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Efficacy of *Althaea officinalis* leaf extract in controlling *Alternaria* spp. pathogenic on *Citrus*

Fatemeh Maghsoodi · Parissa Taheri

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Abstract In this study, inhibitory effects of the aqueous and ethanolic leaf extracts obtained from marshmallow (*A. officinalis*) were evaluated against *Alternaria* spp., pathogenic on *Citrus*. The marshmallow leaf extracts were added to potato dextrose agar medium at various concentrations and antifungal effect of the extracts against three species of *Alternaria* pathogenic on citrus was tested in vitro and in vivo. The ethanolic leaf extract revealed significant inhibitory effect on the mycelial growth of all fungal species and complete inhibition on spore germination of the pathogens at 100 mg mL^{-1} concentration, similar to those of the fungicide tested. Microscopic analysis revealed that the extract caused morphological changes, including abnormal hyphal morphology with a rough surface and shriveled hyphae. The HPLC analysis revealed the presence of phenolic/flavonoid compounds, including chlorogenic acid, quercetin, benzoic acid, apegenin, cinnamic acid, ferulic acid and Kaempferol. Results of enzyme activity assays revealed that the activity of cellulase, amylase, protease and pectinase reduced in the samples treated with the ethanolic extract. The ethanolic leaf extract protected *Citrus sinensis* (orange) fruits against the pathogens and reduced lesion diameter of *Alternaria* disease in a concentration dependent manner. The concentrations of 50 and 100 mg mL^{-1} were the most effective in reducing lesion diameters on citrus fruits campared to the control. Both in vitro and in vivo assays revealed potential of marshmallow ethanolic leaf extract to be used as a safe and eco-friendly protectant of citrus fruits against *Alternaria* spp. as destructive pre- and post-harvest fungal parhogens. Future research seems to be necessary to investigate antifungal effect of the major components detected in marshmallow leaf extract and determine the best formulation to increase its efficiency against phytopathogens.

Keywords *Alternaria* leaf spot · Antifungal activity · Plant extract

F. Maghsoodi · P. Taheri (✉)
Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran
e-mail: p-taheri@um.ac.ir

Introduction

The genus *Alternaria* is distributed worldwide with several saprophytic, endophytic, and pathogenic species, causing pre – and postharvest diseases on cereals, fruits, and vegetables (Garganese et al., 2016). More than 100 plant species have been reported to be affected by various *Alternaria* species (Armitage et al., 2015). *Alternaria* brown spot (ABS) is the most important disease that affects various plant species belonging to the genus *Citrus* and their hybrids (Timmer et al., 2003, Peever et al., 2004). It damages different parts of the host plants such as leaves, branches and new fruits (Martelli et al., 2016). The ABS disease is currently found in several tropical and subtropical citrus – growing regions (Vega and Dewdney, 2014). The symptoms of ABS are characterized by brown to black spots on young leaves surrounded by a chlorotic halo (Akimitsu et al., 2003). Fruit symptoms include light brown, slightly depressed spots to

circular and dark brown areas on the external surface (Vicent et al., 2007) and reduction of fruit quality, which can cause the fruit drop before harvest and the infection develops on fruits postharvest in storage period (Perina et al., 2019). Control of this destructive disease is mainly based on using synthetic fungicides, however, high costs, harmful effects on human health and environment and development of pathogen resistance to various types of fungicides after frequent usage of synthetic chemicals stimulated the investigations of alternative disease management strategies.

Plant-derived compounds, such as essential oils and extracts could be recommended as safe alternatives for synthetic chemicals. Extracts and essential oils obtained from a number of plant species show antimicrobial activity against *Alternaria* spp. Sallam and associates (2011) reported the effect of six plant extracts, including *Ocimum basilicum*, *Azadirachta indica*, *Eucalyptus camaldulensis*, *Datura stramonium*, *Nerium oleander* and *Allium sativum* against *Alternaria solani* in vitro and in vivo. Another study showed antifungal activity of ethanolic extract from root tissues of *Solanum nigrum* against *A. solani,* the causal agaent of black leaf spot of chines cabbage (Muto et al. 2006). Also, inhibitory effects of leaf extract of *Allium cepa* and *A. sativum* against *A. solani*, the causal agent of tomato early blight disease have been reported by Latha et al. (2009). Antifungal activity of essential oil obtained from the fruits of *Cicuta virosa* L. var. *latisecta* in vitro and in vivo against *A. alternata* is reported by Tian et al. (2011). Hamad et al. (2019) reported the antifungal activity of acetone extract obtained from *Tectona grandis* woods against *A. solani*. *A. officinalis,* commonly known as marshmallow is a valuable medicinal plant belonging to Malvaceae family. This plant is native to the southern Europe and parts of Asia (Tahmouzi et al., 2020). *A. officinalis* is widely used traditionally for treating irritation of oral and pharyngeal mucosa, mild gastritis, skin burns, and for insect bites. It is also used against catarrh of the mouth and throat, gastrointestinal tract and urinary tract complain, as well as for inflammation, ulcers, abscesses, burns, constipation, and diarrhea (Korbekandi et al., 2016). Antibacterial effects of *A. officinalis* root extract on two main microorganisms responsible for dental caries, including *Streptococcus mutans* and *Lactobacillus* (in vitro) was reported by (Haghgoo et al., 2017). Also, antimicrobial efficacy of *A. officinalis* seed aqueous extracts against respiratory tract pathogens was reported by Gautam et al. (2015). Ozturk and Ercisli (2007) found that methanol extracts of aerial parts of *A. officinalis* had antibacterial activity against food-borne bacteria, such as *Acidovorax facilis*, *Enterobacter hormachei*, *Kocuria rosae*, *Bacillus* spp. Aminnezhad et al. (2016) reported the antibacterial effect of *A. officinalis* extract on growth and biofilm formation of *pseudomonas aeruginosa*. Although, there are many reports about antimicrobial effects of *A. officinalis* and the mechanisms of function against human pathogens and foodborne pathogens but, our knowledge on the antimicrobial effect of *A. officinalis* and the mechanisms of its action against plant pathogens is limited. It is necessary to investigate effect of essential oils and plant extracts not only on the phytopathogens growth, but also on various types of virulence factors produced by the pathogens, including cell wall degrading enzymes (CWDEs), which are involved in destructive effects of each pathogen on the host plant tissues. Enzymes such as cellulase, pectinase, protease, and amylase are among the most important CWDEs produced by fungal pathogens that effect of essential oils and/or extracts obtained from some medicinal plants on production and activity of these virulence factors have been investigated (Khaledi et al. 2015; Abd-El-Khair and El-Gamal 2011). Therefore, the aims of this study were: (i) to investigate and compare the effect of *A. officinalis* aqueous and ethanolic leaf extract on the mycelial growth, spore germination and germ tube elongation of *Alternaria* spp. pathogenic on citrus, (ii) to determine the potential of very low concentration of the extract without any effect on the fungal growth, in reducing the activity of CWDEs produced by the pathogens, which are involved in the invasion and penetration into the host plant tissue, (iii) to identify more accurately effect of the extract on the hyphal structures, (iv) to determine the efficacy of *A. officinalis* leaf extract in controlling *Alternaria* leaf spot disease on citrus fruits, and (v) to identify the constituents of *A. officinalis* leaf extract via HPLC analysis.

Materials and methods

Fungal isolates

Three species of *Alternaria,* including *A. alternata*, *A. atra* and *A. dumosa,* pathogenic on citrus were obtained from the fungal collection of Department of Plant Protection, Faculty of Agriculture, Ferdowsi University

of Mashhad in Iran. These isolates were cultured on potato dextrose agar (PDA, Merck Germany) medium and incubated at 28 °C for 7 days.

Plant material and preparation of the leaf extract

Mature leaves of marshmallow were collected in August 2018 from different parts of Khorassan-Razavi province in Iran. The plant was identified at botanical herbarium of Ferdowsi University of Mashhad. Fresh leaves were washed and dried at room temperature under shade. Dried leaves were powdered by a blender and the obtained powders were macerated in distilled water (Scavo et al., 2019) and ethanol 70% (Merck, Germany) as solvent with the ratio of 1: 10 (samples: solvent, w/v) for 72 h (Romeo et al., 2015). Then, the crud extract was filtered through double layers of muslin cloth. The solvent in ethanolic extract was removed by using rotary evaporator and the residue was diluted with sterile distilled water to obtain final concentration of 100 mg/mL. Then, the concentrations of 50, 25, 12.5 and 3.5 mg/mL were prepared from it. Finally, the solution was filtered by syringe filter (0.2 μm) and stored inside glass vials at 4 °C until used.

Antifungal activity of marshmallow extracts against *Alternaria* spp. in vitro

The antifungal activities of the aqueous and ethanolic extracts obtained from marshmallow were evaluated against *Alternaria* spp. by poisoned food technique as described by (Ali et al., 2020). The PDA medium containing different concentrations of marshmallow aqueous and ethanolic leaf extract, including 100, 50, 25 and 12.5, 6.25, 3.12 mg/mL were prepared. Then, each mixture was poured into a 6 cm diameter sterilized petri dish plate and left to solidify. The Mycelial plugs (5 mm diameter) were taken from 7 days old cultures of *A. atra, A. alternata, A. dumosa* and placed in the center of each petri dish. The plates were incubated at 28 °C for 7 days. Non-amended PDA plates were used as a negative control and mancozeb 80% wp (3 mg/mL) was use as positive control. All treatments consisted of three replicates and the experiment was performed three times. The percentage of mycelial growth inhibition was calculated by determining radial growth in the treated fungi and controls and using the following formula (Zorzi Tomazoni et al., 2019): Growth inhibition (%) = (dc - dt /dc) × 100; where, dc is the mean colony diameters of the control, and T is mean colony diameters of the treated groups. Based on the higher antifungal effect of the ethanolic leaf extract compared to the aqueous extract, ethanolic extract was used in the rest of experiments.

Spore germination assay

Effects of the ethanolic extract of marshmallow on spore germination and germ tube elongation were performed in Water agar (WA) medium amended with different concentrations of the extract. The concentrations of 100, 50, 25 and 12.5 mg/mL were used for spore germination assay. Then 100 μL of *A. alternata, A. atra, A. dumosa* spore suspension at 1×10^7 spore/mL (estimated by using a haemocytometer slid) were added to each petri dish and spread on the surface of the plate. Then, the plates were incubated at 28 °C for 12 to 24 h. The Petri-dish inoculated without the extract concentration used as negative control and mancozeb 80% (3 mg/mL) was use as positive control. Three replications were used for each treatment and the experiment was repeated three times. A spore was estimated as germinated when the length of germ tube was longer than the spore diameter (Feng and Zheng, 2007). Approximately 100 spores were examined in each replicate for determining germination rate and germ tube length using an optical microscope (Olympus BH2, Tokyo, Japan).

Microscopic analyses

Light microscopy analysis of effect of the extract on the hyphal structure

Hyphal structure of *Alternaria* species grown in PDA plates containing the most effective concentration (100 mg/mL) of the ethanolic leaf extract was investigated 5 days after culturing the fungi. A piece of each mycelial sample was removed from the plate and morphological changes of hyphae exposed to the extract were examined under the light microscope (Olympus, BH_2, Tokyo, Japon). The fungal samples grown in PDA medium without the extract were used as control and mancozeb 80% wp 3 mg/mL was use as positive control.

Scanning electron microscopy (SEM) analysis

The SEM analysis was carried out using the method described by (Chen et al., 2014) with some modifications. Five days old mycelial plugs of *Alternaria* spp. grown on PDA medium amended with the most effective concentration (100 mg/mL) of marshmallow leaf extract were examined under Scanning electron microscopy (SEM). The pieces of each sample were carefully cut and placed on a plate. Then glutaraldehyde (2.5%, Taab, UK) in 0.1 M phosphate buffer (pH 7.2) was added to the fungal samples and fixed overnight at ambient temperature. Each sample was washed twice with the same buffer. The samples were post-fixed again with 1% osmium teroxide solution for 1 to 2 h. Subsequently, the samples were dehydrated with series of graded ethanol ranging from 10 to 90% for 15 min in each dilution and then rinsed with pure ethanol for 20 min. Then, the samples were dried by air dryer. Finally, the samples were sputter-coated with gold layer, observed and photographed by an electron microscope (LEO-Germany,vp 1450) at 15 kv. Colonies grown on PDA without the extract were used as control samples.

Enzymatic assays

Effect of marshmallow ethanolic leaf extract on the activity of extracellular cell wall degrading enzymes produced by the fungal isolates was evaluated by qualitative method (using solid culture media) and quantitative assay using liquid culture and spectrophotometry as described below.

Qualitative enzyme assays

Agar plate assay was applaied to investigate effect of the ethanolic leaf extract on the activity of cellulase, protease, pectinase and amylase produced by *Aternaria* spp. In this expriment, 3.5 mg/mL concentation of the ethanolic extract was used, which had no effect on mycelial growth of the fungal species. It was added into the plates containing autoclaved specific medium of each enzyme and mixed. Mycelial plugs (5 mm diameter) were taken from 7 days old cultures of *A. atra, A. alternata, A. dumosa* and placed on the center of each petridish. Plates containing only PDA were used as control. All plates were incubated at 28 °C for 5 days. Enzyme activity was measured based on the average diameter of the halo zones in three replicates. For determinaing the activity of cellulase produced by *Alternaria*.spp., a specific medium (containing, 10 g Peptone, 10 yeast exract, 5 g NaCl, 1 g KH_2PO4, 18 g agar, 10 g carboxy-methylcellulose, distilled water 1 L, pH =7.0) was used (Zheng et al., 2011). The extract was added to each plate and 5 mm diameter fungal discs were placed on the center of each petri dish. After 5 days incubation at 28 °C, the surface of plates was flooded with 2% congo red solution for 5 to 7 min, after that de-stained with 1 M NaCl for 15 min (Sunitha et al., 2013). Cellulase activity was detected by observing and measuring the diameter of a yellow halo against the red background of the medium.

Protease activity was evaluated by growing the fungi on skim milk medium (10 g skim milk, 1 g yeast extract, 15 g agar, distilled water 1 L) according to the method described by (Wery et al., 2003). At the end of incubation period (for 5 days at 28 °C), a clear halo formed around the creamy zone which indicated protease activity.

For amylase activity, the fungi were grown on glucose yeast extract peptone agar (GYP) medium, containing 0.2% soluble starch as substrate and pH = 6.0, using the method described by (Sunitha et al., 2013). After incubation period surface of the plates was treated with 1% iodin in potassium iodide. Formation of an orange halo around the brown zone revealed amylase activity.

Czapek - Dox agar medium (containing 0.5 g KCL, 3 g $NaNO_3$, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 1 g K_2HPO, 30 g sucrose, and 15 g agar) was used for pectinase activity determination using 2% pectin (PH = 5.6) as the substrate (Hankin et al., 1971). After incubation period of 5 days at 28 °C, surface of the plates was treated with 1% iodin in 2% potassium iodide. Formation of clear halo around the active colonies revealed pectinase activity.

Qantitative cellulase activity assay

Evaluating the activity of cellulase produced by *Alternaria*. spp. in the presence of marshmallow leaf extract was performed using the method of Abdel-Razik (1970). Specific medium of cellulase assay (containing 5 g yeast exract, 5 g peptone, 5 g K_2HPO_4, 4.6 g carboxy-methylcellulose, distilled water 1 L) was prepared and transferred to the tubes containing 3.5 mg/mL concentration of the ethanolic extract, which had no inhibitory effect on the fungal growth. Then, a 5 mm diameter mycelial plug from 7 days old culture of each fungal species was added to each tube separately. The

medium in the absence of the extract was used as control. All tubes were incubated at 28 °C for 10 days. To determine cellulase activity, the samples (tubes amended with fungal mycelial plugs) were first centrifuged at 5000 rpm and 0.5 ml from the surface of supernatant was added to 1 mL of 0.7% CMC (Carboxymethyl cellulose) in 0.05 mol/L acetate buffer with pH = 4.8 and incubated at 50 °C for 60 min (Wood and Bahat, 1988). Then, 2 ml of DNS (3,5 - dinitrosalicylic acid) reagent was added to each reaction mixture and was boiled for 10 min at 100 °C. Finally, the reaction was stopped by adding 1 mL 40% potassium sodium tartrate. The absorbance of each sample was read at 550 nm. The quantity of CMC solution in the medium was measure by using standard curve of glucose.

Inhibitory effect of marshmallow extract on the disease progress in vivo

To investigate effect of the ethanolic extract as preventive treatment to control *Alternaria* disease on orange (*C. sinensis*) fruits, the fruits without physical wounds or apparent infection were selected. They were dipped in sodium hypochlorite solution for 2 min and then rinsed with distilled water and air- dried at room temperature for 2 h. The fruits were wounded (2 mm in diameter and 2 mm deep) with a sterile nail in 4 selected location and each wound was treated with 10 μL of the extract. After 1 h, spore suspension of each fungal pathogen containing 3×10^4 spore/L was added to each wound. Fruits treated with sterile distilled water were used as negative control and mixtures of spore suspension and the fungicide mancozeb 80% wp (3 mg/mL) were used as positive control. The inoculated fruits were kept in plastic boxes containing wet filter papers to maintain high relative humidity (RH) without direct contact with the wet layer and incubated in dark place at 20 °C for 7 days (Nicosa et al., 2015). The lesions diameter (mm) around each selected location on the fruits was measured (Carvalho et al., 2011).

High performance liquid chromatography (HPLC) analysis

The HPLC, as a popular analytical technique, was used in this study for separation, identification and quantification of each constituent of the extract. Analysis of phenolic compounds in the ethanolic leaf extract of marshmallow was performed by using an Agilent 1260 series HPLC system equipped with Zorbax Eclipse C_{18} column (100 mm × 4.6 mm), quaternary gradient pump and UV detector. Separation of compounds was performed using a ternary linear elution of mobile phases A, B and C. Solvent (A) consisted of HPLC grade water 0.2% H3PO4 (v/v), solvent (B) consisted of methanol and (C) consisted of acetonitrile. The column temperature was 30 °C and injection volume for all samples was 20 μL. The UV detector was set at 248 nm to identify phenolic compounds (Abdelkhalek et al., 2020). Identification of phenolics was based on comparison of the retention time of standards and their UV spectra and the results were expressed as miligrams per gram of the crud extract. The standard phenolic and flavonoid compounds used were benzoic acid, chlorogenic acid, Apigenin, *p*-coumaric acid, cinnamic acid, ferulic acid, rutin, quercetin and kaempferol.

Statistical analysis

The data were analyzed by one – way analysis of variance (ANOVA) using SPSS software version 22.0 (SPSS Inc., Chicago, IL, USA). The means were compared by the Duncan's multiple range test at $P < 0.05$. Values in figures are the means of data and bars indicate standard deviations. Each experiment was repeated at least four times and each time performed with four replications.

Results

Antifungal activity of *A. officinalis* leaf extract in vitro

Antifungal activity of the ethanolic and aqueous leaf extracts obtained from marshmallow were evaluated against *Alternaria* spp. pathogenic on citrus. Results of the antifungal assay (Table 1) showed that the ethanolic leaf extract at concentrations of 100, 50, 25, and 12.5 mg/mL inhibited the growth of fungal colonies in a dose-dependent manner and the concentrations less than 12.5 mg/ml showed no significant inhibitory effect against *Alternaria* spp. As showen in Table 1, highest concentration of the extract was more effective against *A. alternata* than *A. atra* and *A. dumosa.* The highest inhibition percentage of mycelial growth at highest

concentration (100 mg/mL) was (% 73.82) for *A. alternata*. Antifungal activity of the extract against *A. alternata* and *A. atra* at the highest concentration was similar to that of the fungicide used as a control. But suppressive effect of the extract on mycelial growth of *A. dumosa* was less than that of mancozeb 80% wp. Application of the aqueous leaf extract showed considerably lower inhibitory effect in concentrations of 50 and 100 mg/mL and using it at lower concentrations had no effect on radial growth of the fungi tested (Table 1). Therefore, the ethanolic leaf extract was selected to be used in the rest of experiments.

Effect of *A. officinalis* leaf extract on *Alternaria* spp. spore germination

Effect of the ethanolic leaf extract on spore germination and germ tube elongation of the tested fungi were are presented in Table 2. The obtained results revealed that treatment with different concentrations of the extract significantly suppressed fungal spore germination and germ tube elongation of *Alternaria* spp. compared to the control. Application of the extract at 50 and 100 mg/mL concentrations completely inhibited spore germination of the fungal isolates (*A. alternata, A. dumosa* and *A. atra*) which was similar to the effect of mancozeb 80% wp.

Microscopic analyses

Light microscopic analysis of marshmallow extract effect on the hyphal structures

Microscopic analysis of vegetative growth of *Alternaria* species on PDA containing 100 mg/mL concentration of marshmallow ethanolic leaf extract and the fungicide as positive control after 7 days revealed degenerative changes in the hyphal morphology (Figs. 1a-f). Hyphal changes in presence of the extract included deformation and reduction of cell wall diameter, rough surface, hyphal fragmentation and granulation of cytoplasm, whereas untreated colonies grown on PDA displayed normal hyphal morphology (Figs. 1g-i).

Electron microscopic (SEM) analysis of hyphal structures

The SEM analysis of the hyphal morphology was used to investigate effect of marshmallow ethanolic leaf extract and the fungicide on the hyphal surface of

Table 1 Antifungal activity of marshmallow leaf extract and fungicide on mycelial growth of three *Alternaria* species on PDA medium after 7 days of inocubation.at 28 °C. Statistical data analysis was carried out using SPSS (ver.22) software. Different letters indicated significant differences according to Duncans multiple range test at the level $P < 0.05$

Treatment	Concentation (mg/mL)	Mycelial growth inhibition (%)		
		Alternaria alternata	*Alternaria dumosa*	*Alternaria atra*
Ethanolic extract	0	–	–	–
	3.12	–	–	–
	6.25	3.06±2.36 g	–	–
	12.5	15.26±1.59 e	7.61±0.45 f	11.73±0.57 e
	25	38.02±0.44 c	28.52±0.64 d	31.81±0.89 c
	50	67.63±2.36 b	54.89±0.88 c	62.46±0.83 b
	100	84.61±0.89 a	70.34±1.63 b	81.8±0.72 a
Aqueous extract	0	–	–	–
	3.12	–	–	–
	6.25	–	–	–
	12.5	–	–	–
	25	–	–	–
	50	13.3±0.2 f	–	5.26±0.2 f
	100	23.43±0.11 d	9.24±0.81 e	19.52±0.43 d
Mancozeb 80%	3	83.74±0.44 a	74.1±1.26 a	81.44±0.9 a

Table 2 Inhibitory effect of marshmallow ethanolic leaf extract and fungicide on spore germination and germ tube elongation of *Alternaria* spp. in water agar (WA) medium after 12 or 24 h incubation at 28 °C. The spores (approximately 100) were randomly observed for germination rate and germ tube length measurement in each treatment. Different letters indicated significant differences according to Duncans multiple range test at the level $P < 0.05$

Treatment	Concentation (mg/mL)	*Alternaria alternata*	
		Germination (%)	Germ tube length (μm)
Ethanolic extract	0	89.33±4.04 d	56.7±2.33 d
	12.5	24±2.6 c	16.33±2.5 c
	25	20.66±1.52 b	7.5±0.5 b
	50	0 a	0 a
	100	0 a	0 a
Mancozeb 80%	3	0 a	0 a
Treatment	Concentation (mg/mL)	*Alternaria dumosa*	
		Germination (%)	Germ tube length (μm)
Ethanolic extract	0	84±2.6 e	54.33±3.78 e
	12.5	34.33±1.52 d	22±1.32 d
	25	28±2 c	14±2 c
	50	6.27±1 b	4.5±0.5 b
	100	0 a	0 a
Mancozeb 80%	3	0 a	0 a
Treatment	Concentation (mg/mL)	*Alternaria atra*	
		Germination (%)	Germ tube length (μm)
Ethanolic extract	0	91.66±1.15 d	59.16±0.7 d
	12.5	28±2.08 c	18.36±0.8 c
	25	21±1 b	8.2±0.34 b
	50	0 a	0 a
	100	0 a	0 a
Mancozeb 80%	3	0 a	0 a

Alternaria spp. Mycelial morphology of the fungi treated with the extract and mancozeb 80% showed changes such as abnormal mycelial morphology with rough, shriveled, and wrinkled surface (Figs. 2a-f). The mycelial growth of *A.alternata*, *A. atra* and *A. dumosa* in PDA medium as the control after 7 days showed regular and normal hyphal morphology with smooth external surface (Figs. 2g-i).

Effect of marshmallow extract on enzymatic activity of *Alternaria* spp.

In the present study, effect of marshmallow ethanolic leaf extract on the activity of CWDEs was investigated for the first time. The clear halo observed around the fungal colonies indicated enzyme activity. According to the data obtained, activity of cellulase and protease produced by *A. alternata* exposed to 3.5 mg/mL concentration of the extract completely were suppressed. Activity of these enzymes in *A. atra* and *A. dumosa* significantly decreased in presence of the extract (Fig. 3a and b). The activities of pectinase and amylase in *A. atra*, *A. alternata*, and *A. dumosa* were significantly reduced in media treated with the extract compared to the controls (Fig. 3c and d).

Quantitative investigation of effect of the extract on cellulase activity

Fungi are the main plant pathogens, which are able to decompose cellulose in plant tissues. Cellulose and hemicellulose are two Major components of plant

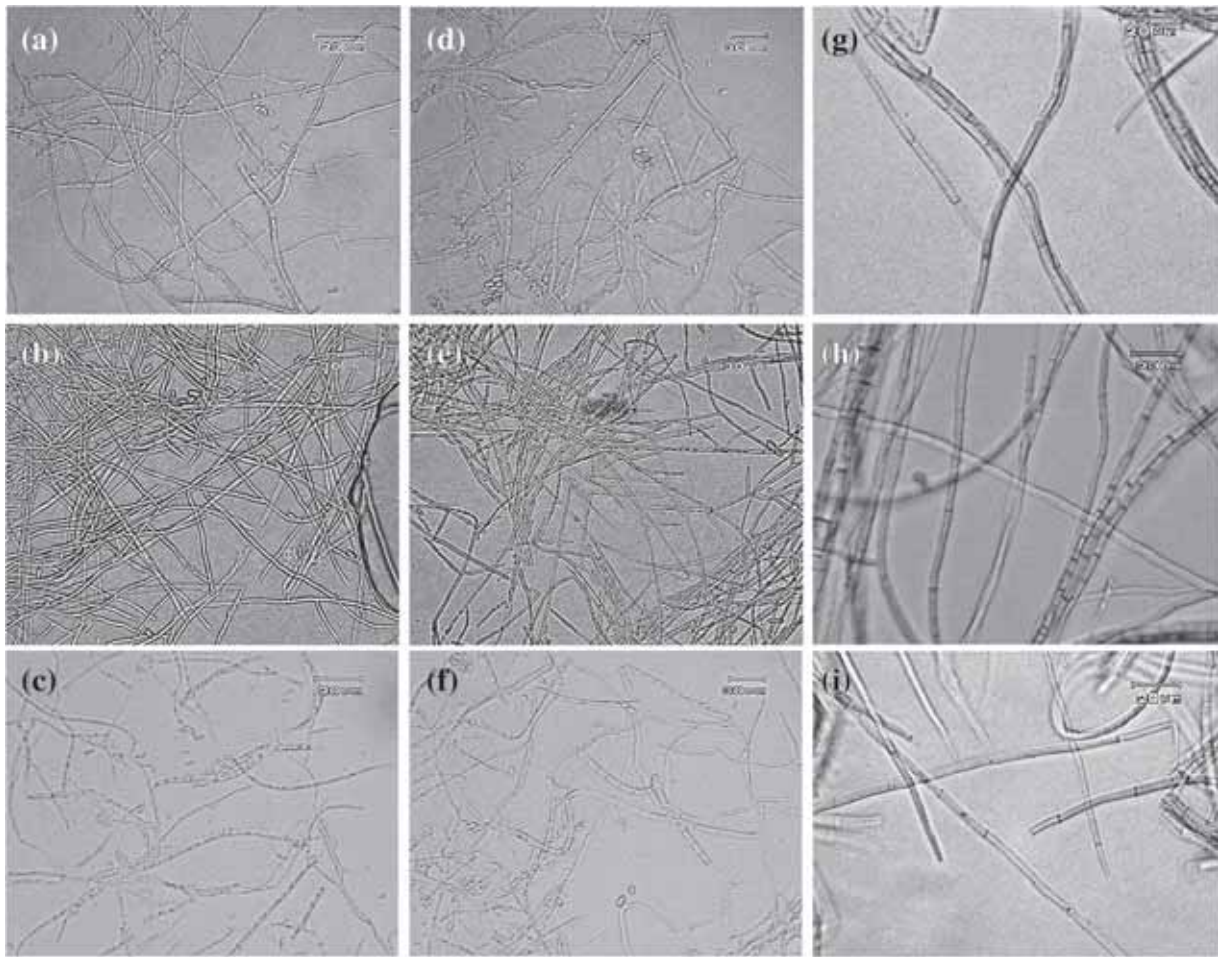

Fig. 1 Effect of marshmallow ethanolic leaf extract on hyphal morphology of *Alternaria* spp. grown on potato dextrose agar (PDA) after 7 days incubation at 28 °C. a-c: The hyphae treated with 100 mg/mL of the ethanolic leaf extract and d-f: the hyphae treated with the fungicide Mancozeb (as a positive control). The main changes were deformation, reduced wall diameter and hyphal fragmentation. g-i: negative control with regular and normal hyphae

material (Wei et al., 2011). Cellulose is an abundant carbohydrate that forms an integral part of the plant cell walls and provides structural integrity (Lynd et al., 2002a and b). It is composed of β-1,4 linked glucan chains (Wei et al.,2011). The obtained data revealed that *Alternaria* spp. were able to produce and secrete a large amount of cellulase the absence of marshmallow ethanolic leaf extract. Maximum cellulase activities of *A. dumosa* and *A. atra* were observed at 192 h post culturing on specific liquid medium (Fig. 4a and c) and *A. alternata* showed maximum cellulase activity at 216 h post culturing (Fig. 4b). For all samples treated with the extract, the cellulase activity was significantly lower than that of the control without the extract treatment (Fig. 4).

Inhibitory effect of marshmallow extract on the disease progress in vivo

According to the data shown in Table 3, the marshmallow ethanolic leaf extract had protective potential and reduced lesion diameter and development of *Alternaria* disease on citrus fruits depending on the concentrations tested. The concentrations of 50 and 100 mg/mL were the most effective in reducing the diameters of lesions on citrus fruits campared to the control. Application of the extract in concentration of 25 mg/mL was not significantly effective in the disease control. The highest effect of extract was observed at concentation of 100 mg/mL against *A. alternata* and *A. atra* with 1.9 and 1.5 mm reductions in lesion diameter respectively.

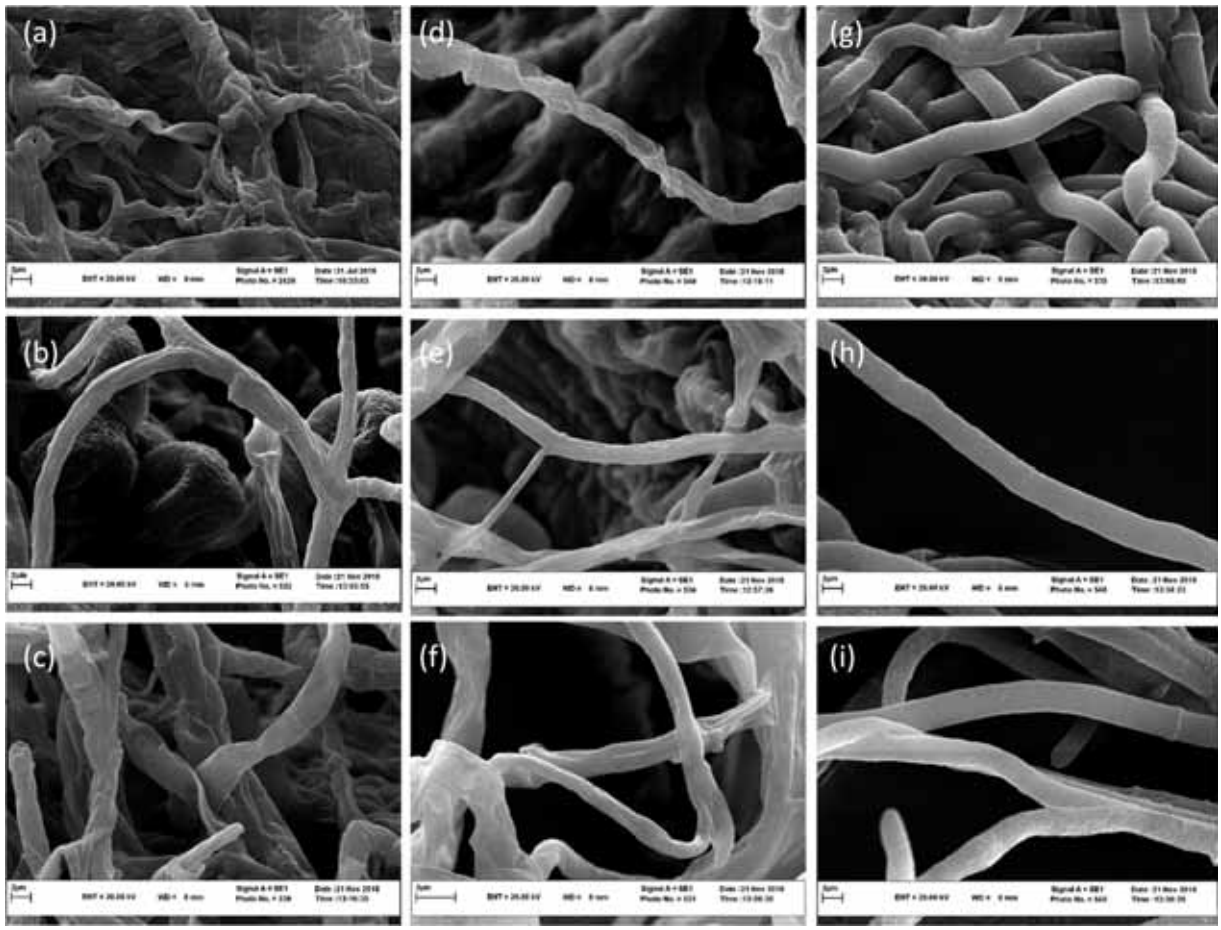

Fig. 2 Scaning electron micrographs of *Alternaria* spp. grown on PDA with or without marshmallow ethanolic leaf extract after 7 days incubation at 28 °C. a: *Alternaria alternata*, b: *A. dumosa* and c: *A. atra* treated with marshmallow ethanolic leaf extract at 100 mg/mL concentration. d: *A. alternata*, e: *A. dumosa* and f: *A. atra* treated with Mancozeb 80% at 3 mg/mL concentration, showed wrinkled and folded hyphae. g: *A. alternata*, h: *A. dumosa* and i: *A. atra* controls with normal and healthy hyphae. Scale bars = 2.0 μm

Reduction of lesion diameters on the fruits treated with the fungicide was statistically similar to effect of the extract at 100 mg/mL concentration (Table 3).

Identification of components present in the ethanolic leaf extract

Phenolic components of marshmallow ethanolic laef extract were identified and quantified by HPLC analysis. As shown in Fig. 5, HPLC chromatograms of the extract obtained under optimum conditions showed fourteen peaks, which were detected at 248 nm. The peaks 2, 4, 5, 6, 9, 12, and 13 were identified and quantified as chlorogenic acid (36.23 mg/g), quercetin (8.38 mg/g), benzoic acid (46.02 mg/g), apegenin (26.59 mg/g), cinnamic acid (20.84 mg/g), ferulic acid (5.21 mg/g) and Kaempferol (33.04 mg/g). Benzoic acid (46.02 mg/g) was found to be the major phenolic constituent of the marshmallow ethanolic leaf extract while the most abundant flavonoid compounds in mg/g were cinnamic acid, chlorogenic acid and Kaempferol.

Discussion

In this study, aqueous and ethanolic leaf extracts of marshmallow were used at different concentrations to investigate their antifungal effects against *Alternaria* species, as the major fungal pathogens causing pre- and post-harvest diseases on citrus. The ethanolic leaf extract showed antifungal activity against all three species of *Alternaria* tested, including *A.dumosa*, *A. atra*,

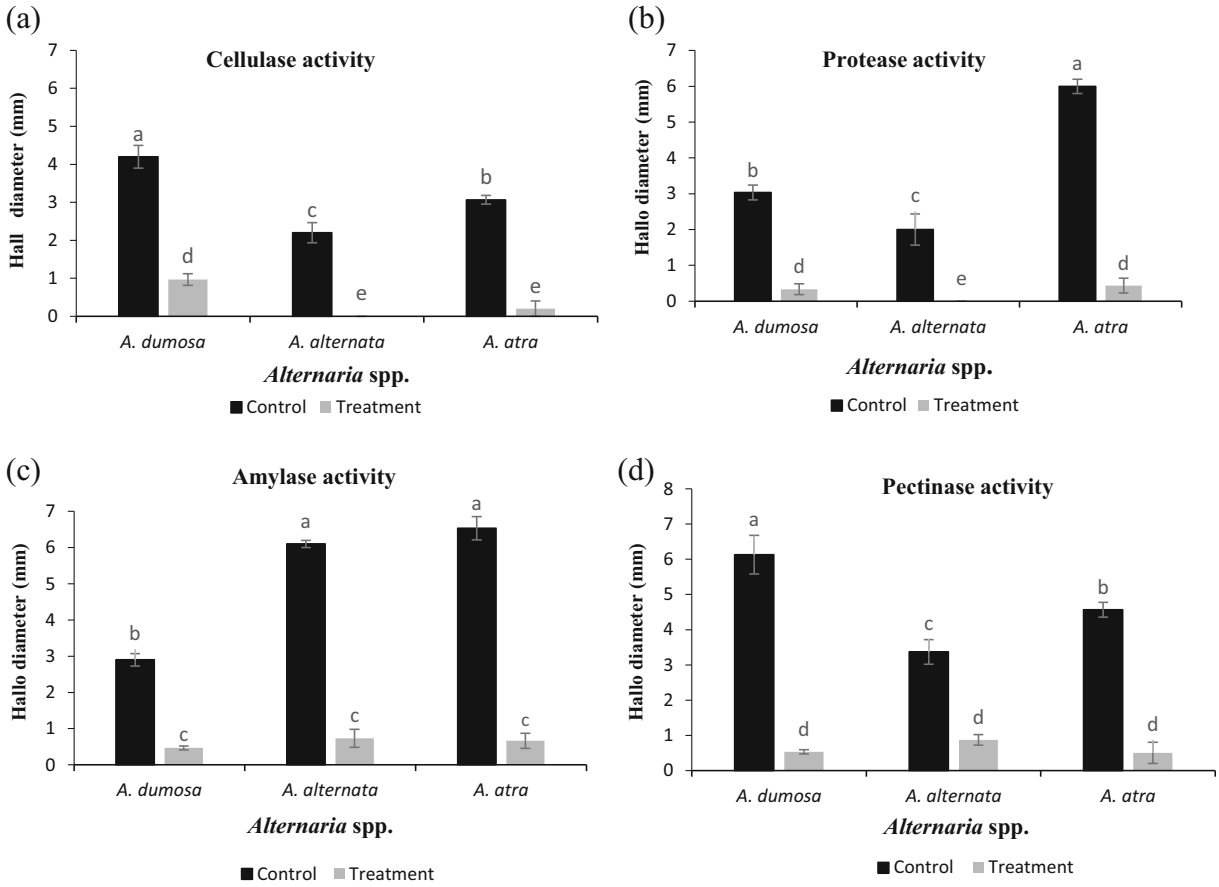

Fig. 3 Qualitative analysis of cell wall degrading enzymes produced by *Alternaria* spp. With or without marshmallow ethanolic leaf extract at 3.5 mg/mL concentration. The fungal cultures were transfered to the plates containing autoclaved specific media of cellulase (a), protease (b), pectinase (c) and amylase (d). Enzymatic activity was estimated after 5 days based on the average diameter of the halo zones in four replicates

and *A. alternata*. The ethanolic extract was effective in reducing the radial growth of *Alternaria* spp., while the aqueous extract did not show a significant effect. Similarly, Mugao et al. (2020) investigated in vitro efficacy of aqueous, ethanolic and methanol extract of ginger, garlic, tick berry, and Mexican marigold in inhibition of *A. solani* growth. They reported that the crude extracts of all plants tested were effective in inhibiting the growth of *A. solani* but ethanol was the best solvent for extracting antifungal metabolites. Tian et al. (2011) demonstrated antifugal activity of *Cicuta virosa* essential oil against *A. alternata*. They found that fungal mycelial growth reduced with increasing concentration of the essential oil, in agreement with the findings of this research. Our data indicated that higher concentrations of the marshmallow extract had stronger antifungal effect on *Alternaria* spp. The ethanolic extract showed significant inhibitory effect on radial growth of fungi tested. Maximum reduction of the fungal growth was between 70.34 to 84.61% in various species by using 100 mg/mL concentration of the extract as the highest concentration tested and these values were approximately equal to the data obtained using the fungicide Mancozeb, indicating high antifungal effect of the extract.

Findings of this study also revealed that the marshmallow extract had strong inhibitory effect on percentage of spore germination and germ tube elongation. Spore germination of each three isolates were completely inhibited at 100 and 50 mg/mL concentrations of the extract which was similar to the effect of fungicide. Similar findings were also reported by Feng and Zheng (2006), who reported that cassia oil at 500 ppm concentration extremely inhibited spore germination of *A. alternata*. The current study is the first report on the antifungal effect of a medicinal plant on spore

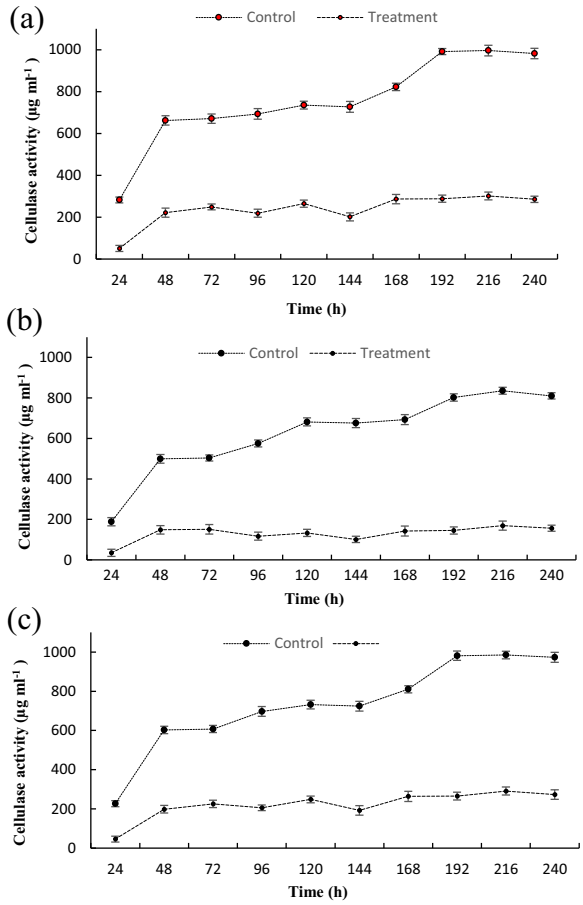

Fig. 4 Effect of marshmallow ethanolic leaf extract at 3.5 mg/mL concentration on cellulase activity of *Alternaria dumosa* (A), *A. alternata* (B), and *A. atra* (C). The bars indicate standard errors (SE)

germination of *A. atra and A. dumosa.* The mechanisms of germination allow many fungi to be pathogenic on plants and germinating spores have the potential to penetrate into the plant tissues and cause destructive diseases (Sephton-Clark et al., 2017).

The findings of this study revealed that marshmallow ethanolic leaf extract caused changes on the hyphal morphology and structure, such as deformation of cell wall, granulation of cytoplasm, shriveled hyphae with rugose surface. Our results are in accordance with previous reports on destructive effects of various essential oils and plant extracts on fungal structures. For example, Castro et al. (2016) reported that treatment with *Cymbopogon flexuosus*, *Eugenia caryophyllus*, *Cinnamomum zeylanicum* essential oils inhibited the growth of *A. alternata* and caused morphological changes, such as disruption of the hyphae. A study performed by Elsherbiny et al. (2015) using electron microscomy analyses revealed that morphological modifications occur in *Fusarium sambucinum* hyphae, including curling, twisting and collapse, disintegration of cell organelles and leakage of cytoplasmic components after treatment with pomegranate peels methanol extract. Similar findings were also reported by Chen et al. (2014), who demonstrated that *Citronella* essential oil caused structural changes in *A. alternata*, such as shriveled hyphae and rough hyphal surface. These changes might also change the activity of enzymes located on the membrane, which are involved in formation of fungal cell wall and lead to abnormal development (Bianchi et al., 1997).

In qualitative evalution of protease, amylase, and pectinase produced by the fungal pathogens, reductions of these enzyme activities in the presence of the extract were observed. Qualitative and quantitative assessment of cellulase activity in the presence of the extract indicated that secretion of cellulase was reduced in the

Table 3 In vivo inhibitory effect of marshmallow ethanolic leaf extract and a fungicide on the diameters of leaf spot on orange fruits inoculated with *Alternaria* spp. (3×10^4. spore/ml) after 7 days. Different letters indicated significant differences according to Duncans multiple range test at the level $P < 0.05$

TreatmentTreatment	Concentration (mg/mL)	Lesion diameter (mm)		
		A.alternata	*A.dumosa*	*A.atra*
Ethanolic extract	0	2.8±0.21 e	4±0.1 d	3.3±0.2 d
	12.5	2.6±0.15 d	3.9±0.8 d	3.2±0.1 d
	25	2±0.1 c	3.4±0.32 c	2.7±0.15 c
	50	1.5±0.05 b	3±0.17 b	2.1±0.11 b
	100	0.9±0.17 a	2.8±0.27 a	1.8±0.1 a
Mancozeb 80%	3	0.96±0.05 a	2.1±0.1 a	1.9±0.15 a

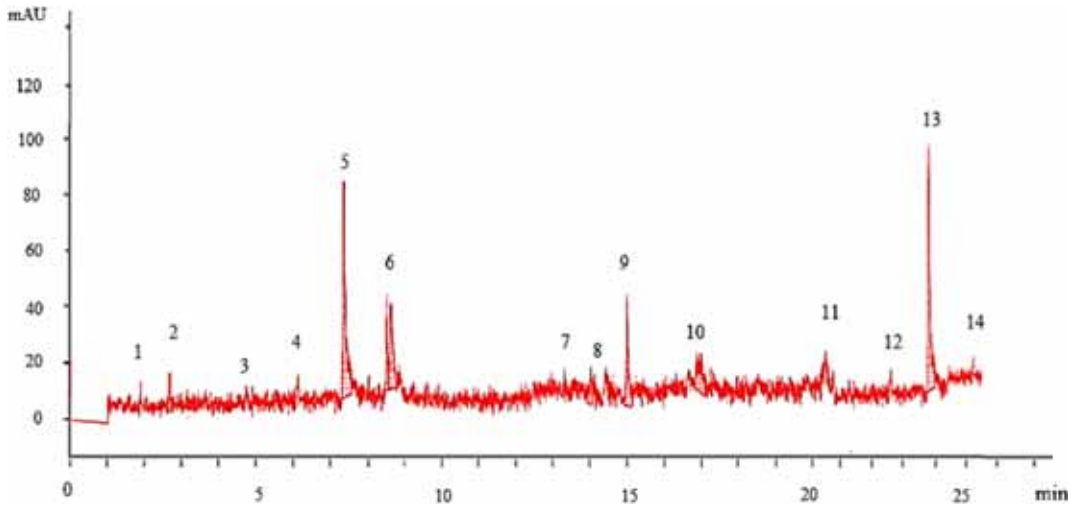

Fig. 5 HPLC chromatograms of marshmallow ethanolic leaf extract. Individual peaks showing phenolics/flavonoids compounds. Peak no. 2. Chlorogenic acid (36.23 mg/g), 4. Quercetin (8.38 mg/g), 5. Benzoic acid (46.02 mg/g), 6. Apegenin (26.59 mg/g), 9. Cinnamic acid (20.84 mg/g), 12. Frulic acid (5.21 mg/g) and 13. Kaempferol (33.04 mg/g)

samples treated with the extract (at 3.5 mg/mL concentration which had no effect on the fungal growth) compared to the control. Similarly, Muhsin et al. (2001) investigated the effect of garlic bulb extract on fungal growth and the activity of amylase, cellulase, phenol oxidase, and protease produced by several fungal species isolated from rhizospheric soil and roots of various plant species, including okra (*Hibiscus esculentum*), tomato (*Solanum lycoperscum*) and wheat (*Triticum aestivum*). These authors reported a high reduction or inhibition of enzymatic activities for the fungi treated with garlic extract compared to the untreated fungal cultures. Khaledi et al. (2015) reported the effect of *Mentha piperita*, *Bunium persicum*, *Thymus vulgaris* essential oils at low concentrations without any effect on fungal growth to decrease activity of cellulase and pectinase secreted by *Rhizoctonia solani* and *Macrophomina phaseolina*, which is in accordance with the findings of this study.

Since various types of CWDEs are important virulence factors of *Alternaria* spp., the marshmallow extract can reduce development of the disease caused by *Alternaria* spp. on citrus which might be due to its inhibitory effect on the activity of CWDEs produced by these fungi. In the in vivo assay, significant reduction in lesion diameters and development of the brown spot disease caused by *Alternaria* spp. was observed by treating orange fruits with the extract. This finding is consistent with previous reports on the in vivo antifungal effect of various essential oils and plant extracts against *Alternaria* species. In a previous study performed by Carvalho et al. (2011), the extracts obtained from different plant species were tested for their effect on *A. alternata* in vitro and in vivo. The most promising extract was obtained from *Anadenanthera colubrina*, that reduced the brown spot disease on Murcott tangor fruits to levels obtained using synthetic fungicides (Carvalho et al., 2011), which was similar to the findings of present study. Investigations of De Lima et al. (2016) using garlic extract and orange essential oil showed their antifungal effects on *A. dauci* and *A. alternata* pathogenic on carrot, because their low concentrations were able to sufficiently reduce the incidence of these fungi and they do not affect carrot seeds germination and emergence. Therefore, plant extracts and essential oils offer a novel and effective alternative method for controlling Alternaria diseases, which their control is currently based on using hazardous chemical fungicides.

In this study, phytochemical screening of phenolic and flavonoid compounds in marshmallow ethanolic leaf extract was done via HPLC analysis and the obtained data revealed the presence of phenolics and favonoid compounds, including chlorogenic acid, quercetin, benzoic acid, apegenin, cinnamic acid, ferulic acid and kaempferol. Similarly, in a previous study performed by (Kadhum et al., 2019) using HPLC analysis, identification, and quantification of flavonoid components in the aerial parts of marshmallow was performed and they reported the presence of quercetin, rutin, apiginine, isorhmntein, scopoletin, coumarins, and Kaempferol in

ethanolic extract of marshmallow. Rutin was reported as the most abundant compound in their study (Kadhum et al., 2019), but in the present research benzoic acid was found to be the main constituent of marshmallow ethanolic leaf extract. Furthermore, the presence of phenolics and flavonoids in methanolic extract of marshmallow is reported by Mehreen et al. (2016). They reported that flavonoid fraction of marshmallow exhibited antimicrobial activity. It seems that the antifungal activity of marshmallow ethanolic leaf extract was due to more abundant ingredients of the analyzed extract and these metabolites are responsible for antifungal activity of the extract. The difference in chemical composition of the extract in this study and previous studies may be attributed to several factors, such as environmental conditions, geographical location, symbiosis with various microorganisms, climatic effects on the plants, harvest season, age of the plant parts, the parts of the plant used, time of collection, nutritional status of the plants and genetic factors (Ma et al., 2019a and b).

In the next step, it seems to be necessary to make the ethanolic extract of marshmallow or its main antifungal components as commercial products with suitable formulation to be practically applied in plant protection. Marshmallow extract and its major ingredients might be used by foliar application to protect plant tissues against the pathogen or decrease the disease development. Furthermore, these natural products could be applied as a component of the wax that is frequently used on the fruits belonging to the genus *Citrus*.

In conclusion, the present study showed that ethanolic leaf extract of marshmallow has strong potential for controlling *Alternaria* leaf spot disease. Many synthetic chemical fungicides have negative side effects, including environmental pollution, damage to human and animal health and beneficial microorganisms, and loss of efficacy due to increased pathogen resistance to fungicides. Therefore, application of natural antimicrobials can improve food safety, which leads to improved environment and human health. Thus, the ethanolic leaf extract of marshmallow can be used as a safe alternative to reduce application of hazardous synthetic fungicides and can be recommended as a protectant coating for citrus fruits and integrated with other disease management strategies.

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Declarations

Ethical statement This manuscript complies to the ethical rules applicable for this journal.

Conflict **of interest** The authors have no conflict of interest to declare.

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