	0.01	
A	2.63	2.22	0.274	1.15	0.42	*0.020	2.82	1.47	0.081	0.40	0.03	*<0.001
B	-6.97	2.08	*0.012	-1.13	0.43	*0.023	-5.26	1.49	*0.004	0.10	0.03	*0.012
AB	-4.84	5.53	0.410	0.29	0.72	0.689	-0.89	2.51	0.728	-0.12	0.05	*0.047
A^2	-7.82	3.99	0.090	-2.53	0.74	*0.006	-12.61	2.57	*<0.001	-0.05	0.06	0.360
B^2	-1.74	4.19	0.691	0.45	0.56	0.440	2.43	1.96	0.241	-0.05	0.04	0.260
A^2B	0.33	7.82	0.967									
AB^2	4.13	6.13	0.522									
A^3	-6.43	7.98	0.446									
B^3	3.95	4.48	0.407									

Model terms with P<0.05 are significant and are assigned an asterisk (*); A: Temperature; B: Time; CE: coefficient estimate and SE: standard error.

Table 5. Significance of the nutrient quality parametric model terms for the dried pumpkin seeds

Factor	Protein digestibility			Trypsin inhibitor activity			Resistant starch		
	CE	SE	P-value	CE	SE	P-value	CE	SE	P-value
Intercept	66.58	0.26		5.60	0.05		1.34	0.01	
A-Temperature	6.39	0.58	*< 0.001	-1.57	0.13	*< 0.001	0.14	0.01	*< 0.001
B-Time	0.49	0.59	0.420	-0.04	0.13	0.731	0.01	0.01	0.710
AB	1.65	0.99	0.126	-0.40	0.22	0.092	0.03	0.02	0.258
A^2	2.72	1.03	*0.022	-0.84	0.22	*0.003	0.03	0.02	0.284
B^2	1.35	0.78	0.111	-0.37	0.17	0.051	0.03	0.02	0.087

Model terms with P<0.05 are significant and are assigned an asterisk (*); A: Temperature; B: Time; CE: coefficient estimate and SE: standard error.

In vitro protein digestibility and trypsin inhibitor activity

High drying temperature and long drying time resulted in high protein digestibility and low trypsin inhibitor activity (Fig. 2). R^2 values imply that 97.3% and 97.9% of variation in protein digestibility (equation 9) and trypsin inhibitor activity (equation 10) was attributed to the independent variables, temperature (A) and drying time (B).

$$\text{Protein digestibility (\%)}_{\text{Seeds}} = 66.58 + 6.39A + 0.49B + 1.65AB + 2.72A^2 + 1.35B^2 \text{ (model P value} < 0.001, R^2 = 0.973, \text{lack of fit P value } 0.169) \quad (9)$$

$$\text{Trypsin inhibitor activity (mg/g Protein DMB)}_{\text{seeds}} = 5.60\text{-}1.57A\text{-}0.04B\text{-}0.40AB\text{-}0.84A^2\text{-}0.37B^2 \text{ (model P value} < 0.001, R^2 = 0.979, \text{lack of fit P value } 0.362) \quad (10)$$

For both *in vitro* protein digestibility and trypsin inhibitor activity, temperature was the only significant variable (Table 5). There was a steep increase in *in vitro* protein digestibility (Figure 2A) and reduction in trypsin inhibitor activity as drying temperature increased from 60 to 80°C. In the temperature ranges of 40 to 60°C and 60 to 80°C, trypsin inhibitor activity reduced by 1.02% and 2.14% respectively with the steepest increase between 70 and 80°C (Fig. 2B). The observed pattern is in agreement with Damodaran (1996) who reported that during denaturation of most globular proteins, the monitored parameter remains fairly unchanged until the critical point of the denaturant is reached and then the parameter changes

abruptly. Roychaudhuri et al. (2003) also reported soybean Kunitz trypsin inhibitor to get denatured at approximately 65°C. Protein digestibility increased by 3.82% and by 7.99% as temperature increased from 40 to 60°C and 60 to 80°C, respectively (Fig. 2A). The steep increase between 60 to 80°C can be attributed to denaturation of the pumpkin seed proteins and the trypsin inhibitor since denaturation is accompanied by increased digestibility (Damodaran, 1996). The protein digestibility of the raw seeds (62.59%±0.15) was however lower than the values of 72% observed in seeds of fluted pumpkin (Fagbemi et al., 2005).

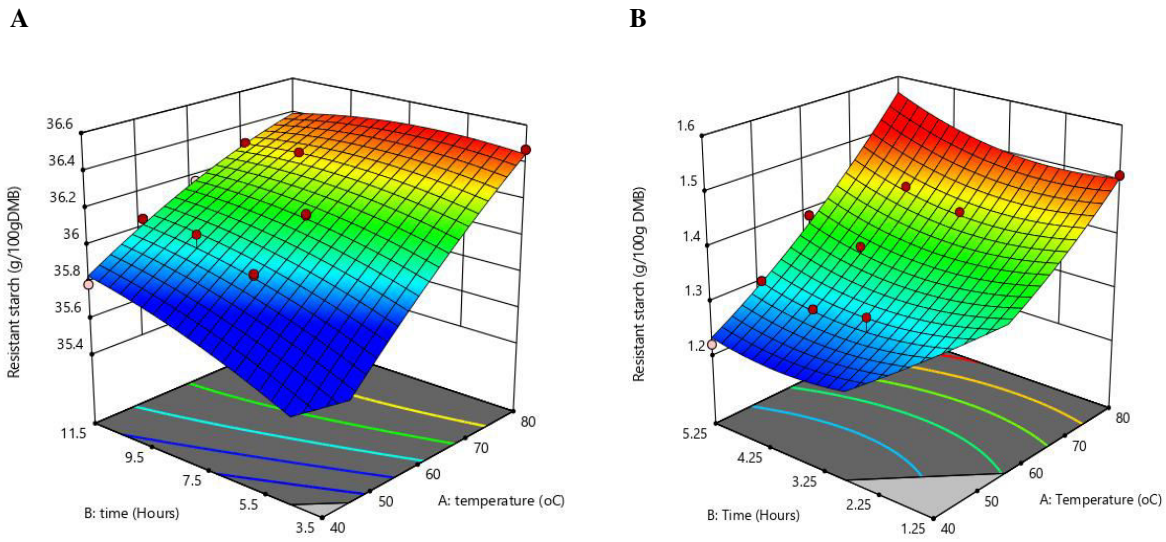

Fig. 1. Resistant starch content (%) of pumpkin flesh (A) and seeds (B) at different drying regimen.

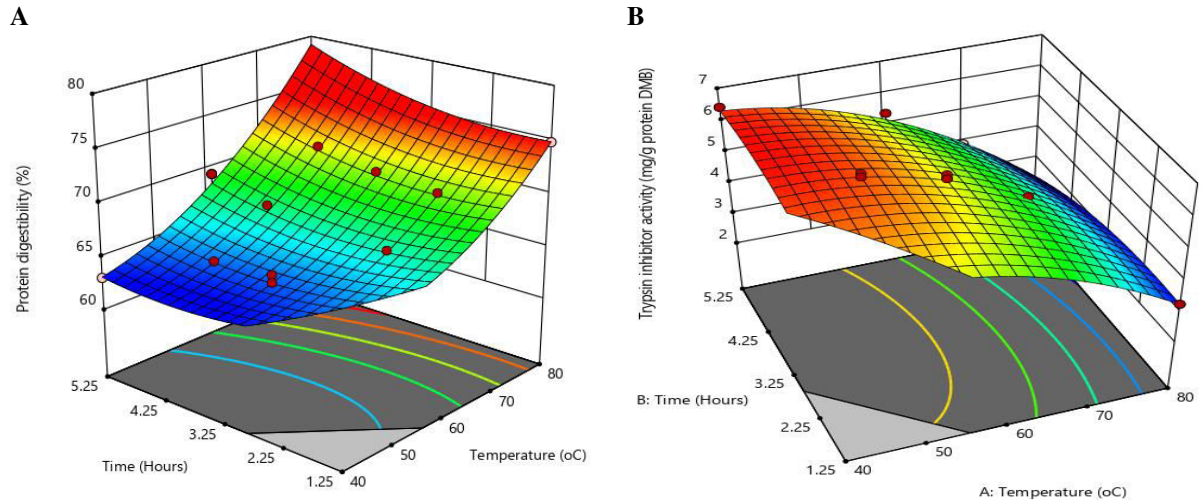

Fig. 2. *In vitro* protein digestibility and trypsin inhibitor activity and of pumpkin seeds at different drying regimen.

Ascorbic acid content of the flesh and seeds

R^2 values imply that 91.1% and 89.0% of the observed variation in ascorbic acid of the flesh (equation 11) and seeds (equation 12) was attributed to the independent variables, temperature (A) and drying time (B).

$$\text{Ascorbic acid (mg/100gDMB)}_{\text{flesh}} = 43.46 + 2.82A - 5.26B - 0.89AB - 12.61A^2 + 2.43B^2 (\text{model p value } < 0.001, R^2 = 0.911, \text{lack of fit P value } 0.182) \quad (11)$$

$$\text{Ascorbic acid (mg/100g DMB)}_{\text{Seeds}} = 14.42\text{-}0.24A\text{-}0.21B + 0.29AB\text{-}0.29A^2\text{-}0.11B^2 (\text{model P value} < 0.001, R^2 = 0.890, \text{lack of fit P value } 0.21) \quad (12)$$

Ascorbic acid content of the flesh was highest at drying conditions of 60°C: 5.5 hours (Figure 3A) whereas that of seeds was highest at drying conditions of 60°C: 2.25 hours (Fig. 3B). The quadratic temperature related term A^2 (mostly) and time, significantly and negatively affected the ascorbic acid content of the flesh (Table 4). For the seeds however, both temperature and time were significant and negatively affected the ascorbic acid content (Table 6). For both the flesh and seeds, ascorbic acid content was low at drying temperatures below and above 60°C. It was however lowest at temperatures below 60°C for the flesh, ascribed to the long period samples were exposed to the drying heat coupled with high activity of the enzyme ascorbic acid oxidase. Ascorbic acid oxidase activity increases upon damaging tissues (Gregory, 1996) for example during cutting of the pumpkin flesh into small pieces to be dried and it has optimum activity at 40°C.

Total carotenoids content of the flesh and seeds

R^2 values imply that 90.1% and 93.3% of sample variation for total carotenoids content of the flesh (equation 13) and seeds (equation 14) was attributed to the independent variables, temperature (A) and drying time (B).

$$\text{Total carotenoids (mg/100gDMB)}_{\text{flesh}} = 11.57 + 1.15A - 1.13B + 0.29AB - 2.53A^2 + 0.45B^2 (\text{model P value} < 0.001, R^2 = 0.901, \text{lack of fit P value } 0.132) \quad (13)$$

$$\text{Total carotenoids (mg/100g DMB)}_{\text{Seeds}} = 7.00 + 0.37A\text{-}0.01B + 0.19AB\text{-}0.23A^2 + 0.20B^2\text{-}0.18A^2B\text{-}0.16AB^2\text{-}0.58A^3\text{-}0.15B^3 (\text{model P value}0.002, R^2 = 0.933, \text{lack of fit P value } 0.988) \quad (14)$$

For both the flesh and seeds, temperature positively influenced the total carotenoids content, implying that an increase in drying temperature led to increased retention of total carotenoids (Fig. 4). Potosí-Calvache et al. (2017) also reported an increase in carotenoids retained in *Cucurbita moschata* as drying temperature increased from 45 to 55°C followed by a decline as drying temperature increased from 55 to 65°C. The observed carotenoid retention at high drying temperatures was probably attributed to the less drying time associated with high temperatures and the inactivation of oxidative enzymes. Besides Rodriguez-Amaya and Kimura (2004) reported degradation of carotenoids by oxidative enzymes to be more of a problem than thermal decomposition during processing and that rapid processing at high temperature was a good alternative.

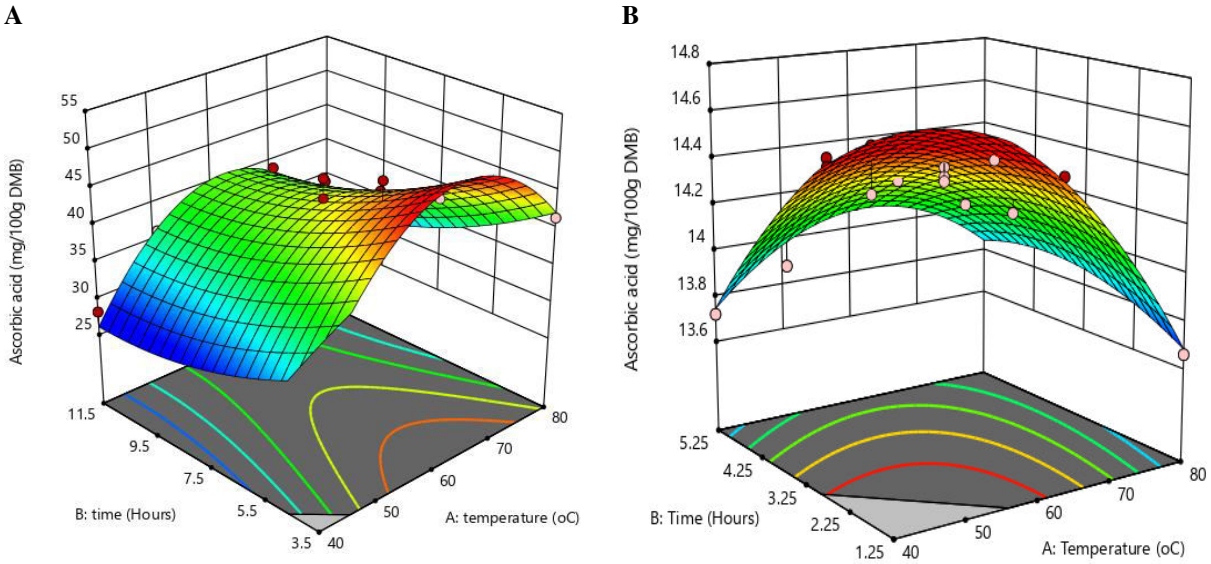

Fig. 3. Ascorbic acid content of pumpkin flesh (A) and seeds (B) at different drying regimen.

Table 6. Significance of the bioactivity parametric model terms for the dried pumpkin seeds

		Factor									
		Intercept	A	B	AB	A^2	B^2	A^2B	AB^2	A^3	B^3
TAA	CE	44.87	2.17	0.04	0.85	-1.59	0.84	-0.66	-0.81	-2.77	-0.71
	SE	0.14	0.45	0.42	1.13	0.81	0.85	1.59	1.25	1.62	0.91
	P-value		*0.001	0.918	0.472	0.091	0.355	0.689	0.536	0.132	0.459
AA	CE	14.42	-0.24	-0.21	0.29	-0.29	-0.11				
	SE	0.04	0.08	0.09	0.14	0.15	0.11				
	P-value		*0.015	*0.031	0.068	0.082	0.338				
TC	CE	7.00	0.37	-0.01	0.19	-0.23	0.20	-0.18	-0.16	-0.58	-0.15
	SE	0.02	0.07	0.06	0.18	0.13	0.14	0.26	0.20	0.27	0.15
	P-value		*0.001	0.889	0.324	0.123	0.196	0.514	0.454	0.064	0.345
AT	CE	6.47	-0.62	-0.21							
	SE	0.03	0.07	0.06							
	P-value		*< 0.001	*0.006							
BT	CE	2.90	-0.18	-0.09							
	SE	0.01	0.02	0.02							
	P-value		*< 0.001	* 0.001							
AT^*	CE	3.35	-0.18	-0.09							
	SE	0.01	0.02	0.01							
	P-value		*< 0.001	*< 0.001							

Model terms with P<0.05 are significant and are assigned an asterisk (*); A: Temperature; B: Time; CE: coefficient estimate and SE: standard error. (Total antioxidant activity: TAA; Ascorbic acid: AA; Total carotenoids: TC; Alpha tocopherol: AT; Beta tocopherol: BT; and Alpha tocotrienol: AT^*.)

Tocopherols and tocotrienol contents of the seed samples

R^2 values imply that 83.9%, 87.1% and 87.4% of sample variation for alpha tocopherol (equation 15), beta tocopherol (equation 16) and alpha tocotrienol (equation 17) was attributed to the independent variables, temperature (A) and drying time (B).

$$\alpha - \text{Tocopherol (mg/100gDMB)}_{\text{Seeds}} = 6.470 - 0.62A - 0.20B \text{ (model P value} < 0.001, R^2 = 0.839, \text{lack of fit P value } 0.498) \quad (15)$$

$$\beta - \text{Tocopherol (mg/100gDMB)}_{\text{Seeds}} = 2.90 - 0.18A - 0.08B \text{ (model P value} < 0.001, R^2 = 0.871, \text{lack of fit P value } 0.498) \quad (16)$$

$$\alpha - \text{Tocotrienol (mg/100gDMB)}_{\text{Seeds}} = 3.35 - 0.18A - 0.08B \text{ (model P value} < 0.001, R^2 = 0.874, \text{lack of fit P value } 0.517) \quad (17)$$

Temperature and time, significantly and negatively influenced the alpha tocopherol, beta tocopherol and alpha tocotrienol content (Table 6) implying that increasing the drying temperature led to more reduction in tocopherols and tocotrienol, graphically presented in the 3D response surface plots (Fig. 5). Their reduction can be explained by the fact that thermal induced degradation of vitamin E increases with increase in temperature (Gregory, 1996).

Total antioxidant activity

R^2 values imply that 90.7% and 93.5% of sample variation for total antioxidant activity of the flesh (equation 18) and seeds (equation 19) was attributed to the independent variables, temperature (A) and drying time (B).

$$\text{TAA(\% DPPH scavenged)}_{\text{Flesh}} = 35.22 + 2.63A\text{-}6.97B\text{-}4.84AB\text{-}7.82A^2\text{-}1.74B^2 + 0.33\,A^2B + 4.13AB^2\text{-}6.43A^3 + 3.95B^3 \text{(model P value } 0.006, R^2 = 0.907, \text{lack of fit P value } 0.06) \quad (18)$$

$$\text{TAA(\% DPPH scavenged)}_{\text{Seeds}} = 44.87 + 2.17A + 0.04B + 0.85AB - 1.59A^2 + 0.84B^2 - 0.66A^2B - 0.811AB^2 - 2.77A^3 - 0.71B^3 \text{ (model P value } 0.002, R^2 = 0.935, \text{lack of fit P value } 0.955) \quad (19)$$

The 3D plot (Figure 6) showed an increase in retention of total antioxidant activity as drying temperatures increased from 40 to 60 °C (flesh) and 40 to 70°C (seeds) after which total antioxidant activity reduced with further increase in drying temperature. Time significantly and negatively affected the total antioxidant activity of the flesh (Table 4) probably because ascorbic acid, which was the dominant antioxidant, was significantly and negatively affected by time. In addition, ascorbic acid as an antioxidant is known for regenerating other antioxidants like tocopherols. Besides, total antioxidant depends on synergistic and redox interactions among the different molecules, in addition to the levels of antioxidants in food (Pellegrini et al., 2003). However, for the seeds, temperature was the most influential and positively affected the total antioxidant activity (Table 6). Ekorong et al. (2015) also found the effect of drying temperature to outweigh the effect of time on the total antioxidant activity of dried mango seed kernels.

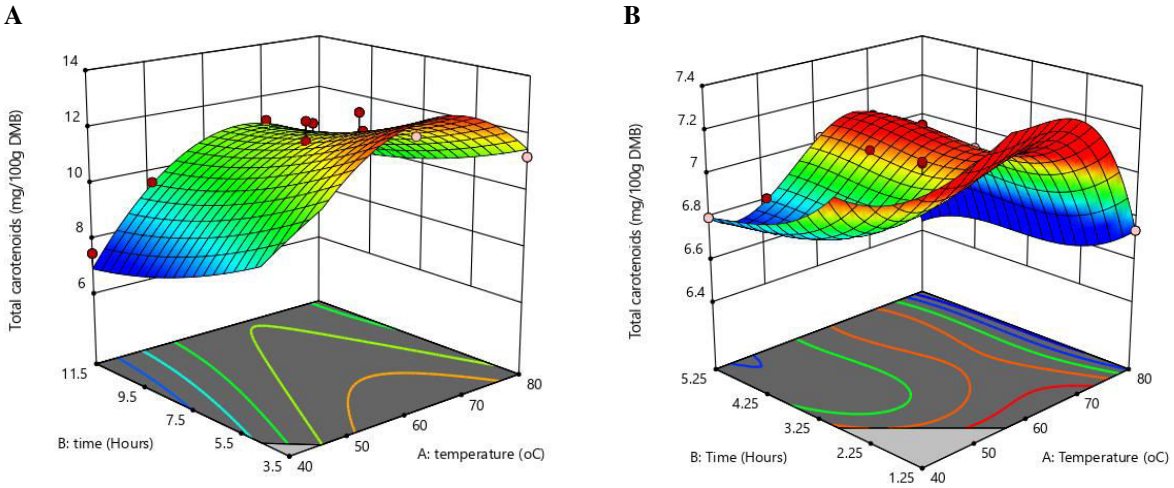

Fig. 4. Total carotenoids of pumpkin flesh (A) and seeds (B) at different drying regimen.

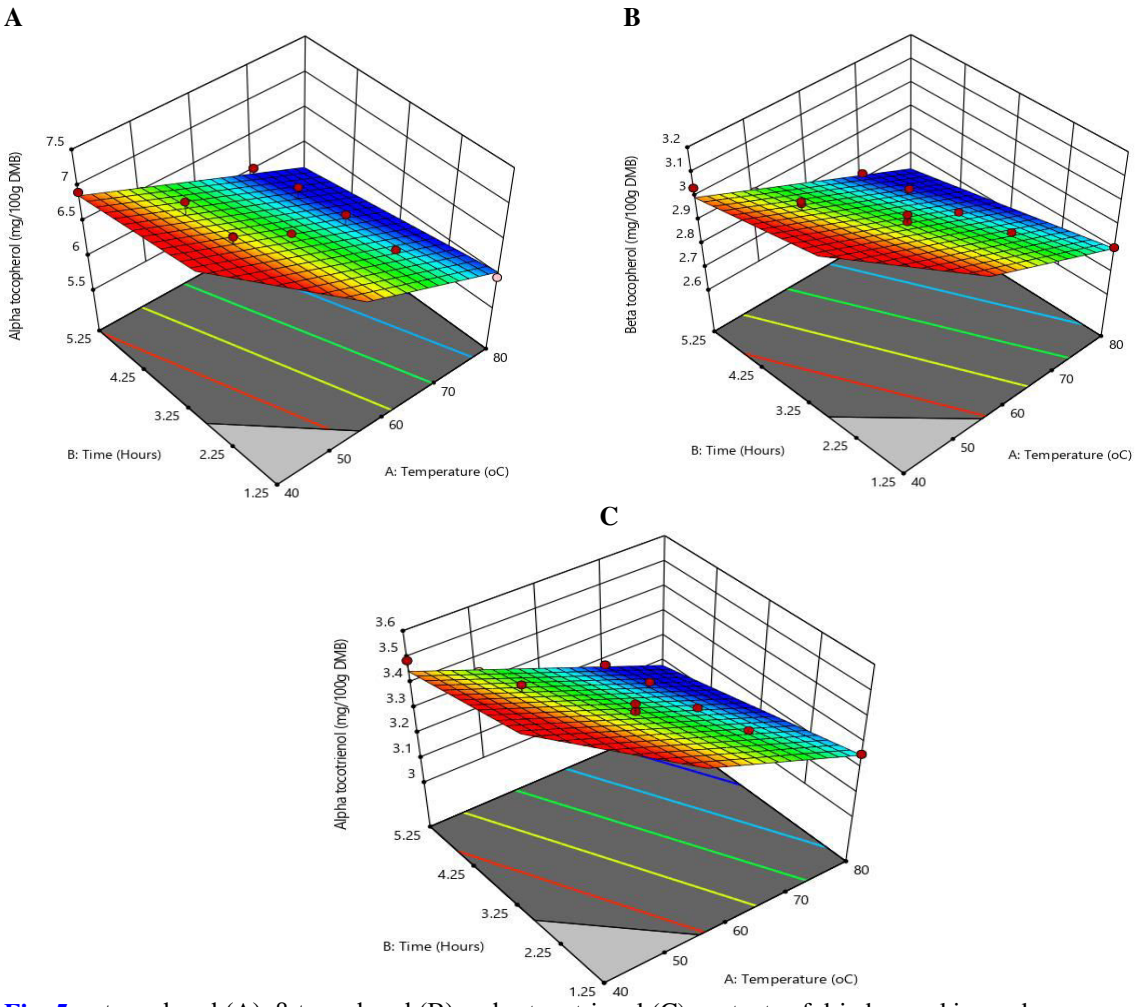

Fig. 5. α-tocopherol (A), β-tocopherol (B) and α-tocotrienol (C) contents of dried pumpkin seeds.

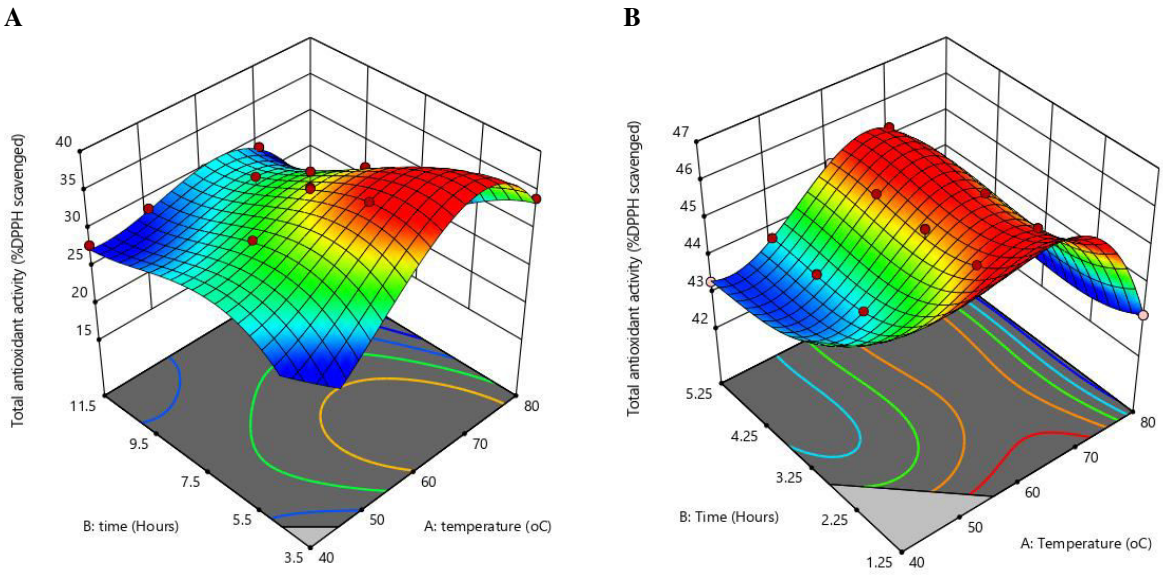

Fig. 6. Total antioxidant activity of pumpkin flesh (A) and seeds (B) at different drying regimen.

CONCLUSION

The increase in resistant starch content at the optimised drying conditions 57°C; 6.9 hours (flesh) implies better nutritional quality and nutraceutical value of flour from flesh especially in conferring health benefits like reducing the chances of developing Type-2 diabetes mellitus, obesity, and cardiovascular diseases. Increase in *in vitro* protein digestibility and reduction in trypsin inhibitor activity of seeds at the optimised drying conditions of 60°C; 3.15 hours implies that the body will utilise better the protein of the pumpkin seed flour. In addition, the body shall obtain maximum cumulative capacity of the components in flours to scavenge free radicals thus delaying the onset and progression of diseases like cancer.

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Conflicts of interest
The authors have no conflict of interest.

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Enhancing the shelf life of tomato fruits using plant material during storage

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ABSTRACT

Purpose: Postharvest losses of fruits and vegetables are major problem for countries that agriculture is one of the source economy and that losses in tomato have reported from 20 to 50%. It is one of the very perishable fruit and it changes continuously after harvesting. The aim was to evaluate the effect of guava (*Psidium guajava*) and physic nut (*Jatropha curcas*) leaf extract on fresh ripe tomato fruits. **Research method:** Two tomato varieties obtained (UTC and Tandilo) from Gombe Main Market and were then treated with aqueous and ethanolic extract of *Psidium guajava* and *Jatropha curcas* leaves to extend their shelf life and maintain the quality of tomato fruits during storage. The experiment was laid out using completely randomized block design, (CRBD). Number of days to deterioration was recorded on visual eating quality. The recorded data were analyzed using analysis of variance. **Findings:** Ethanolic extracts of *P. guajava* and *J. curcas* was highly effective on UTC variety. The postharvest decay that was the main quality factor in this experiment significantly reduced in Tandilo and UTC tomato fruits after using 2.5g/1000ml ethanolic J. curcas leaves extract by 24 and 25 days respectively; and 22 and 23 days after using 2.5g/1000ml ethanolic P. guajava leaves extract. **Limitations:** No limitations were founded. **Originality/Value:** These findings indicate that application of leaves extracts proved to be effective in extending the shelf life of test tomato fruits.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most widely cultivated and extensively consumed horticultural crop (Abrar et al., 2016). In Nigeria, is one of the most important vegetable fruit grown for consumption in every home, consumed both fresh and in paste form and a cheap source of vitamin A, C, E and minerals which protect the body against diseases, Babatola et al. (2008) and Hosea et al. (2017). Tomato and tomato base products are believed to be healthy food owing to its low calories, cholesterol-free and good fiber source (Zamora et al., 2005). Tomato is believed to be an important functional food in preventing and curing malignant diseases like prostate cancer, breast cancer (Canene-Adams et al., 2005), lungs cancer (Feskanich et al., 2000) and other diseases like cataracts, heart disease (Rao & Rao, 2007), diabetes, hyperglycemia (Subash et al., 2007), inflammation, arthritis, immune system decline, brain dysfunction (Petr & Erdman, 2005), and maintenance of body homeostasis (Kennedy et al., 2006).

The storage and preservation of tomato is vital to the economy of homes, farmers and country considering the important role played by tomato in health and food security (Irokanula, et al., 2015). Deterioration of fresh tomato fruits can results from physiological breakdown due to ripening process, loss of water, injury, temperature or microorganism invasion (Babatola, et al., 2008). Postharvest losses of fresh fruits and vegetable including tomatoes are estimated to be 20-25% in developing countries (Neeta et al., 2010).

Due to high perishable nature of tomato fruits, many of them rot before they reached various areas of country where they are not cultivated and demand is high. Owing to the lack of knowledge on postharvest handling, packing and preservation, the fruits lose their quality (Ahmed & Tariq, 2014). This result in quantitative and nutritional losses to farmers, consumers as well as rural and urban dwellers far from areas of production who have to pay more to get them (Ejale & Abdullah, 2004). Commercially, preservation of vegetable products is difficult in the tropics because of poor transportation networks and high environmental temperatures that favor decay rather than storage. Many synthetics have been used to preserve tomato fruits but consumers are becoming very concerned on the use of synthetics on horticultural crops like tomatoes. The use of plant materials as preservatives apart from extending shelf life of foods, are less toxic to humans and animals than synthetic preservatives. These attributes of plant in preservation of food enhances the economic value of such foods (Irokanula et al., 2015). Attempt has been made to investigate on the use of plants as bio-preservatives, which are multipurpose, cheap, easy to use and have tremendous uses as food and medicine. This study seeks to investigate the preservative activities of *Psidium guajava* and *Jatropha curcas* leaves extract on postharvest shelf life and quality of tomato fruits.

MATERIALS AND METHODS

Collection of tomato fruits and plant materials

Two fresh tomato fruits varieties, Tandilo and UTC, were collected from Gombe Main Market and used in the study. Collected tomato fruits were transported to the Department of Biological Sciences Laboratory, Gombe State University for treatment and observation. Leaves of *P. guajava* and *J. curcas* were collected from the University Botanical Garden, transported to the laboratory and cleaned, air dried and grounded into powder.

Preparation of plant extract

Powdered leaves of *P. guajava* and *J. curcas* (50 g) each was dissolved in 500 ml of ethanol and distilled water in a 1 liter capacity flask (Olamifin, 2002). Flasks were allowed to stand for 7 days and shaken at regular intervals. The solutions were filtered using filter paper. Filtrates were evaporated in water bath to obtain the extracts.

Preparation of aqueous and ethanolic extracts concentration

Ethanolic and aqueous extracts (0.5g in 1000 ml and 2.5g in 1000ml respectively) were prepared as explained by Adoum et al. (1997). The solutions of extracts were kept in refrigerator for daily use.

Qualitative analysis of plant materials

Phytochemical screening of bioactive constituents on extracts (aqueous and ethanol) was carried out as explained by Akponah et al. (2013).

Shelf life determination of tomato fruits

The experiment was laid in complete randomize block design with nine treatment and ten replication each. Fresh, ripe, firm and smooth tomato fruits were grouped into nine, each group containing ten (10) tomato fruits, first and second group were surface washed daily with 0.5g.ml^{-1} aqueous extract of *P. guajava* and *J. curcas* respectively, third and fourth group with 2.5g.ml^{-1} aqueous extract of *P. guajava* and *J. curcas* respectively, fifth with ethanolic extract of *P. guajava* (0.5g.ml^{-1}) concentration, sixth group with ethanolic extract of *P. guajava* (2.5g.ml^{-1}) concentration, group seven with ethanolic extract of *J. curcas* (0.5g.ml^{-1}) concentration, group eight with ethanolic extract of *J. curcas* (2.5g.ml^{-1}) concentration and the ninth group with distilled water for 3 minutes respectively. Each group was allowed to dry and kept in cup board for shelf life determination. Tomato fruits were recorded spoilt or deteriorated on rating of 4 (excellent), 3 (good), 2 (fair), 1 (poor) and 0 (very poor) (Mustapha et al., 2005).

Statistical analysis

The result of preservative effect of guava and jatropha leaves extracts on tomato fruits was analyzed using Analysis of Variance (ANOVA) at 5% level of significance.

RESULTS

Phytochemical constituents of leaves extract of *P. guajava* and *J. curcas*

The result for phytochemical screening is presented in Table 1. The result showed that alkaloids, tannins, steroid, were presents in ethanolic extracts of *P. guajava* while saponins, flavonoids, glycoside and phenols are absent. Aqueous extracts of *P. guajava* shows that alkaloids, tannins, saponins and steroids are presents while flavonoids, glycosides and phenols are absent. Alkaloids, tannins, saponins, flavonoids and steroids were present in both ethanolic and aqueous extracts of *J. curcas* leaves while glycosides, phenols are absent in both ethanolic and aqueous extracts of *J. curcas* leaves.

Shelf life determination of tomato fruits

The result showed that Tandilo and UTC variety recorded 14 and 15days respectively after treatment with 0.5g.1000ml^{-1} aqueous extracts of *P. guajava* leaves, while Tandilo and UTC variety recorded 18 and 20days respectively after treatment with 0.5g.1000ml^{-1} ethanolic extract of *P. guajava* (Fig. 1).

Table 1. Phytochemical constituents of *P. guajava* and *J. curcas* Leaves

	Ethanolic extract		Aqueous extract	
	P. guajava	*J. curcas*	*P. guajava*	*J. curcas*
Alkaloids	+	+	++	+
Tannins	++	++	++	+++
Saponins	++	+	+++	++
Flavonoids	+	-	-	+
Steroids	++	+++	++	++
Glycosides	-	-	-	-
Phenols	-	-	-	-

Key: +++ = Abundantly present; ++ = strongly present; + = Present; - = absent.

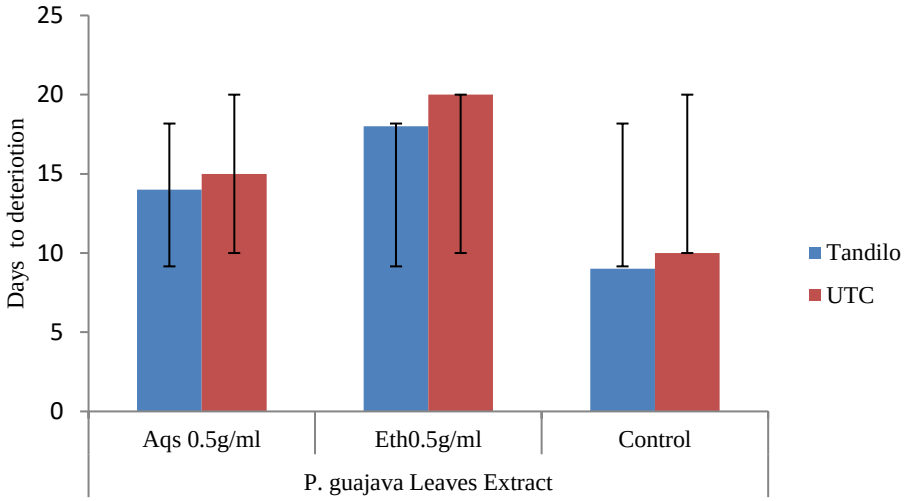

Fig. 1. Effect of P. guajava extract (0.5 g/ml) on shelf life of tomato fruits.

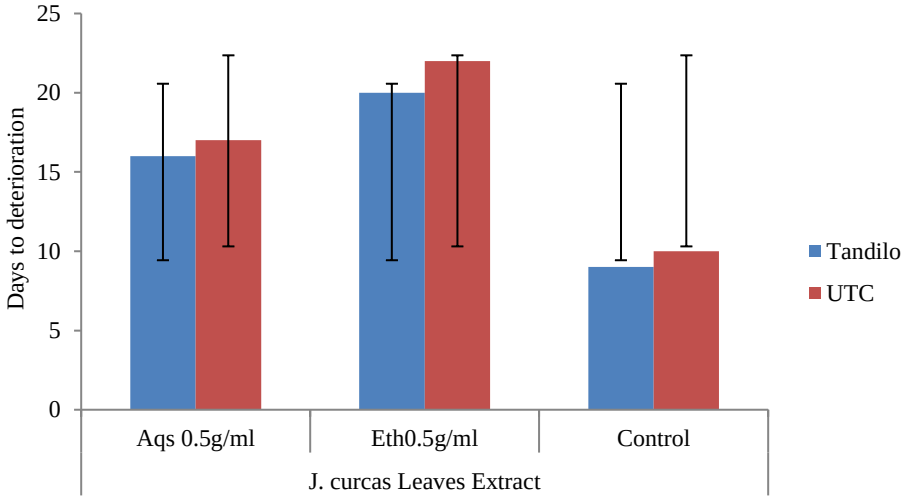

Fig. 2. Effect of J. curcas extract (0.5 g/ml) on shelf life of tomato fruits.

Tandilo and UTC variety recorded 16 and 17days respectively after treatment with 0.5g/1000 ml aqueous extract of *J. curcas* leaves, while after treatment with 0.5g.1000 ml^{-1} ethanolic extract of *J. curcas* leaves, Tandilo and UTC variety recorded 20 and 22 days respectively (Fig. 2). After treatment with 2.5g.1000ml^{-1} aqueous extract of *P. guajava*, Tandilo and UTC variety recorded 18 and 20 days respectively while, Tandilo and UTC variety recorded 22 and 23 days respectively after treatment with 2.5g.1000ml^{-1} ethanolic

extract of *P. guajava* leaves (Fig. 3). At 2.5g.1000ml^{-1} aqueous extract of *J. curcas*, Tandilo and UTC recorded 19 and 22 days while after treatment with 2.5g.1000ml^{-1} ethanolic extract of *J. curcas*, Tandilo and UTC recorded 24 and 25 days respectively. Control Tandilo recorded 9days and UTC recorded 10 days (Fig. 4).

DISCUSSION

The phytochemical analysis of *P. guajava* aqueous and ethanolic leaf extract showed presence of alkaloids, saponins, tannins, flavonoids, and steroids. Bansode and Chavan (2014), confirmed the presence of flavonoids, tannins, phenols and terpenoid in their work of screening for screening of guava for effective phyto-medicines and study on its antimicrobial effect against selected enteric pathogens. Also, Okunrobo et al. (2010) and Lincy et al. (2016) reported that *P. guajava* leaves contains secondary metabolites which include alkaloids, saponins, flavonoids, glycosides, anthraquinones, vitamins, reducing sugar, tannins, terpenoids, carbohydrates and amino acids.

Qualitative analysis of secondary metabolites in *J. curcas* leaf was confirmed in this study. Nwokocha et al. (2011) reported the presence of alkaloids, tannins, saponins, flavonoids and phenols in all parts of *J. curcas* plant. Present study confirmed the presence of metabolites with the exception of phenols and flavonoids in aqueous extracts. Also, tannins were found to be abundantly present. Qasim et al. (2017) also stated that secondary metabolites were present in different quantities in leaf of *J. curcas* when extracted using different solvent.

The surface washing of tomatoes with aqueous extract of *P. guajava* and *J. curcas* leaves at different concentration has enhanced shelf life and decreases deterioration of tomato varieties. This maybe as a result of bioactive component (alkaloids, tannins, saponins and steroids) present in the leaf extracts. Also, factors that aided in increasing the shelf life of tomatoes treated with aqueous extract is the treatment time and method employed in applying the extracts. This agrees with Bukar and Magashi (2013), who reported preservative activities of aqueous suspension of *Balanites aegyptiaca*, *Guiera senegalensis* and *Parkia biglobosa* leaves on tomatoes, oranges and pepper. They also reported that duration of treatment and vehicle used in treatment application of extract help in extending shelf life.

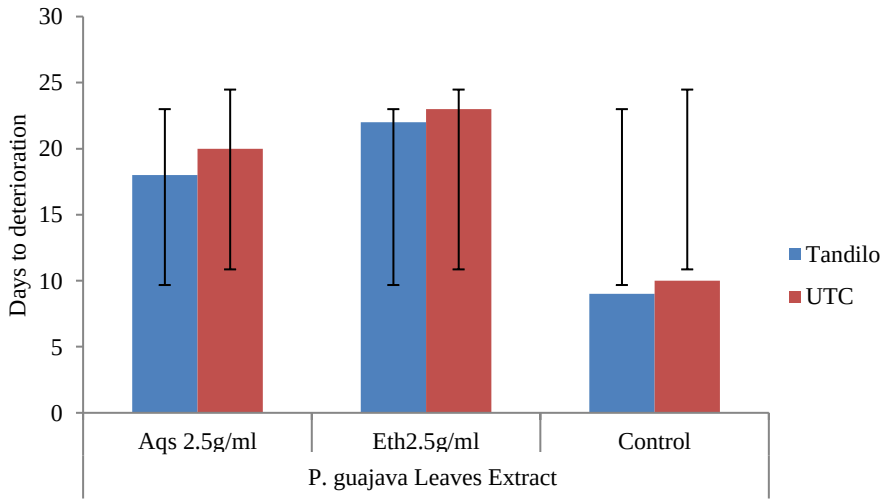

Fig. 3. Effect of P. guajava extract (2.5 g/ml) on shelf life of tomato fruits.

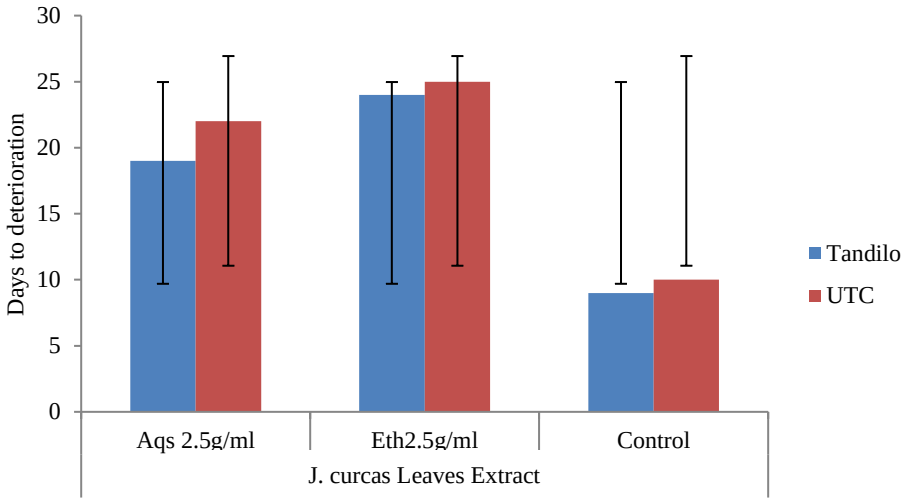

Fig. 4. Effect of plant extract (2.5 g/ml) on shelf life of tomato fruits.

Significant control of deterioration and shelf life enhancement of tomato varieties was observed in ethanolic extract of *P. guajava* and *J. curcas* at different concentration. This shows that tomatoes on retail can be preserved for up to 25 days while still maintaining firmness, flavor and texture. Irokanulo et al. (2015), reported similar result that tomatoes can be stored up to 30 days when covered with leaf and stem bark powder of *Moringa oleifera* and still remain fresh; and Jaiswal et al. (2018), that Aloe vera gel formulation has the ability to extend the shelf life of tomatoes to 30 days.

Sedighe et al. (2014) and Safiyaa et al. (2016) found a high record of deterioration and lowest shelf life in control treatment. Same result was observed in present study. Control treatment deteriorated at 9 and 10 days for Tandilo and UTC varieties respectively. Same result was observed by Bukar and Magashi (2013); but different from Hosea et al. (2017) who reported 19 days.

Extract application at $0.5g.1000ml^{-1}$ and $2.5g.1000ml^{-1}$ prove to be effective in increasing shelf life and decreasing rate of deterioration on tomatoes, because it can act as a barrier to oxygen and moisture which can speed up deterioration rate. This is because leaf extracts of *P. guajava* and *J. curcas* contains various compounds that can delay or inhibit microorganisms that are responsible for food born diseases in humans potentially. Jaiswal et al. (2018), reports same result in his work for development of Aloe vera based edible coating for tomato.

Surface washing of tomatoes not only prevents spoilage and increase shelf life and marketability, but also prevents fungal attack. Raheja and Thakore (2002) reported same results that extract from medicinal plants like *Alium sativum, Azadirachta indica, Mentha arvensis* and *Psoralea corylifolia* were found to be effective in preserving fruits from attack by pathogenic and environmental factors. Singh et al. (1999) also stated that, plants extracts are an alternative to commercial fungicides in preservation of plants products.

Among tomato varieties, UTC had the highest shelf life, this in an indication that UTC variety can withstands biotic and abiotic challenges better than Tandilo. Hosea et al. (2017) reported same result. Treatments of tomato varieties through aqueous and ethanolic extract of *P. guajava* and *J. curcas* leaves can serve in decreasing deterioration rate and increase shelf life of tomatoes as well as eliminating or reducing pathogenic and food spoilage microorganisms and enhancing food safety.

CONCLUSION

Understanding the postharvest treatment that could be a pesticide free method to reduce plant pathogens, control insect infestation and maintain fruit quality is important. Ultimately, the uses of plant materials as food preservatives apart from extending shelf life of foods are less toxic to humans and animals than synthetic or chemical preservatives. These attributes of plant materials in food preservation enhance the economic value of such foods. The present result obtained shows that extracts of *P. guajava* and *J. curcas* were able to extend shelf life and quality of tomato. This provides information on the use of plant leaves extract in postharvest preservation of fruits.

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Conflict of interest
There is no conflict of interest between the authors.

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Essential oil components, phenolic content and antioxidant activity of *Anthriscus cerefolium* and *Anthriscus sylvestris* from Iran

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ABSTRACT

Purpose: The Apiaceae family (Umbelliferae) is one of the largest families of flowering plants. The genus *Anthriscus* of this family is considered of high importance because of its use in folk medicines and flavoring throughout the world. Three species of this genus are represented in the Flora of Iran. The main objective of this study was to evaluate the essential oil composition, phenolic content and antioxidant activity of *Anthriscus cerefolium* and *Anthriscus sylvestris.* **Research Method:** The essential oil samples were isolated by hydrodistillation in a Clevenger type apparatus and analyzed using GC and GC–MS methods. The antioxidant activity and total phenolic content were determined by DPPH scavenging assay and Folin-Ciocalteu method, respectively. **Findings:** Oxygenated monoterpenes constituted the principal fraction of essential oils obtained from *A. cerefolium* (rich in estragole), while aliphatic esters were detected to be the main class of compounds isolated from *A. sylvestris* (rich in chrysanthenyl acetate). Among the essential oils and methanolic extracts from two *Anthriscus* species at vegetative stage the highest antioxidant activity was observed for essential oil of *A. sylvestris* (IC50=71.3 $\mu g.ml^{-1}$) followed by essential oil of *A. cerefolium* (IC50=115 $\mu g.ml^{-1}$). In addition, the amounts of total phenolic contents of *A. cerefolium* and *A. sylvestris* methanolic extracts at full flowering stage (76.7 and 74.6 mg $GAE.L^{-1}$) were determined. Other important group of compounds and their biological properties needs to be studied in *Anthriscus* species due to their potential pharmacological and food industry value**. Research limitations:** No limitations were founded. **Originality/Value:** Since the essential oil of *A. sylvestris* at vegetative stage demonstrated the noticeable antioxidant ability which makes it well qualified to be used as natural ingredients to synthetic antioxidants in food industry.

INTRODUCTION

The family of the *Apiaceae* (Umbelliferae) is one of the most important families of flowering plants comprises over 3700 species scattered throughout the world, mainly in the northern temperate regions and high altitudes in the tropics. Several species of the Apiaceae are well known as a source of essential oils which are used for different purposes including nutrition, medicine, beverages, spices, repellents, staining, cosmetics, fragrances and industrial uses (Sayed-Ahmad et al., 2017; Zengin et al., 2019). The genus *Anthriscus Pers.* (commonly known as beaked parsley, rough chervil, beaked chervil) with 12 species growing in Europe, eastern North America, Africa, New Zealand and temperate parts of Asia, belongs to the family Apiaceae, subfamily Apioideae, tribe Scandicineae (Pavlović et al., 2011; Hendrawati et al., 2012). Three species of this genus [*A. cerefolium* (L.) Hoffm, *A. sylvestris* (L.) Hoffm., and *A. nemorosa* (M. B.) Speng.] are represented in the Flora of Iran (Mozaffarian, 2007). The chervil plants are generally well known for their strong and distinctive flavors and in some cases providing important nutrients which can fortify the consumer's diet to enhance its nutritional value. Some of the *Anthriscus* species are used as flavoring agent and spice for culinary purposes. The young aerial parts of the chervil are nearly always used fresh as vegetable, but can be preserved by drying, deep freezing or by making a pesto-like preparation (Pavlović et al., 2011; El Gendy et al., 2015). Aerial parts, roots and fruits of several *Anthriscus* Pers. species have been traditionally used in folk medicine of many countries for the treatment of asthma, bronchitis (Kim et al., 2019), hypertension (Fejes et al., 2000), rheumatism, gastrointestinal ailments, inflammation, and stomach-ache (Bagci et al., 2016). The species are also used as hematinic, tonic (Lim et al., 1999), antipyretic, antitussive, diuretic, analgesic, cough remedy (Bagci et al., 2016) detoxifying agent (Baser et al., 1998). In recent years, reports concerning the cytotoxic (Lai et al., 2018), antimicrobial (Pavlović et al., 2011; Lai et al., 2018), antioxidant (Lim et al., 1999; Lai et al., 2018), memory-enhancer, anxiolytic and antidepressant (Bagci et al., 2016), antilipoperoxidant (Fejes et al., 2000), anticancer (Ikeda et al., 1998; Lim et al., 1999), anti- allergic (Kim et al., 2019), apoptotic (Jeong et al., 2007), insecticidal (Kozawa et al., 1982), allelopathic (Lyytinen & Lindström, 2019) properties of various *Anthriscus* species are also available in the literatures.

Recently, an increasing interest in scientific researches concerning discovery of natural products including the plant extracts, essential oils of aromatic plants and their components that can be applied to the food, cosmetic, perfume and pharmaceutical industries has gained an increasing interest (Bakkali et al., 2008; Shahwar et al., 2012). Likewise, scientific research reveals that the antioxidant capacity of biologically active compounds which isolated from plant gives beneficial effect to human health. For that reason, antioxidant activity is widely used as a parameter to describe nutritional health food or plants and their natural active components (Lobo et al., 2010).

Nowadays, the use of natural substances such as medicinal plant extracts are considered as an alternative to the employ of synthetic preservatives to prolong the storage stability of food products owing to their notable antioxidant, since consumers are keeping away from consumption of products with harmful synthetic additives or chemicals because of their potentially health hazard and side effects such as carcinogenicity and toxicity (Riahi et al., 2013). Previous studies have demonstrated that antioxidant activity of plant extracts may be associated with their level of phenolic compounds (Tungmunnithum et al., 2018). Therefore, international attention has been directed toward discovery of phenolic compounds in crude extracts of plant materials as functional and beneficial ingredients that can be applied to the food industry, due to their ability to postpone oxidative degradation of lipids and thereby

improve the quality and nutritional value of food (Trumbeckaite et al., 2011; Riahi et al., 2013).

The *Anthriscus* species have been demonstrated to possess important groups of compounds, such as flavonoid glycoside (Dall'Acqua et al., 2006; Žemlička et al., 2014), flavolignans (Ikeda et al., 1998), lignans, coumarins (Jeong et al., 2007), Furanocoumarins, furanocoumarin ethers (Fujioka et al., 1999) and cyclopropane fatty acids (Kuiper & Stuiver, 1972). Moreover, chemical composition of essential oil obtained from different *Anthriscus* species were presented by various studies (Lemberkovics et al., 1994; Bos et al., 2002; Nickavar et al., 2009; Pavlović et al., 2011; Kiliç, 2017; Lai et al., 2018).

Previous studies suggest that chemical composition of plants can be influenced by a number of intrinsic and external factors such as genetic background, physiological condition of plant, climatic and agronomic conditions, type of plant part, growing stage of the plant, method of extraction and postharvest processing and storage conditions (Ramezani et al., 2009; Norouzi & Norouzi, 2018). In compliance with this variation, the biological activities of plant can be expected to vary, based on chemical composition variability. Thus, the findings of investigation of plant chemical composition at different phenological stages and its coherence with the biological activities can be considered to select the optimal harvesting time of this plant for relevant industries to be used in foods, cosmetics and pharmaceuticals (Djouahri et al., 2017). So the aim of this study was to assess the volatile components, phenolic content and antioxidant activity of two *Anthriscus* species: A. *cerifolium* (called isti-ot) and *A. sylvestris* (called jajigh), the former is one of the most popular vegetation in the region used as vegetable in the spring and fall seasons.

MATERIALS AND METHODS

Plant materials

The fresh aerial parts of two *Anthriscus* Pers. Species (*A. cerefolium* & *A. sylvestris*) were collected during vegetative (March) and full flowering (may) stages in 2018 from Lajayer Rural District, Germi, Ardabil province, Iran, GPS coordinates: 38°58'02.3"N 48°15'07.3"E. The plant materials were air dried at room temperature in shadow (until reached final moisture content of 10% wet basis) and were ground to fine powder, used for further extractions.

Isolation of the essential oils

The essential oils of all air-dried samples (100 g) were isolated by hydrodistillation employing a Clevenger-type apparatus for 3 h. The oil was dried over anhydrous sodium sulfate, and then, was kept in a sealed dark vial at 4°C until further analysis. Experiments were carried out in the University of Mohaghegh Ardabili.

Essential oil analysis procedure

GC analysis of the essential oils was performed using a Shimadzu GC-9A gas chromatograph, equipped with flame ionization detector (FID) and DB-5 fused silica column (30 m×0.25 mm i.d., film thickness 0.25 µm). Oven temperature was programmed from 50 to 240°C at the rate of 3°C.min^{-1}; initial and final temperatures were held for 5 and 10 minutes, respectively. Detector (FID) temperature was 265°C and injector temperature was 250 °C. Helium was used as carrier gas with a linear velocity of 32 cm.s^{-1}. The percentages of compounds were calculated by the area normalization method. GC–MS analysis were carried out in an Varian 3400 GC/MS system equipped with a DB-5 fused silica column (30 m×0.25 mm i.d., film thickness 0.25 µm);oven temperature was 50–240°C at a rate of 4°C.min^{-1}, transfer line

temperature 290°C, carrier gas, helium, with a linear velocity of 31.5 $cm.s^{-1}$, split ratio 1:60, ionization energy 70 eV, scan time 1 s, and mass range 50–550 $m.z^{-1}$. The components of the essential oils were identified by comparison of their mass spectra with those of a computer library or with authentic compounds, and confirmed by comparison of their retention indices calculated relative to homologous series of n-alkanes (C5-C24), either with those of authentic compounds or with data reported in the literature (Adams, 2007).

Methanolic extract

25 g of the powdered samples were extracted in Soxhlet apparatus with methanol (MeOH) at 60°C for 4 h. The solvent evaporated at reduced pressure using a rotary evaporator and extracts stored in the dark at +4 °C until further tests.

Determination of antioxidant activity by DPPH scavenging assay

The scavenging effect of DPPH free radical was measured according to modified method of Kondo et al. (2002). The reaction system consisted of 0.1 ml extract or essential oil at different concentrations (5, 10, 20 and 30 $\mu g.ml^{-1}$) and 2 ml of DPPH methanolic solution (0.21 mM). The reaction mixture was shaken and left for 30 min at room temperature in the dark, and the absorbance was measured at 517 nm in a spectrophotometer. Ascorbic acid was used as was used as reference. Inhibition of free radical DPPH in percent (I%) was calculated in following way: $I\%=[(A_0 - A_1)/A_0]\times100$, where A_0 is the absorbance of the control reaction (containing all reagents except the extract) and A_1 is the absorbance in the presence of the sample. The concentration of sample required to decrease the initial DPPH absorbance by 50% was calculated as IC_{50}.

Determination of total phenolic content

The total phenolic contents were determined by using Folin-Ciocalteu method. A volume of 1 mL of the plant extract (1 $mg.ml^{-1}$) was mixed with 1 ml of 10% Folin-Ciocalteu's reagent (diluted 1:10 with de-ionized water) and were neutralized with 4 mL of sodium carbonate solution (7.5%, $w.v^{-1}$). After 30 min incubation at room temperature with intermittent shaking, the absorbance was measured at 760 nm. Gallic acid was used as a reference standard for plotting calibration curve ($Y= 0.0031x+0.1$, $R^2= 0.976$). Total phenols were expressed in terms of gallic acid equivalents (mg gallic $acid.l^{-1}$).

RESULTS AND DISCUSSION

Essential oil composition

The constituents of the hydrodistillated essential oil isolated from two *Anthriscus* species at different phenological stages were analyzed by GC-MS. The essential oil components along with retention indices and their relative percentages are presented in Table 1, where the compounds are given in order of their elution from the DB-5 column.

A hundred and four components were detected in the essential oils obtained from different samples representing 93.40% to 98.20% of the total oil composition. Both quantitative and qualitative differences were observed among all essential oils.

Oxygenated monoterpenes were the main class of compounds extracted from vegetative and flowering stages of *A. cerefolium* (93.83% and 88.13%, respectively), while aliphatic esters were detected to be the main fraction of essential oils obtained from *A. sylvestris* at vegetative (66.90%) and flowering (42.48%) stages.

Sesquiterpene hydrocarbons were the second fraction of the essential oils extracted from *A. cerefolium* at vegetative and flowering stages. Among groups of the chemical compounds,

the oxygenated monoterpenes with 7.5% of the total oil were the second predominant fraction of essential oil isolated from *A. sylvestris* collected before flowering phase, whereas monoterpene hydrocarbons were second fraction of the essential oil of this species at flowering stage.

Table 1. Chemical composition and characteristics of essential oils isolated *Anthriscus* species at various phenological stages

Compounds	RI	Percentage (%)			
		A. cerefolium		*A. sylvestris*	
		Vegetative stage	Flowering stage	Vegetative stage	Flowering stage
3-methyl-2-butenal (prenal)	746		0.01	0.18	
n-Hexanal	798		0.07		0.08
(Z)-2-hexenal	851		0.03		
(E)-2-Hexenal	854	0.02			
Nonane	860	0.05	0.09		0.17
Heptanal	903		0.01		
α-Pinene	940		0.03	1.33	2.5
2-Heptenal	954		0.04		
Camphene	955			0.14	0.34
Benzaldehyde	962		0.03		
β-Sabinene	978				0.23
β-Pinene	982	0.29	0.43		14.24
2,6-Dimethyl 2,6-octadiene	989			0.39	0.24
1-Decene	990			0.74	0.31
β-Myrcene	991		0.26	2.10	
2-Pentylfurane	993	0.03			
Dehydro-1,8-cineole	994	0.52			
2,3,6-Trimethylpyridine	1003			0.40	
α-Phellandrene	1005				0.27
cis-2-(2-Pentenyl)furan	-		0.03		
Hepta-2(E),4(E)-dienal	-		0.01		
Corylon	1024			0.51	
o-Cymene	1027			0.59	0.26
p-Cymene	1029			0.39	0.20
Limonene	1032	0.12	0.18	0.92	1.93
trans-β-Ocimene	1052		0.05		3.72
3,5-Octadien-2-ol	-		0.02		
Benzeneacetaldehyde	1044		0.01		
4-Methyl-1,5-Heptadiene	1048			0.12	
2-Octenal	-		0.01		
2,2-Dimethyl 3,4-pentadienal	-		0.03		
Hexylvinylcarbinol	1059	0.03	0.04		
3-Nonanone	-	0.12	0.03		
γ-Terpinene	1061				0.16
α-Terpinolene	1063	0.07	0.07		0.09
3-Decyne	1092				8.64
cis-4-Undecene	-	0.09	0.14		
Undecane	-	1.14	1.74	0.31	0.29
Nonanal	1101		0.07		
2,6-Dimethyleneoct-7-en-3-one	1107				0.23
5-Undecene	-		0.02		
2-Nonen-1-ol, (Z)	-		0.03		
cis-Verbenol	-			7.10	
3,4-Heptadiene	-		0.05		
1-(4-Methyl-3-cyclohexen-1-yl)ethanol	1149				1.08
2,4-Dimethylbenzaldehyde	1179				0.50
4-ethylbenzaldehyde	1181			0.20	
3-Nonanol	1091	0.06			
Estragole	1195	59.01	53.96	0.40	1.31
4-Ethyl-3-heptene	-	0.18	0.22		

1-Cyclohexene-1-carboxaldehyde	-		0.02		
β-Safranal	1202			1.90	3.00
p-Cumic aldehyde	1240				0.25
Chrysanthenyl acetate	1262		0.65	64.85	41.5
2-Octyne	-	0.07	0.36		
Anethole	1281		0.05		
Isobornyl acetate	1286			0.59	0.31
(E)-5-Tridecene	-	0.10	0.17		
Tridecane	-	0.08	0.14		
2,4-Nonadienal	-		0.03		
Bicycloelemene	1327		0.06		
Citronellyl acetate	1351				0.24
α-Cubebene	1352		0.07		0.13
β-Bourbonene	1385		0.04		
β-Elemene	1392		0.08		
o-tert-Butylphenol	-			1.66	
Methyleugenol	1401	34.3	34.12		
β-Caryophyllen	1418	0.51			2.31
Geranyl propionate	1445			0.40	0.18
β-Ionone	1470				0.26
Germacrene D	1483	0.33	1.25		0.75
α-Caryophyllene	1455	0.03	0.08		0.31
trans-β-Farnesene	1459		0.13	0.70	5.15
α-Amorphene	1482		0.04		
β-Ionone	-		0.07		
Geranyl ester	1475			0.40	0.18
Phenylethyl 2-methylbutyrate	1477		0.04		
alpha-Bergamotene	1434		0.34		
Bicyclogermacrene	1494		0.31		
α-selinene	1495			0.27	0.49
Zingiberene	1497	0.41			
α-Farnesene	1507				0.51
β-Bisabolene	1509	0.16	0.18	0.04	
delta-Cadinene	1512		0.16		
Tridecanal	-			0.66	
Myristicine	1520	0.03	0.03		0.18
β-sesquiphellandrene	1523	0.06			
Elemol	1551		0.02		
Elemicine	1554				0.14
α-Dendrolasin	1571			0.17	0.12
spathulenol	1578		0.21		
Caryophyllene oxide	1583		0.13	5.45	0.95
Neryl acetate	1381	0.05	0.05		0.07
Butanoic acid	1582	0.17	0.12		
calamenene	1537		0.04		
τ-Cadinol	1638		0.03		
Longiborneol	1592		0.02		
Iso-Caryophyllene	-		0.02		
Humulene oxide II	1609			0.39	
Myristic aldehyde	1611				0.46
Perhydrofarnesyl acetone	1830				0.51
Neophytadiene	1838	0.17	0.30	0.10	0.44
trans- α-Bergamotol	1698				0.30
n-Hexadecanoic acid	1972			1.10	0.05
Total (%)		98.20	97.07	93.40	95.53
Aldehyde		0.19	0.56	2.59	5.23
Monoterpene hydrocarbons		0.48	1.02	5.86	24.41
Oxygenated monoterpenes		93.83	88.13	7.50	1.31
Sesquiterpene hydrocarbons		1.50	2.80	1.01	9.65
Oxygenated Sesquiterpene		0.17	0.53	6.01	1.37
Aliphatic esters		0.05	0.70	66.90	42.48
Aromatic esters			0.04	0.20	
Alcohol		0.09	0.09		1.08
Other		1.89	3.20	3.33	10.00

Relative amounts of different classes of compounds of essential oils of different *Anthriscus* species were reported by pervious researchers. Monoterpene hydrocarbons were the main group of constituents of essential oil produced by hydrodistillation of *A. cerefolium* fresh herb, while a phenol was the main component of oil isolated by supercritical fluid extraction from its fresh herb (Simandi et al., 1996). According the study of Hendawy et al. (2019) regarding the essential oil of *A. cerefolium*, cultivated in Egypt under different locations, oxygenated compounds were shown to be principal compound group of oils. In other study, oxygenated monoterpenes were the most noticeable fraction of all essential oil of *A. cerefolium* L. cultivated in Egypt and underwent at different treatments of NK fertilizers (El Gendy et al., 2015).

Monoterpene hydrocarbons fraction was identified as the main group of components in essential oil of fresh leaves, DCM extract of fresh leaves and DCM extract of air dried leaves of *A. sylvestris*, while sesquiterpene hydrocarbon fraction dominated DCM extract of freeze dried leaves (Kiliç, 2017). Sesquiterpene hydrocarbons constituted the principal fraction of essential oils obtained from the roots (Pavlović et al., 2011) and aerial parts (Bagci et al., 2016) of *A. nemorosa*.

In the present study, some compounds are only observed at one of the phenological stages. It is believed that synthesis of specific components in plants has relevance to the phenological stage in which the plants are (Norouzi & Norouzi, 2018). There were 21 similar components in essential oils of *A. cerefolium* at different phenological stages, while 19 components were in common to essential oil of *A. sylvestris* at vegetative and flowering stages. Furthermore, the essential oils of flowering stage in both species were more complex and therefor had more compounds in comparison to the essential oils of vegetative phase. This could be explained by the ability of older plant to produce alternative defensive mechanisms, since the constituents of essential oil play an important role in plants defense reactions (Ochoa-López et al., 2015). It can also be related to low rate of biosynthesis of essential oil components during the vegetative stage that may be due to partial inactivation of enzymes necessary to the biosynthesis of certain compounds (Ochoa-López et al., 2015).

In present study, twenty-eight compounds were identified in essential oil of *A. cerefolium* at vegetative stage, accounting for 98.20% of total oil. The main components of this essential oil were estragole (59.01%) and methyleugenol (34.3%). In a similar way, among 62 compounds existed in the essential oil of *A. cerefolium* during reproductive phase, estragole (53.96 %) and methyleugenol (34.12%) were as the main ones. The effect of different phenological stages on the composition of essential oil may be due to its effect on enzyme activity and metabolism of essential oil production (Sellami et al., 2009).

According the study of Baser et al. (1998) the essential oil of *A. cerefolium*, grown wild in Turkey was characterized by methylchavicol,1-allyl-2,4-dimethoxybenzene, undecane and β-pinene as the main constituents. It has been documented that essential oil isolated from *A. cerefolium* by hydrodistilation was found to contain methylchavicol (80%) andl-allyl-2,4-dimethoxybemene (16%), while the oil isolated by supercritical fluid extraction contained a much lower level of methyl chavicol (21.1%) and a higher amount of l-allyl-2,4-dimethoxybenzene (57.4%) (Simandi et al., 1996). The main components of essential oil obtained from aerial parts of this species in Turkey were caryophyllene, γ-cadinene, trans-pinocarveol, spathulenol and caryophyllene oxide (Kiliç, 2017).

In another study, volatile constituents of chervil plant (*A. cerefolium*), cultivated in Egypt under different nutritional conditions were investigated. The oil compositions of all samples were distinguished by methyleugenol as the main component followed by estragole, 2-allyl-1,4-dimethoxybenzene, (-)-zingiberene and 1-nonene (El Gendy et al., 2015). Similar findings

were also observed by Hendawy et al. (2019) in plants cultivated in 4 different locations in Egypt.

The composition of essential oil of *A. sylvestris* was compeletly different from that of the *A. cerefolium*. Low percentages were observed for Estragole in the essential oil of *A. sylvestris* at vegetative (0.4 %) and flowering (1.31%) phases. Methyleugenol as the second main component of *A. cerefolium* samples was not present in *A. sylvestris* volatile oil. In the other hand, Chrysanthenyl acetate which was the major constituents of the essential oil of the *A. sylvestris* during ontogenesis, couldn't be detected in vegetative stage and was detected in low amount in the essential oil of flowering phase (0.65%) of *A. cerefolium*.

Essential oil obtained from vegetative stage of *A. sylvestris* contained 31 compounds representing 93.40% of the total oil composition and was dominated by Chrysanthenyl acetate (64.85%) and cis-Verbenol (7.1%). GC-MS analysis of *A. sylvestris* essential oil at flowering phase showed that among 46 components which represented 95.53% of the total oil, Chrysanthenyl acetate (64.85%) and β-Pinene (14.24%) were synthesized and accumulated as the major components.

The essential oil of *A. sylvestris* flower have been reported previously to contain Phenol, o-cresol, eugenol, β-myrcene, d-limonene, γ-terpinene, p-cymene, benzyl alcohol, phenethyl alcohol, l-linalool, β-farnesene and d-sabinyl acetate, while phenol, cresol (o-, m-, p-), guaiacol, eugenol, p-cymene, α-pinene, β-myrcene, d-limonene, γ-terpinene, terpinolene, β-farnesene, cis-3-hexen-1-ol, benzyl alcohol, phenethyl alcohol, sabinyl acetate, l-α-fenchyl acetate and chrysanthenyl acetate were identified from the leaves essential oil (Kurihara & Kikuchi, 1979). (-)-sabinen was shown to have highest percentage among volatile compounds from flowers, buds and leaves of *A. sylvestris* (Borg-Karlson et al., 1993). In another study Myrcene, α-pinene and β-pinene were the main components of the essential oil of this species (Valterová et al., 1997). Moreover, the chemical composition of essential oil and dichloromethane extracts of leaves and roots from *A. sylvestris* were analysed by GC and GC–MS. The results showed that β-Phellandrene, β-Myrcene in fresh leaves essential oil, β-Myrcene and trans-Sabinyl acetate in extract of fresh leaves, β-Phellandrene and Z-β-Ocimene in both essential oil and extract of fresh root were reported as major constituents (Bos et al., 2002).

Free radical scavenging activities

The radical scavenging effect of the essential oils and methanolic extracts from two *Anthriscus* species at vegetative stage was investigated using DPPH as reagent (Fig. 1). The concentration of sample required to inhibit 50% of radicals (IC_{50}) is a parameter widely used to measure the antioxidant activity. The lower IC_{50} value demonstrates stronger antioxidant activity (Roby et al., 2013). In current study, the highest antioxidant activity was obtained for essential oil of *A. sylvestris* (IC_{50}=71.3 $\mu g.ml^{-1}$) followed by essential oil of *A. cerefolium* (IC_{50}=115 $\mu g.ml^{-1}$). IC_{50} values for extract of *A. cerefolium* and *A. sylvestris* were 982 and 1733 $\mu g.ml^{-1}$, respectively.

It has been recommended that antioxidant activity of phytochemicals is related directly to the presence of active major components. Whereas, other compounds with lower amounts could play an important role in antioxidant capacity due to synergistic or antagonistic effects between the volatile components (Mastelic et al., 2008; Norouzi & Norouzi, 2018).

Fejes et al. (2000) assessed the In vitro antioxidant activity of aqueous extracts from different vegetative parts (root, herb) of *A. cerefolium* by various test methods. Based on their results, both root and herb extracts possessed DPPH radical scavenging capacity. However, the exact compounds which demonstrate radical scavenging activity are still unclear.

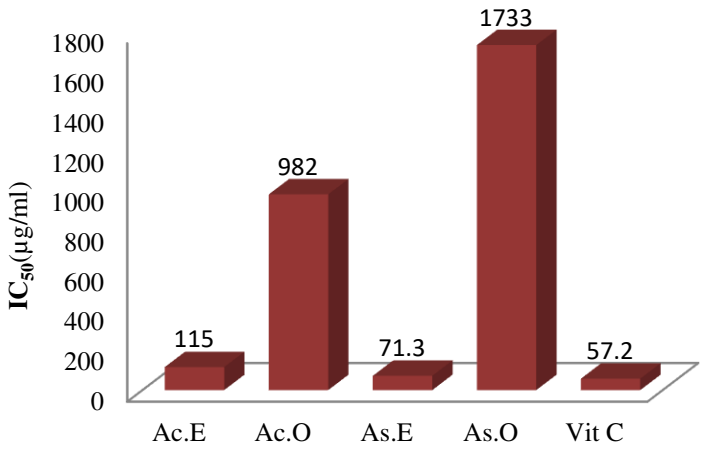

Fig. 1. IC50 (µg.ml^{-1}) of the essential oils and methanolic extracts from two *Anthriscus* species at vegetative stage. Note: Ac.E, extract of *A. cerefolium*; Ac.O, essential oil of *A. cerefolium;* As.E, extract of *A. sylvestris*; As.O, essential oil of *A. sylvestris*

Findings by Milovanovic et al. (1996) highlighted that antioxidant activity of ethanolic extract of *A. sylvestris* was superior to apigenin, quercetin, or a tocopherol mixture. The antioxidant activity of the crude methanol extract of *A. sylvestris* was found to be related to luteolin-7-O-glucoside and chlorogenic acid (Dall'Acqua et al., 2006).

Total phenolic content

The total phenolic contents of *A. cerefolium* and *A. sylvestris* at full flowering stage were determined spectrophotometrically according to the Folin-Ciocalteu procedure which were 76.7 and 74.6 mg Galic acid equivalent per 1 liter of extract, respectively. Verma et al. (2007) and Ayan et al. (2007) suggested previously that flowering stage conduct the way in which phenolic compounds reach the highest levels. Accumulation of phenolics during the late vegetative phase can be attributed to the fact that during this stage, the plant protection is mainly secured by phenolic compound which are highly synthesized during this stage (Sellami et al., 2009).

CONCLUSION

Characterization of volatile compounds of two *Anthriscus* species growing wild in Iran revealed that changing in phenological stages can influence the pathway of the essential oils biosynthesis. Besides, climatic factors such as temperature, sunlight, relative humidity, rainfall and water etc. differ throughout various growth phases, and they can also cause some variation in essential oil composition. Moreover, essential oil of *A. sylvestris* at vegetative stage demonstrated the noticeable antioxidant ability (analogous to reference standard) which makes it well qualified to be used as functional ingredients and natural alternatives to synthetic antioxidants in food industry, since increasing attention has been directed toward finding naturally occurring antioxidant.

Conflict of interest

The authors declare no conflict of interest to report.

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Callosobruchus maculatus (Fab.) (Coleoptera: Chrysomelidae) infestation and tolerance on stored cowpea seeds protected with *Anchomanes difformis* (Blume) Engl. extracts

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ABSTRACT

Purpose: The study was conducted to evaluate the infestation and tolerance of *Callosobruchus maculatus* to stored cowpea treated with *Anchomanes difformis* extracts. **Research Method**: Different concentrations of *A. difformis* extracts were admixed with 20g of uninfested cowpea seeds in 250ml plastic dishes. Ten unsexed adult *C. maculatus* were released into each treatment and toxicity was assessed at 24 hrs interval days after infestation, number of eggs laid on cowpea seeds was counted. At the end of the experiment, seeds were reweighed, number of emerged adult and damaged seeds were recorded. **Findings**: All extracts were significantly toxic to *C. maculatus* as concentration increased. Ethyl acetate (EA) extract exerted highest mortality ranged 90-100%, while methanol recorded highest longevity. Maximum reduction in egg laid was observed with EA extract (51.44-74.45%), followed by methanol extract at 100 (58.69%) and 150 µl (69.56%). While maximum adult emergence inhibition was observed at 150 µl (93.33%) with EA. Cowpea seeds in control dishes suffered the heaviest infestation (31.76%) compared to EA and acetone extracts that recorded zero infestation at 100 and 150 µl concentration respectively. Susceptibility of cowpea seeds treated with extracts was significantly lower compared to control with 100% infestation. **Limitations**: No hindrances was encountered during the study. **Originality/Value:** The study revealed that *A. difformis* possesses oviposition deterrent and adult emergence inhibition properties against *C. maculatus* that can be utilize for the management of *C. maculatus* in stored cowpea seeds. Further studies are recommended for exploring the active compound responsible for its insecticidal activities and toxicological effect using albino rats as a model.

INTRODUCTION

Cowpea production and storage is severely hampered by *Callosobruchus maculatus* (Fab.) (Coleoptera: Chrysomelidae) infestation leading to enormous nutritional and economic loss. Infestation of the bruchids starts in the field but it is difficult to detect it at the time of harvest, but its infestation is generally manifested in storage where substantial loss occurs when seeds are stored for longer period.

Cowpea bruchid, *C. maculatus* as an economically important insect pest of stored cowpea causes 20-50% losses in storage (Gosh & Durbey, 2003). Astronomical losses attributable to the insect during post-harvest storage is the possible reasons for importing cowpea from neighbouring West African countries to compliment local production in spite of Nigeria been the largest producer of cowpea.

Successful management of stored grain insects is the final component of the struggle to limit postharvest losses of agricultural produce due to insect infestation and diverse measures have been employed to control these species. The use of synthetic insecticides and fumigants remains the main and most effective means of protecting and controlling stored food grains against insect infestation and grains/seeds damage (Adesina et al., 2012). Their continuous and indiscriminate use has over the years been associated with numerous problems that include environmental pollution (Assad et al., 2006), development of resistant strain (Nisha et al., 2018), toxicity to non-target organisms (Dennis, 1981) and insecticide residues in food (Shazali et al., 2003). The various problems caused by the persistent use of pesticides have gradually led to an increasing interest in the development of alternative pest control methods, such as the use of biopesticides.

Plants with insecticidal properties could be regarded as potential alternatives to chemical pesticides. Indeed, various plants or plant extracts are used to control agriculturally important insect pests. A wide range of medicinal plants are toxic, repellent, ovicidal or antioviposition, while some have antifeedant properties, insect growth and development regulators several of which were regarded as insecticides (Khoshnoud et al., 2008; Boulogne et al., 2012; Addisu et al., 2014; Adesina et al., 2015). The insecticidal proprieties of plants lie mainly on their secondary metabolites or bioactive compounds (Pettersen, 1984).

Anchomanes difformis (Blume) Engl. (Family Araceae) commonly known as forest Anchomanes, children's umbrella and God's umbrella is native to tropical Africa (Burkill, 1985). In Nigeria, the plant is known as Chakara by the Hausa, Olumahi by the Igbo and Abirisoko or ogirisako by the Yoruba (Egwurugwu et al., 2016). *Anchomanes difformis* is a perennial, herbaceous and deciduous plant with stout prickly stem growing about 2metres high and whitish horizontal tuber/rhizome that measures 50-80 cm long, and 10-20 cm in diameter.

Anchomanes difformis is used in African traditional medicine in the treatment of various ailments among which are dysentery, diabetes, gonorrhoea, oedema, jaundice, scabies, hypertension, respiratory diseases and as poison antidote, diuretic, laxative and ease child birth (Burkill, 1985; Oyetayo, 2007; NsondeNtandou et al., 2017; Ahmed, 2018). Studies also indicates that *A. difformis* leaves, tuber and roots extracts possessed analgesic, antibacterial, antimalarial, antioxidant, anti-inflammatory, and antipyretic properties (Oyetayo, 2007; Adeleke & Adetunji, 2010; Eke et al., 2013; Abubakar et al., 2013; Abiodun et al., 2014; NsondeNtandou et al., 2017; Ahmed, 2018).

There appears to be a scarcity of empirical information on the utilization of *A. difformis* extracts for their insecticidal activities. However, Akinkurolere (2007) and Adebo et al. (2018) reported the efficacy of *A. difformis* powder for the management of stored product insects. The present work is carried out to determine the toxicity of *A. difformis* extracts against pulse beetle, *C. maculatus*.

MATERIALS AND METHODS

Experimental location

The study was conducted under ambient laboratory conditions (30±2 °C temperature, 65±5% relative humidity and 12L:12D photo regime) in the Department of Crop, Soil and Pest Management Technology, Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria (Latitude 5° 12' N and Longitude 5° 36' E).

Cowpea seeds

Drum variety of cowpea used for the study was procured from Oja Ulede, Owo, Ondo State, Nigeria. On receipt, the seeds without no visible signs of beetle eggs and presence of adults or exit holes were handpicked and sterilized in an oven at 100 °C for an hour (Adesina & Idoko, 2013). This was done to terminate any developmental stages of *C. maculatus* that might be in the seeds (Idoko & Adesina, 2013). Thereafter, the seeds were allowed to cool to avoid mouldiness (Olotuah et al., 2007).

Insect culture

The initial culture of *C. maculatus* used in this study were obtained from infested stock purchased from Oja Ulede market, Owo, Ondo State, Nigeria. From this initial culture, new cultures of the insect were reared on Sokoto white variety (a local susceptible variety) of cowpea in one liter plastic jars covered with muslin cloth to allow for aeration and oviposition in the laboratory under ambient conditions (25-30 °C, 70-75% relative humidity). First generation of *C. maculatus* adults that emerged from these were used in the experiment.

Plant collection and extraction

Tubers of *A. difformis* were harvested from abandoned farmland within Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria. The tubers were cut into small pieces, air dried at room temperature. Thereafter, the dried piece of tubers was pounded in a mortal with pestle and passed through a uniform size sieve. The powder was stored in airtight container till need for extraction. About 250 g of *A. difformis* powder was subjected to cold extraction by soaking in 400 ml of hexane, ethyl acetate, acetone and methanol respectively for 48 hrs. Then the extracts were decanted and concentrated in a rotary evaporator to make it solvent free. The residues were stored in vial bottles and tested for insecticidal activities by contact and fumigant toxicity.

Effect of *A. difformis* extracts on *C. maculatus* adult mortality and longevity

Different concentrations (50, 100 and 150 µl) of the extracts were mixed with 20 g of uninfested and wholesome susceptible drum cowpea seeds in 250 ml plastic dishes with the aids of micro pipette. The treated cowpea seeds were stirred using a glass rod to ensure proper coating of the seeds with the extract. The seeds were then air-dried for some minutes to evaporate the solvent (Talukder & Howse, 1994). There was also control experiment with no addition of plant extract and each treatment was replicated three times. Ten unsexed newly emerged 2-3 days old *C. maculatus* from the culture were released into each treatment and toxicity of the extracts was assessed at 24 hrs interval for 4 days, adult insects were considered dead after failure to respond to probing with sharp safety pin. Percentage adult survival was calculated using Abbott (1925) formula.

Effect of *A. difformis* extracts on *C. maculatus* oviposition and fecundity

After 7 days of infestation, the number of eggs laid by female beetles on the cowpea seeds in each treatment were counted and recorded separately. All the eggs laid in different Petri

dishes were examined and the viable eggs were identified. Viable eggs were recognized by their morphological feature (Lima et al., 2004), since they become opaque as a function of their residue discharged by the larvae during penetration. This was used to calculate the percentage reduction of egg laid (1) and hatched egg (2) as follows:

$$\% \text{ Reduction of egg} = \frac{\text{No of laid in control} - \text{no of egg laid in treated dish}}{\text{no of eggs laid in control dish}} \times \frac{100}{1} \quad (1)$$

$$\% \text{ Egg hatchability} = \frac{\text{mean no of hatched eggs}}{\text{mean no of eggs laid}} \times \frac{100}{1} \quad (2)$$

The experiment was kept undisturbed on the laboratory workbench for 30 days to allow for the emergence of the first filial (F_1) offspring. The number of emerged adult from each treatment was used to calculate the percentage reduction of adult emergence (3):

$$\% \text{ Reduction in adult emergence} = \frac{\text{no of emerge adult from control dish} - \text{no of emerge adult from treated dish}}{\text{no of emerge adult from control dish}} \times \frac{100}{1} \quad (3)$$

Effect of *A. difformis* extracts on *C. maculatus* infestation and tolerance on stored cowpea

At the end of the experiment, the seeds were sieved and reweighed to get the final weight which was used to determined percentage weight loss. Thereafter, numbers of seeds with adult exit hole(s) were sorted, counted and recorded. This was used to calculated percentages infestation (4) and tolerance (5).

$$\text{Percentage infestation} = \frac{\text{number of seeds with emergent/adult exit or eggs (or both)}}{\text{Total number of seed observed}} \times 100 \quad (4)$$

Where: Nh = number of seeds with emergent/adult exit or eggs (or both) and No = Total number of seed observed.

$$\% \text{ Pest tolerance} = \frac{\text{number of undamaged seeds} - \text{number of damaged seeds}}{\text{Total number of undamaged}} \times 100 \quad (5)$$

Statistical analysis

Each treatment was replicated three times and arranged in a Completely Randomized Design (CRD). Data obtained were subjected to analysis of variance (ANOVA); where significant differences were obtained ($P \leq 0.05$), means were separated with Duncan New Multiple Range Test (DNMRT). Data in percentages were arcsine transformed prior to analysis (Sokal & Rohlf, 1981).

RESULTS

Effect of *A. difformis* extracts on *C. maculatus* adult mortality and longevity

All extracts were toxic to *C. maculatus* and there was significant difference in the mortality recorded as concentration increased over exposure period and in relation to the solvents used for the extraction (Table 1). At 24-96 hrs exposure periods, ethyl acetate (EA) extract at all concentrations exerted the highest mortality ranged 90-100% mortality, closely followed by hexane (45-96.67% and acetone extracts (39.25-96.67%). Among the extracts, *A. difformis* extracted with methanol recorded the least *C. maculatus* mortality and highest longevity (18.43-86.67%).

Table 1. Effect of *A. differmis* extracts on *C. maculatus* adult mortality and longevity

Exposure Time (hrs)	Solvent	Concentration (µl)		
		50	100	150
24	Hexane	45.00 ±2.89^{c}†	45.67 ±0.67^{d}	71.56 ±0.00^{d}
	Methanol	18.43 ±0.01^{b}	33.17 ±0.34^{b}	56.83 ±0.04^{c}
	Acetone	39.25 ±0.02^{c}	39.32 ±0.09^{c}	50.75 ±0.02^{b}
	Ethyl Acetate	90.00 ±5.77^{d}	90.00 ±0.00^{e}	96.67 ±3.33^{e}
	Control	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
48	Hexane	63.43 ±0.01^{d}	71.67 ±0.11^{d}	74.89 ±3.33^{b}
	Methanol	19.77 ±0.67^{b}	39.22 ±0.01^{b}	71.23 ±0.33^{b}
	Acetone	50.70 ±0.07^{c}	56.53 ±0.26^{c}	71.57 ±0.01^{b}
	Ethyl Acetate	93.33 ±3.33^{e}	96.67 ±3.33^{e}	96.67 ±3.33^{c}
	Control	3.33 ±3.33^{a}	3.33 ±3.33^{a}	3.33 ±3.33^{a}
72	Hexane	71.04 ±0.52^{d}	71.57 ±0.01^{c}	93.33 ±3.33^{c}
	Methanol	30.15 ±0.15^{b}	63.43 ±0.01^{b}	77.37 ±2.91^{b}
	Acetone	62.44 ±1.00^{c}	93.33 ±3.33^{d}	96.67 ±3.33^{c}
	Ethyl Acetate	93.33 ±3.33e	96.67 ±3.33^{d}	100.00 ±0.00^{c}
	Control	13.33 ±3.33^{a}	13.33 ±3.33^{a}	13.33 ±3.33^{a}
96	Hexane	90.00 ±0.00^{d}	93.33 ±3.33^{bc}	96.67 ±3.33^{bc}
	Methanol	43.33 ±3.33^{b}	83.33 ±3.33^{b}	86.67 ±3.33^{b}
	Acetone	71.54 ±0.02^{c}	96.67 ±3.33^{c}	96.67 ±3.33^{bc}
	Ethyl Acetate	93.33 ±3.33^{d}	100.00 ±0.00^{c}	100.00 ±0.00^{c}
	Control	20.00 ±5.77^{a}	20.00 ±5.77^{a}	20.00 ±5.77^{a}

†Mean with the same alphabet down the column are not significantly different using Duncan New Multiple Range Test (DNMRT) at p >0.05.

Effect of *A. differmis* extracts on *C. maculatus* oviposition and fecundity

Anchomanes differmis extracts significantly reduced the number of egg laid by *C. maculatus* on the treated cowpea seeds. The numbers of egg laid were significantly reduced with increased in the extracts concentrations (Table 2). Maximum reduction in egg laid was observed in cowpea seeds treated with ethyl acetate extract (51.44-74.45%), followed by methanol extract (ME) at 100 (58.69%) and 150 µl (69.56%). Percentage reduction of egg laid between hexane and methanol were not significantly different at 50 and 150 µl, while cowpea seeds in control dishes recorded zero percent egg laid reduction (Table 2).

Percentage egg hatchability

Percentage egg hatchability significantly decreased with increased in the extracts concentration. Also the various extracts significantly suppressed egg hatching (Table 2). Ethyl acetate extract significantly recorded the lowest percentage egg hatched at 50 and 100 µl (26.58 and 16.67%) respectively. While at 150 µl no egg was hatched from both ethyl acetate and acetone extracts (Table 2). Meanwhile, 62.32% of the eggs laid in control were hatched.

Percentage adult emergence reduction

Cowpea seeds treated with the various extracts significantly inhibited adult emergence, reduction in adult emergence increases as the concentration of the extracts increased (Table 2). Maximum adult emergence inhibition was observed at 150 µl (93.33%) with ethyl acetate and minimum inhibition at the same concentration was observed in hexane extract (63.43%). However, non-significant difference was observed in the reduction of adult emergence at 50 µl between methanol, acetone and ethyl acetate extracts, at 100 µl between acetone and ethyl

acetate extracts and at 150 µl between hexane and methanol extracts respectively. Adult emergence in control dish does not experience any inhibition as they recorded zero percentage adult emergence inhibition (Table 2).

Effect of *A. differmis* extracts on *C. maculatus* infestation and tolerance on stored cowpea

Percentage infestation

Result in Table 3 shows that percentage *C. maculatus* infestation on treated cowpea seeds was extracts and concentration dependent. The various extracts significantly suppressed *C. maculatus* infestation as their concentration increases. Cowpea seeds in control dishes suffered the heaviest infestation (31.76%) compared to those treated with ethyl acetate extract that suffered 5.63% infestation at 50 µl concentration and recorded zero infestation at 100 and 150 µl concentration respectively. While acetone extract completely suppressed infestation at 150 µl concentration.

Percentage pest tolerance

Susceptibility of the cowpea seeds treated with *A. differmis* extracts was significantly lower compared to those in control that were more susceptible (100%) to *C. maculatus* infestation followed by hexane extract. Ethyl acetate treated seeds had the lowest significant percentage tolerance to *C. maculatus* infestation followed by acetone extract. While hexane and methanol extracts exhibited no significant percentage tolerance at 50 and 150 µl, respectively (Table 3).

Percentage weight loss

The percentage weight loss caused by *C. maculatus* during storage was significantly reduced (p<0.05) in seeds treated with extracts from *A. differmis*. The results indicated that control had the highest significant weight loss (12.61%) and ethyl acetate had the least weight loss (2.33, 1.67 and 0.67%) while hexane, methanol and acetone extracts were not significantly different (p>0.05) and had similar effect on weight loss.

Table 2. Effect of *A. differmis* extracts on *C. maculatus* oviposition and fecundity

	Solvent	Concentration (µl)		
		50	100	150
% Reduction of egg laid	Hexane	30.05 ±0.02^{b}†	32.71 ±0.01^{b}	67.53 ±0.01^{b}
	Methanol	28.27 ±1.33^{b}	58.69 ±0.00^{d}	69.56 ±2.96^{b}
	Acetone	44.08 ±3.35^{c}	55.73 ±0.00^{c}	67.77 ±0.09^{b}
	Ethyl Acetate	51.44 ±0.67^{d}	58.93 ±0.33^{d}	74.45 ±0.67^{c}
	Control	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
% Egg hatchability	Hexane	43.36 ±0.02b^{c}	42.81 ±0.01^{c}	42.23 ±0.33^{c}
	Methanol	40.27 ±0.13^{b}	38.40 ±0.05^{c}	15.00 ±2.08^{b}
	Acetone	47.55 ±0.08^{c}	32.00 ±1.15^{b}	0.00 ±0.00^{a}
	Ethyl Acetate	26.58 ±4.30^{a}	16.67 ±3.33^{a}	0.00 ±0.00^{a}
	Control	62.32 ±1.67^{d}	62.32 ±1.67^{d}	62.32 ±1.67^{d}
% Reduction adult emergence	Hexane	33.17 ±0.04^{b}	44.00 ±1.00^{b}	63.43 ±0.01^{b}
	Methanol	44.67 ±0.33^{c}	58.67 ±1.33^{c}	64.60 ±0.30^{b}
	Acetone	47.49 ±3.33^{c}	62.00 ±1.00^{d}	80.00 ±5.77^{c}
	Ethyl Acetate	42.86 ±3.59^{c}	61.89 ±0.00^{d}	93.33 ±3.33^{d}
	Control	0.00 ±0.00^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}

†Mean with the same alphabet down the column are not significantly different using Duncan New Multiple Range Test (DNMRT) at p >0.05.

Table 3. Effect of *A. differmis* extracts on *C. maculatus* infestation and tolerance on stored cowpea

	Solvent	Concentration (µl)		
		50	100	150
Percentage infestation	Hexane	15.57 ±0.01^{b}†	10.31 ±0.01^{c}	8.32 ±0.01^{c}
	Methanol	21.19 ±0.06^{c}	6.01 ±0.01^{b}	5.63 ±0.11^{b}
	Acetone	15.23 ±0.09^{b}	10.45 ±0.02^{c}	0.00 ±0.00^{a}
	Ethyl Acetate	5.63 ±0.11^{a}	0.00 ±0.00^{a}	0.00 ±0.00^{a}
	Control	31.67 ±1.67^{d}	31.67 ±1.67^{d}	31.67 ±1.67^{d}
% Pest tolerance	Hexane	81.81 ±0.06^{c}	79.45 ±0.08^{d}	73.75 ±0.03^{c}
	Methanol	83.51 ±0.20^{c}	69.55 ±0.08^{c}	67.17 ±0.04^{c}
	Acetone	73.33 ±0.67^{b}	62.44 ±1.00^{b}	28.00 ±4.16^{b}
	Ethyl Acetate	48.33 ±4.41^{a}	36.67 ±3.33^{a}	13.33 ±3.33^{a}
	Control	100.00 ±0.00^{d}	100.00 ±0.00^{e}	100.00 ±0.00^{d}
% Weight loss	Hexane	7.04 ±0.00^{b}	6.29 ±0.00^{c}	2.33 ±1.20^{b}
	Methanol	5.74 ±0.00^{b}	4.33 ±0.33b^{c}	2.67 ±0.33^{b}
	Acetone	5.41 ±0.33^{b}	3.67 ±0.33^{ab}	1.33 ±0.67^{b}
	Ethyl Acetate	2.33 ±0.33^{a}	1.67 ±0.33^{a}	0.67 ±0.67^{b}
	Control	12.61 ±1.33^{c}	12.61 ±1.33^{d}	12.61 ±1.33^{a}

†Mean with the same alphabet down the column are not significantly different using Duncan New Multiple Range Test (DNMRT) at p >0.05.

DISCUSSION

The use of plant parts and products to control agriculturally important insect pest have been an age long practice among resource poor African farmers, this indicates that naturally occurring bioactive compounds extracted from locally available medicinal and aromatic plants have potentials for managing stored products insect infestation (Golob & Webley, 1980; Ileke & Ariyo, 2015).

Treatment of stored cowpea seeds with crude *A. differmis* extracts at various concentrations significantly reduced the adult insect longevity, egg production and hatchability, which in turn influenced the number of adults emerged and resulted in seed damage and loss in weight in relation to the increased in the extract concentrations. This indicated that *A. differmis* extracts were lethal to adult *C. maculatus* and could serve as a bioinsecticide which could be due to the presence of some bioactive compounds. The consequential high adult mortality observed could be due to high toxic effect of the plant extracts. Aniszewski (2007) postulated that toxic secondary metabolites found in botanicals can block ion channels, inhibit enzymes, or interfere with neurotransmission, loss of coordination, and death. The higher insect mortality caused by ethyl acetate extract was the maximum as compared to the mortality caused by acetone and hexane extracts while the least insect mortality in extract treated cowpea was observed in methanol extract. This shows that the effectiveness of the plant materials hinges on the plant active constituents; these might possibly get into the body system of the insect and interfere with the normal development causing mortality of the insect. The study equalled to the finding of Shabnam (2009); Achio et al. (2012) and (Ojianwuna et al., 2016) who reported that high mortality (40-100%) of *Rhyzopertha Dominica* (F) and *Sitophilus granuriuss*, *Macrotermes bellicosus* was evoked when exposed to different doses/concentrations of *Capsicum annuum*, *Zingiber officinale*, *Dennettia tripetala* and *Allium sativum* products respectively.

The extracts mode of action based on the bioassay might be: through contact of the various extract with the body wall of the insects causing irritation of the skin (Williams et al., 2004); inhalation resulting in inflammation of pulmonary tissue and damage to respiratory cells (Reilly et al., 2007) and metabolic disruption, membrane damage and nervous system dysfunction (Ojianwuna et al., 2016). The finding is in consonance with the finding of Shabnam (2009) who reported that *A. sativum* and *Curcuma longa* significantly reduced *Tribolium castaneum* larval and adult emergence as well as weight loss in infested stored grains.

Oviposition deterrent activity showed that female beetles preferred to lay eggs in the control dish or in the dish that contain extracts of lower concentration. The extracts of highest concentration were least preferred by the female beetles for oviposition. It is noteworthy that all the extracts showed more than 50% of deterrent activity even at 100µl with the exception of hexane extract (32.71%). This aligned with study of Elango et al. (2009), who reported the oviposition activity indices of acetone, ethyl acetate, and methanol extracts of *Aegle marmelos*, *Andrographis lineata* and *Cocculus hirsutus* against *Anopheles subpictus*. The results from this study also agrees with that of Rao and Sharma (2007) and Adesina and Ofuya (2015) who observed significant ovicidal effect of ethyl acetate and hexane extracts of custard apple seed on rice moth and *Secamone afzelii* methanol and hexane extracts on *C. maculatus* in reducing the number of eggs laid per female beetles.

The potential of the extract to reduce the egg laying ability by the female beetles may be attributed to the presence of toxic bioactive chemicals present in the plant (Adesina & Ofuya, 2015) which prompted alterations in the physiology and behavior of the insect species reflected by their egg-laying capability (Prathibha et al., 2014). Mehra and Hiradhar (2002) and Rajkumar and Jebasan (2009) opined that plant extracts that demonstrated significant oviposition deterrent activity were insect repellent. The reduction in number of eggs laid at higher doses of *A. differmis* extracts can be attributed to the interruption of vitellogenesis and damage to the egg chambers during various life stages of *C. maculatus* (Pandey & Khan, 1998). Dhar et al. (1996) reported that oviposition was possibly regulated by the volatile compounds absorbed through cuticle

The percentage of adult emergence reduction increases with increased concentration of extract. In the present trial, it was observed that the number of emerged adult insects was directly proportional to the number of hatched eggs. Insecticidal activity of the plant extract might be due to the presence of various bioactive compounds which may jointly or independently contribute to inhibition of adult emergence (Arivoli & Tennyson, 2011). Plant extracts have the prospective to impede the growth of various developmental stages during insect life history such as interruption of larval development, extend pupal duration, inhibit moulting, cause morphological defects and mortality during moulting and melanization processes of insect (Shaalan et al., 2005; Arivoli & Tennyson, 2011)

The significant reduction in adult emergence from treated cowpea seeds could be ascribed to the ovicidal properties of the plant, which leads to egg mortality, reduction in number of hatched eggs or larval mortality which caused the larvae from maturing to adult. This shows that *A. differmis* extracts undoubtedly have oviposition deterrent, ovicidal, and lavicidal properties. Jayakumar (2003) reported that plant extracts have obvious effects on postembryonic survival of the insect and resulting reduction in adult emergence in all the concentrations of different plants. The worthy inhibitory effects *A. differmis* extracts on the procreative cycle in which the F_1 progeny was reduced by more than 50% give a glimmer of optimism for use as stored grains protectants.

The non-tolerance and low susceptibility of cowpea seeds treated with the extracts to *C. maculatus* infestation was concentration dependent. Seed treated with 100 and 150µl ethyl acetate recorded zero percent infestation. Result from this trial agrees with Adesina and

Mobolade-Adesina (2016) who reported significant protection of cowpea seeds treated with *S. afzelii leaf* extracts to insect infestation. He opined that the protection confers on the treated seeds might be due to the high insect mortality rate and inability of the eggs to hatch; thereby reducing metabolic activities of insects. The significant protection recorded may also be due to the repellent activity of the plant extracts.

Weight loss indicated the quantitative loss in stored grains due to larvae feeding showing a direct relationship between insect population and weight loss. In present findings, all the extracts provide a significant reduction in seed damage and weight loss compared with the untreated seeds. The significant reduction in seed damage and lower weight loss is due to reduced oviposition and number of hatched eggs; consequently, reduced larval feeding, thus lowered the percentages of seeds damaged and seed weight losses. This supports the findings of Wahedi et al. (2013); Adesina and Mobolade-Adesina (2016) where neem seed extract and *S. afzelii* leaves extract significantly prevented emergence of F1 adults of *C. maculatus* and subsequent weight loss done due to pest respectively.

CONCLUSION

The findings of the present research have lead credence to the use plant material as phyto-insecticide for the control of *C. macuatus* against stored cowpea seeds. The study revealed that *A. differmis* possesses oviposition deterrent and adult emergence inhibition properties against *C. maculatus*. Among the extracts tested, ethyl acetate extract exhibited best result as insecticidal product for the management of *C. maculatus* in stored cowpea. Further studies are recommended for exploring the active compound responsible for such activities and its toxicological effect on albino rats.

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Conflict of interest
The authors declare no conflict of interest to report.

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