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Research article

Isolation, characterization and control of *Botrytis* spp. pathogenic on strawberry in Iran

Fatemeh Maghsoodi, Parissa Taheri *, Saeed Tarighi

Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

This study was performed to identify *Botrytis* species pathogenic on strawberry and investigate effect of *Ferula gummosa* essential oil (EO) against *Botrytis* spp. The infected plant samples were collected in Khorasan Razavi province from strawberry fruits with gray mold disease symptoms. Following purification of the fungi, of the 54 isolates, 53 isolates were identified as *B. cinerea* and 1 isolate belonged to *B. pelargonii* based on morphological and molecular (ITS and RPB2 sequences) identification. The EO obtained from *F. gummosa* at 125, 250, 500, 750 and 1000 μg mL^{-1} concentrations showed significant antifungal effect on mycelial growth of *B. cinerea* and *B. pelargonii* in a dose dependent manner. Also, the EO at all concentrations tested strongly inhibited spore germination of *B. cinerea* and *B. pelargonii.* Treatment with the EO at EC50 concentration significantly reduced the sclerotia production of *B. cinerea* and B. *pelargonii*. Light and electron microscopy observations showed that *F. gummosa* EO at EC50 concentration caused morphological changes in the fungal structures. This EO reduced the activity of cell wall degrading enzymes, such as cellulase and pectinase produced by both fungal species. A total of 22 compounds were identified in the EO by gas chromatography-mass spectrometry. The major compounds of *F. gummosa* EO were β-Pinene (% 37.7), γ-Terpinene (% 21) and a-pinene (% 12). Moreover, the *F. gummosa* EO at 500, 750 and 1000 μg mL^{-1} concentrations considerably reduced the disease severity and infection of strawberry fruits by *B. cinerea* and *B. pelargonii.* According to the results of this study, inhibitory effect of *F. gummosa* EO was impressive in controlling strawberry postharvest gray mold disease. This is the first report on inhibitory ability of *F. gummosa* EO against strawberry postharvest gray mold disease, which can be suggested as a preserver coating for the fruits to extend their shelf life during storage period.

1. Introduction

Strawberry (*Fragaria ananassa* Duch.) is an important soft fruit that is popular all over the world, known for its high nutritional value, soothing fragrance, delicious taste and quick return to growers [1]. But ripe strawberry fruits are very sensitive to storage due to their physical characteristics and high respiratory rate [2]. *Botrytis* is a necrotrophic fungus with a wide range of hosts that is economically important, worldwide [3]. Symptoms of the disease vary, but the most common symptoms are soft rots and water-soaked parenchyma tissues, followed by gray masses of conidia. These signs cause the fruits to decay and reduce their marketing [2].

Previously, classification of *Botrytis* was mainly relied on cultural and morphological characteristics coupled with host specificity

* Corresponding author.
E-mail address: p-taheri@um.ac.ir (P. Taheri).

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[4–6]. Features such as colony color, conidium size, and sclerotial shape and size are useful in distinguishing some species, but many species are morphologically identical, and culture conditions can significantly affect these characters [5–7].

Morphological observations can now be complemented with molecular characteristic [8]. Internal transcribed spacer (ITS) sequence is utilized for genetic identification and determination of phylogenetic relationships [9]. However, although the ITS region is useful for identifying other fungal species, it has limited value in recognizing *Botrytis* species due to its fails to sufficiently define species placement [10]. Therefore, conserved genes, such as Heat-shock protein 60 (HSP60), DNA – dependent RNA polymerase subunit II (RPB2) and glyceraldehyde-3- phosphate dehydrogenase (G3PDH), have been used for differentiation between *Botrytis* species [11,8].

The generalist species of this genus is *Botrytis cinerea*, a highly destructive postharvest pathogenic fungus with a wide host range [6]. According to Dean et al. (2012), *B. cinerea* is ranked as the second most significant plant pathogen globally [8]. This fungal pathogen can infect various fruits and vegetables [7,8,12–16] and many other plants, with unprecedented economic consequences. Plant pathogens, such as *B. cinerea*, invade plant cell walls to colonize tissues by producing various cell wall degrading enzymes (CWDEs) like pectinase, cellulase, and laccases, which help degradation the cell wall and utilize it for nutrients [17].

Currently, the main strategy for control of gray mold is using synthetic chemical fungicides, such as iprodione and carbendazim [14]. However, the persistent use of synthetic fungicides can lead to the emergence of resistant fungal strains and may occasionally be hazardous to humans, which limits their use in managing postharvest diseases [18]. Therefore, alternative natural products should be developed for preserving postharvest quality and extend the shelf life of strawberries. For this purpose, natural substances such as essential oils and plant extracts can be utilized in plant protection against pathogens.

Essential oils (EOs) are a mixture of plant products and volatile bioactive materials that are mainly composed of terpenes, sesquiterpenes and oxygenated compounds including alcohols, aldehydes, and phenols [19–21]. The antifungal activity of the EOs is related to the existence of these compounds and the main antifungal mechanisms of EOs include cytoplasmic membrane destruction and damaging to DNA and mitochondria [22]. Different studies have investigated antifungal activity of many plant substance particularly plant EOs and their constituents [2,19,22–30] as a safe strategy and ecofriendly approaches to control *B. cinerea*.

Ferula gummosa, known as Barijeh in Persian, is a pharmaceutical plant belongs to the Apiaceae family [31]. Its stem and root produce a milky latex or oleo-gum-resin called galbanum, which can be released naturally or manually [32]. Galbanum consists of essential oil, resins, and gums, and is recognized for its antimicrobial and anti-inflammatory properties [32]. Pharmacological studies have confirmed the antimicrobial efficacy of EOs from various parts of *F. gummosa*. For example, its EO revealed strong activity against several bacteria [33,34] and some plant pathogenic fungi, including *B. cinerea*, *Colletotrichum gloeosporioides*, and *Aspergillus niger* [35]. To the best of our knowledge, there are no reports about the inhibitory effect of this EO on gray mold disease of strawberries under *in vitro* or *in vivo* conditions, despite being a promising control strategy considering its antimicrobial potential. So, according to the importance of *Botrytis* spp. fungus as a major destructive postharvest pathogen of strawberry and advantage of finding a safe and novel strategy like *F. gummosa* EO for controlling gray mold disease and preserve strawberries fruits during storage, the study was conducted. The objectives of this study were (I) morphologically and molecularly identify *Botrytis* spp. isolated from naturally infected strawberry fruits, (II) to assess the antifungal effects of the EO obtained from *F. gummosa* on the mycelial growth, spore germination, germ tube elongation, and sclerotia production of *Botrytis* spp. pathogenic on strawberry fruits, (III) to evaluate the potency of very low concentration of the EO (without affecting the fungal growth) in reducing the activity of CWDEs of fungal pathogens (IV) to determine the effect of this EO in controlling the diseases caused by *B. cinerea* and *B. pelargonii* on strawberry fruits, and (V) to detect constituents of the EO via GC-Mass analysis.

2. Materials and methods

2.1. Fungal isolate collection

During 2022 to 2023, diseased strawberry fruits with typical gray mold symptoms were collected from strawberry storage facilities in Khorasan Razavi province, in the northeast of Iran. For pathogen isolation, the infected fruits were immersed in 0.1 % sodium hypochlorite (NaOCl) for 1 min, rinsed three times with sterilized distilled water, and allowed to air dry. Then, parts of the diseased samples were placed on potato-dextrose-agar (PDA) petri plates and incubated at 20 °C in the dark for 14 d. Finally, the hyphae with similar cultural morphology were cut from the edge of fungal colonies, transferred to a new PDA plate and purified by single-spore culture method [36]. These fungi were preserved on PDA medium for short-term and lyophilized filter papers method was used for long-term storage.

2.2. Morphological identification of Botrytis spp

Purified isolates of *Botrytis* spp. were cultured on potato dextrose agar (PDA) medium and features such as colony color, presence or absence of sclerotia, sclerotia form and size, conidiophores, conidium size and shape were used for morphological identification [5–7]. The fungal isolates were examined under an Olympus microscope (Bh2, Tokyo, japan) to observe characteristics of conidiophores and conidia.

2.3. DNA extraction and molecular identification of the fungi

One isolate of each species was selected for molecular identification. The genomic DNA of representative isolates was extracted from the fungal mycelia cultured in the Potato Dextrose Broth (PDB) medium for 10 d after incubation at 20 °C using Genomic DNA

isolation kit IV (DENA Zist Asia, Iran) according to the manufacturer's instructions. The ribosomal DNA - internal transcribed spacer (rDNA-ITS) region and the conserved gene RNA polymerase II (RPB2), which are commonly used for species differentiation in *Botrytis* genus, were amplified by polymerase chain reaction (PCR) using primer set ITS1/ITS4 [9], and RPB2-f/RPB2r [11]. The PCR reaction was prepared in a 25 μL volume, containing 8.5 μL of sterile ddH_2O, 12.5 μL of PCR Master Mix (AMPLIQON, Denmark), 1 μL of 10 pM each of primers and 2 μL of template DNA (50 ng). The PCR amplification programs were the same described by Ref. [37]. The PCR products were visualized by electrophoresis on a 2 % agarose gel and sequenced by Macrogen (Seoul, South Korea). Sequences were analyzed using the basic local alignment search tool (BLAST) in the National Center for Biotechnology Information (NCBI). The MEGA7.0 software and neighbor-joining method were used to construct the phylogenetic tree with bootstrap values of more than 70 %.

2.4. Pathogenicity test

To confirm pathogenicity of the fungal isolates, on the ripe strawberry fruits, initially single spore isolates of *Botrytis* spp. grown on PDA for 10 d at 20 °C were prepared. Then, conidial suspension of each isolate was adjusted to 1×10^6 conidia/mL and inoculation of the surface-sterilized healthy fruits was done by spraying 5 μL drop of the conidial suspensions. For the control, fruits were inoculated with sterilized distilled water. Finally, the fruits were placed on moist paper towels in plastic box for 7 d at 20 °C and relative humidity (RH) of more than 90 %. One box contained up to six inoculated strawberries and constituted three replicates. Disease severity was determined using the scores described by Bestfleisch et al. [38],: 0 (no symptoms), 1 (symptoms <10 %), 2 (symptoms 11%–25 %), 3 (fruit rot 26%–50 %) and 4 (full fruit rot >50 %). Disease index (DI) was calculated using the formula described by Taheri and Tarighi [39].

2.5. Plant material and extraction of the EO

For the EO extraction, galbanum of *F. gummosa* was collected from Mashhad, Khorasan-Razavi province, Iran. Identification of the samples was confirmed by botanical herbarium of Ferdowsi University of Mashhad. After authenticating the specimens, 200 g of each were air dried at room temperature and hydrodistillation was done using a Clevenger-type apparatus for 4 h. The isolated oil was dried with anhydrous sodium sulfate and kept in sterile dark vials at 4 °C, until used.

2.6. Antifungal activity of the EO

Antifungal activity of the EO obtained from *F. gummosa* was evaluated by the agar dilution method [12]. In this method, final 125, 250, 500, 750 and 1000 μg mL^{-1} concentrations of the EO were prepared and mixed with autoclaved PDA medium. Then, each mixture was added to a 6 cm diameter sterile plates and solidification at room temperature. Mycelial plugs (5 mm diameter) were cut from the margins of *B. cinerea* and *B. pelargonii* cultures and placed in the center of each petri plate. The PDA plates without the EO were used as control. All tested petri dishes were incubated at 20 °C for 3 d. Three replicate plates were used for each treatment and the experiment was performed three times. After incubation time, the percentage of inhibiting radial growth was measured according to the following equation I (%) = $[(C - T/C)] \times 100$, which C and T represent the radial growth (mm) in control and treatment respectively [21].

2.7. Determining minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and effective concentration for 50 % of maximal inhibitory effect (EC50)

The agar dilution method was used as previously described to determine the MIC values. Different concentrations of the EO were prepared, mixed with PDA medium and supplemented by mycelial discs of *B. cinerea* MDS55 and *B. pelargonii* MDS19 isolates. Then, all plates were incubated at 20 °C for 48 h. Three replicate plates were used for each concentration. The MIC was defined as the lowest concentration of the EO, that was capable of completely inhibiting the fungal growth [40]. For determining the MFC values, fungal colonies at concentrations above the MIC values were transferred into the fresh PDA medium and incubated for 72 h. The lowest concentration of the EO with no colony growth after subculturing was defined as the MFC values [40]. The EC50 (effective concentration for 50 % of maximal inhibitory effect) values were determined according to the relations between concentrations of the EO and radial mycelial growth [41].

2.8. Effect of the EO of F. gummosa on spore germination and germ tube elongation

Investigating inhibitory effect of the EO obtained from *F. gummosa* on spore germination of *B. cinerea* and *B. pelargonii* was performed using an assay described by Soylu et al., [23]. The PDA media were amended with 125, 250, 500, 750 and 1000 μg mL^{-1} concentrations of the EO. Then, for each treatment, an aliquot of 50 μL of *B. cinerea* and *B. pelargonii* spore suspension adjusted to 10^4 conidia mL^{-1}, (calculated using a hemocytometer) was spread on to the plates and incubated at 20 °C for 10–12 h. The PDA plates without the EO were used as control. A spore was considered as germinated when the length of its germ tube was exceeded than its smallest diameter. There were three replicates for each treatment and in each replicate approximately 100 spores were examined under Olympus microscope (BH2, Tokyo, Japon). The total spore germination percentage was calculated using the following formula [42]: Total germination rate (%) = (*Germinated spores/Total spores*) × 100.

2.9. Effect of the EO on sclerotia production and germination rate

Lowering of the temperature increases sclerotial formation and reduces sporulation in *Botrytis* genus [43]. So, in this test we used a lower temperature. Effect of the EO obtained from *F. gummosa* on sclerotia production and germination of *Botrytis* isolates were evaluated. Berifly, each mycelial plugs (5 mm diameter) of *B. cinerea* and *B. pelargonii*, were added to PDA media treated with the EC50 concentration of the *F. gummosa* EO and incubated at 8 °C for 30 d. Then, the sclerotia produced on each PDA plate were counted [44].

Sclerotia germination was assessed by transferring ten sclerotia from each PDA plate supplemented with the EO to a fresh PDA plate without addition of the EO and the mycelial growth from the sclerotia medium was investigated after 3 d incubation at 20 °C [45]. Nontreated PDA was used as the control in each of the experiments. Each treatment had three replicates and the whole of experiment was performed three times.

2.10. Light microscopic analyses

The inhibitory effect of *F. gummosa* EO at the EC50 concentration on the hyphal structures of *B. cinerea* and *B. pelargonii* was investigated under a light microscope. For this poupous, fungal samples treated with and without the EO as control were prepared and incubated at 20 °C. After 3 d, a thin plug (at least three blocks of 5 mm diameter for each treatment) of each of fungal colony was prepared, and morphological changes of the hyphae were observed.

2.11. Scanning electron microscopy analyses (SEM)

For the SEM analysis, the 5 mm diameter fungal blocks (at least three blocks for each treatment) were prepared as described previously. The samples were then pre-fixed in 3 % glutaraldehyde in 0.1 M phosphate buffer saline (pH 7.2) for 2 h at room temperature, washed twice with 0.1 M phosphate buffer saline and then refixed in 1 % osmium tetroxide in the same buffer for 2 h at room temperature. Subsequently, the samples were dehydrated using various dilutions of ethanol ranging from 10 to 90 % and finally in absolute ethanol [46]. After dehydration, the samples were air dried, placed on stubs and covered with a thin layer of gold using a sputter coater. Finally, the samples were observed individually and photographed by an electron microscope at accelerating voltage of 18 and 20 kV (LEO - Germany, vp 1450).

2.12. Qualitative enzyme assays

Evaluation of the inhibitory effect of *F. gummosa* EO at 0.1 EC50 concentration (that had no effect on the fungal growth) on cellulase, amylase, protease and pectinase activity of *B. cinerea* and *B. pelargonii* was performed by agar plate assay. First, specific medium of each enzyme was prepared, supplemented with the EO and transferred to petri dishes. Then, 5 mm mycelial plugs of each isolate were placed in petri dishes and incubated at 20 °C for 3–5 d. The petri dishes without the EO were used as control. The average diameter of the halo zones in three replicates from three independent assays was used to determine the enzymatic activity.

Details of each enzyme assay are described below:

Cellulase activity was measured according to the method described by Lingappa and Lockwood [47]. After incubation time, cellulase medium was amended with 0.2 % Congo red and destained with 1 m NaCl solution. Visible yellow halo around the fungal colonies revealed cellulase activity.

Amylase activity was measured following the method of [48]. At the end of incubation, the surface of amylase assay media was flooded with 1 % iodine in 2 % potassium iodide. Halo around the fungal colonies showed amylase activity.

Protease activity was determined by growing the fungal isolates on skim milk medium described according by Ref. [49]. After incubation time, the clear halo formed around the fungal colonies indicated proteas activity.

Pectinase activity was evaluated using recipe define by Ref. [50]. After incubation time, the pectinase assay media were supplemented with 1 % iodine in 2 % potassium iodide. Development of hyaline halos around the colonies were recorded as enzyme production.

2.13. Quantitative assay of cellulase and pectinase activities

Quantitative assessments of cellulase and pectinase produced by *B. cinerea* and *B. pelargonii* in the presence of the EO obtained from *F. gummosa* were performed spectrophotometrically during 10 d after incubation. The specific medium of each enzyme was prepared, supplemented with *F. gummosa* EO at 0.1 EC50 concentration without any effect on the fungal growth and inoculated with 5 mm diameter mycelial plug from fresh cultures of each fugal isolate. After inoculation, all flasks were incubated at 20 °C for 10 d. The specific medium of each enzyme without the EO was used as control. Three replicates were used for each treatment and each experiment was done three times.

The specific medium, containing the substance described by Ref. [51] was used for investigating cellulase activity. After incubation time, 0.5 mL from the surface of centrifuged crud enzyme extract was mixed with 1 mL of 0.7 % carboxymethyl cellulose (CMC) in 0.05 % mol L^{-1} sodium acetate buffer with pH = 4.8 and incubated for 60 min at temperature of 37 °C. Then, reaction was stopped by adding 2 mL of 3,5 dinitrosalicylic acid (DNS) to each reaction mixture and boiling at 100 °C for 5 min. After cooling the mixture, 40 % sodium potassium tartrate was added, and absorbance of the sample was measured at 550 nm [17]. The standard curve of glucose was used. For determination of the amount of reduced sugar released, one unit of cellulase activity was defined as the amount of enzyme

that catalyzes 1.0 μ mol of glucose per min during the hydrolysis reaction [52].

For pectinase activity, each of the fungal isolates were grown on specific culture medium of pectinase with f recipe described by Ref. [53]. After incubation time, the medium was centrifuged and 1 mL from the surface of crud enzyme extract was added to 1 mL 1 % pectin in 0.1 mol L^{-1} with pH = 6 sodium acetate buffer and incubated at 40 °C for 10 min. Then, for stopping the reaction, 2 mL of DNS was added and boiled for 10 min at 100 °C. The samples were cooled at room temperature and absorbance of each sample was measured at 540 nm. The measurement of pectinase activity was based on the amount of reducing sugar (D - galacturonic acid) released in the culture supernatant. The amount of D-galacturonic acid was determined using DNS colorimetric method of Colowich [54].

2.14. *In vivo efficacy of the EO on the disease control*

Protective and curative effects of the EO obtained from *F. gummosa* were investigated in controlling gray mold disease on artificially inoculated strawberry fruits at concentrations that showed higher inhibitory effect *in vitro* assay. Protective effect determination was done as described by Zamani – Zadeh et al. [55] with some modifications. Initially, fresh and healthy strawberry fruits were sterilized using 0.1 % sodium hypochlorite (NaOCL) for 1 min and washed three times with sterilized distilled water. Then, strawberry fruits were wounded by using a medicinal syringe and immersed in *F. gummosa* EO at each of 500, 750 and 1000 μg mL^{-1} concentrations. After the liquid was dried, 20 μL of conidial suspensions equal to 1×10^6 conidia mL^{-1} was sprayed onto the surface area of fruits. Then, the fruits were placed in sterile plastic box with humidity over 85 % and kept at 20 °C temperature for 10 d.

Curative assay was conducted by Xu et al. [56] method with some modifications. Initially, fruits were surface-sterilized and wounded as described above. Then, conidial suspensions equal to 1×10^6 conidia mL^{-1} was sprayed onto the surface area of fruits. Inoculated fruits were kept in a covered plastic box for 12 h prior to the treatment. Then, the fruits were immersed in *F. gummosa* EO at mentioned concentrations and left at ambient temperature until the surfaces were air-dried. Finally, all fruits were kept in humidified plastic boxes at 20 °C for 10 d. In both protective and curative assay, controls were immersed in sterile distilled water. Treatments and

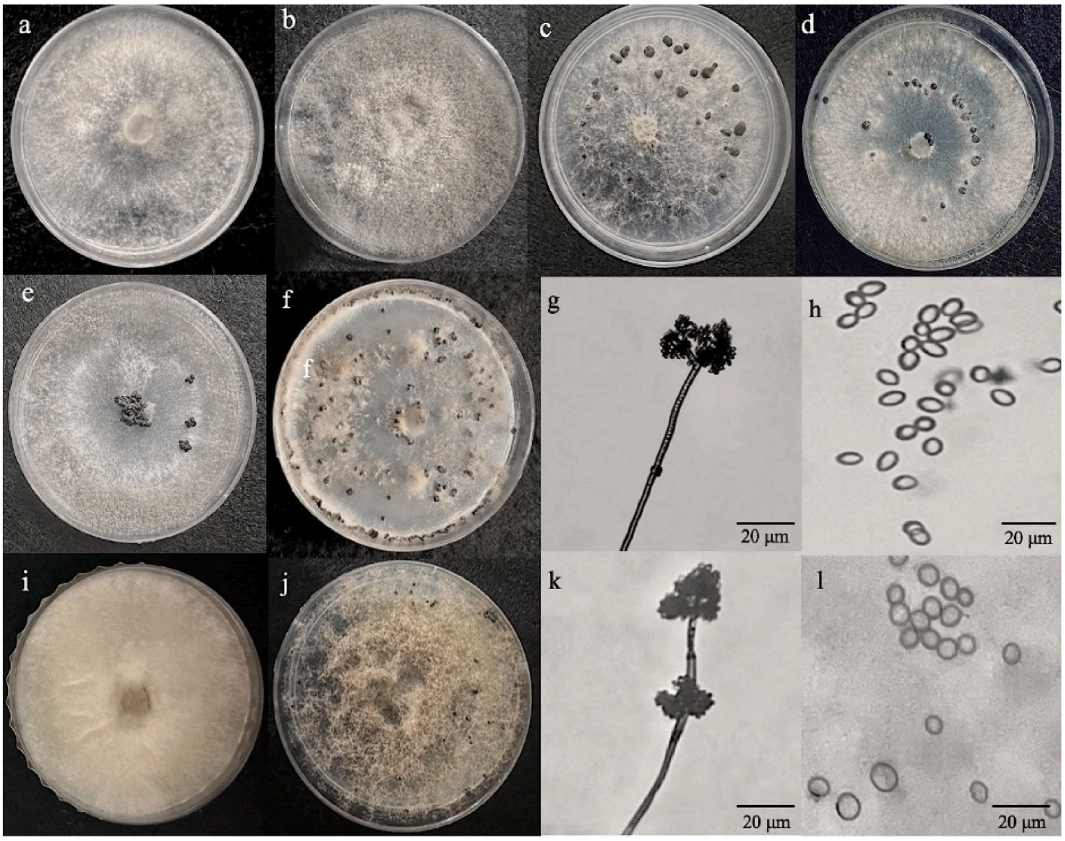

Fig. 1. Morphological characteristics of *Botrytis* spp. isolates obtained from strawberry fruits. Colony cultured of *Botrytis cinerea* on potato dextrose agar (PDA) medium after 5 days incubation at 20 °C (a), conidial (b) and conidial-sclerotial (c–f) morphological type of the *B. cinerea* isolates after 7–14 days incubation at 20 °C, Pattern of sclerotia formation of *B. cinerea* on PDA after 7–14 days were irregular form (c), on concentric rings (d), centrally placed large sclerotia (e), in the edges of the plates (f), Conidiophore (g) and Conidial morphology (h), Colony cultured of *Botrytis pelargonii* on PDA medium after 5 days incubation at 20 °C (i), Sporulation and formed sclerotia of *B. pelargonii* on PDA medium after 7–14 days incubation at 20 °C (j), Conidiophore (k) and Conidial morphology (l) of *B. pelargonii.*

controls were replicated three times, using six fruits by box and one box per repeat.

After 10 d incubation, the disease severity was determined using the previously mentioned method and percentage of inhibitory effect was determined using the formula: Inhibition rate (%) = $[(C - T/C)] \times 100$

where C and T represent the mean disease index in the control, and main disease index in relevant treatment, respectively [42].

2.15. Gas chromatography-mass spectrometry analysis

The GC–MS analysis of the EO obtained from *F. gummosa* was performed by using Agilent 7890A gas chromatograph (GC) connected with an Agilent 5977A mass spectroscopy detector with electron ionization energy of 70 eV. The gas chromatograph was equipped with a HP-5MS capillary column (30 m × 0.25 μm × 0.25 μm). Helium served as the carrier gas, maintaining with a constant flow rate of 1 mL min^{-1} and with an injection volume of 0.4 μL. The column temperature was set at 70 °C for 10 min and then gradually raised to 200 °C for 3 min, the injector temperature was set at 250 °C [57]. The total run time of the GC was 41.5 min. The relative percentage amount of each component was computed by comparing its average peak area to the total peak areas. The components were identified by comparing their mass fraction patterns with those stored in the spectrometer database using the National Institute of Standards and Technology (NIST) library.

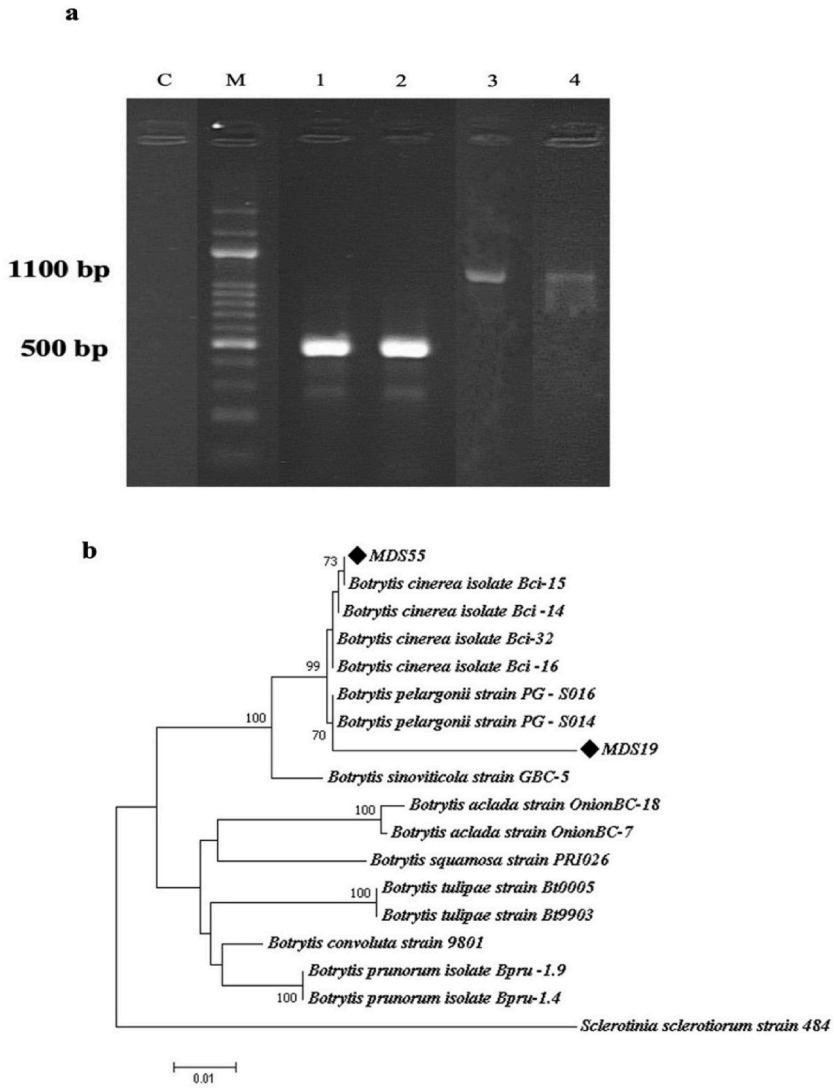

Fig. 2. PCR amplification products of the ITS and RPB2 gene of *Botrytis* spp. obtained from strawberry. Lane C: control sample; Lane M, molecular marker; Lane 1 and 2: amplify the ITS region of the *Botrytis cinerea* and *Botrytis pelargonii* respectively; 3 and 4: amplify the RPB2 gene of the *B. cinerea* and *B. pelargonii* respectively (a), The unadjusted version of this figure is provided as a supplementary file. Phylogenetic tree was constructed based on the ITS and RPB2 sequence of representative isolates MDS55 (*B. cinerea*) and MDS19 (*B. pelargonii)* (b), using the neighbor-joining method in the MEGA 7. With Bootstrap values ≥ 70 from 1000 replicates.

2.16. Statistical analysis

Statistical analysis was performed to determine differences between the treatments using SPSS software program (Ver. 12). The average comparisons were done using Duncan test at $P < 0.05$. Values in figures are the means of data and the bars indicate standard deviations. Each experiment was carried out using completely randomized design in triplicate, with three independent repetitions for each experiment.

Table 1
Disease index (DI) of *Botrytis* spp. isolates obtained from Mashhad, Khorassan-Razavi province, located in the northeast of Iran.

Number	Species	Isolate code	Region	Disease Index (DI)
1	*B. cinerea*	MDS1	Mashhad	72.91 ± 2.94 cd
2	*B. cinerea*	MDS35	Mashhad	93.74 ± 2.48 a
3	*B. cinerea*	MDS15	Mashhad	89.58 ± 1.15 ab
4	*B. cinerea*	MDS9	Mashhad	64.58 ± 2.15 d
5	*B. cinerea*	MDS7	Mashhad	81.25 ± 4.41 bc
6	*B. cinerea*	MDS3	Mashhad	64.58 ± 2.15 d
7	*B. cinerea*	MDS6	Mashhad	72.91 ± 2.94 cd
8	*B. cinerea*	MDS2	Mashhad	68.74 ± 3.1 d
9	*B. cinerea*	MDS17	Mashhad	81.25 ± 4.41 bc
10	*B. cinerea*	MDS25	Mashhad	89.58 ± 1.15 ab
11	*B. cinerea*	MDS21	Mashhad	89.58 ± 1.15 ab
12	*B. cinerea*	MDS41	Mashhad	64.58 ± 2.15 d
13	*B. cinerea*	MDS12	Mashhad	91.66 ± 2.94 ab
14	*B. cinerea*	MDS26	Mashhad	81.25 ± 4.41 bc
15	*B. cinerea*	MDS55	Mashhad	91.66 ± 2.94 ab
16	*B. cinerea*	MDS34	Mashhad	91.66 ± 2.94 ab
17	*B. cinerea*	MDS42	Mashhad	72.91 ± 2.94 cd
18	*B. cinerea*	MDS18	Mashhad	81.25 ± 4.41 bc
19	*B. cinerea*	MDS33	Mashhad	89.58 ± 1.15 ab
20	*B. cinerea*	MDS13	Mashhad	64.58 ± 2.15 d
21	*B. cinerea*	MDS29	Mashhad	93.74 ± 2.48 a
22	*B. cinerea*	MDS46	Mashhad	81.25 ± 4.41 bc
23	*B. pelargonii*	MDS19	Mashhad	89.58 ± 1.15 ab
24	*B. cinerea*	SB39	Sabzevar	68.74 ± 3.1 d
25	*B. cinerea*	SB5	Sabzevar	64.58 ± 2.15 d
26	*B. cinerea*	SB22	Sabzevar	54.16 ± 3.5 e
27	*B. cinerea*	SB38	Sabzevar	54.16 ± 3.5 e
28	*B. cinerea*	SB4	Sabzevar	68.74 ± 3.1 d
29	*B. cinerea*	SB14	Sabzevar	72.91 ± 2.94 cd
30	*B. cinerea*	SB26	Sabzevar	64.58 ± 2.15 d
31	*B. cinerea*	CHT56	Chenaran	93.74 ± 2.48 a
32	*B. cinerea*	CHT66	Chenaran	68.74 ± 3.1 d
33	*B. cinerea*	CHT52	Chenaran	81.25 ± 4.41 bc
34	*B. cinerea*	CHT45	Chenaran	89.58 ± 1.15 ab
35	*B. cinerea*	CHT58	Chenaran	93.74 ± 2.48 a
36	*B. cinerea*	CHT62	Chenaran	72.91 ± 2.94 cd
37	*B. cinerea*	CHT63	Chenaran	72.91 ± 2.94 cd
38	*B. cinerea*	CHT54	Chenaran	81.25 ± 4.41 bc
39	*B. cinerea*	CHT57	20aran	72.91 ± 2.94 cd
40	*B. cinerea*	CHT28	Chenaran	81.25 ± 4.41 bc
41	*B. cinerea*	CHT36	Chenaran	89.58 ± 1.15 ab
42	*B. cinerea*	CHT48	Chenaran	81.25 ± 4.41 bc
43	*B. cinerea*	CHT18	Chenaran	68.74 ± 3.1 d
44	*B. cinerea*	CHT8	Chenaran	89.58 ± 1.15 ab
45	*B. cinerea*	CHT53	Chenaran	68.74 ± 3.1 d
46	*B. cinerea*	NNP69	Neyshabur	91.66 ± 2.94 ab
47	*B. cinerea*	NNP11	Neyshabur	72.91 ± 2.94 cd
48	*B. cinerea*	NNP16	Neyshabur	81.25 ± 4.41 bc
49	*B. cinerea*	NNP74	Neyshabur	72.91 ± 2.94 cd
50	*B. cinerea*	NNP70	Neyshabur	81.25 ± 4.41 bc
51	*B. cinerea*	NNP50	Neyshabur	89.58 ± 1.15 ab
52	*B. cinerea*	NNP21	Neyshabur	68.74 ± 3.1 d
53	*B. cinerea*	NNP24	Neyshabur	72.91 ± 2.94 cd
54	*B. cinerea*	NNP15	Neyshabur	68.74 ± 3.1 d

3. Results

3.1. Morphological identification of the fungal isolates

Fifty-four isolates of *Botrytis* spp. were obtained from symptomatic strawberry fruits. Based on morphological characteristics described by Jarvis [5]. and Mirzaei et al. [7], a total of 53 isolates were identified as *B. cinerea* and 1 isolate detected as *B. pelargonii*. The colonies of *B. cinerea* isolates grew very rapidly on PDA medium during 5 d incubation at 20 °C and produced cottony – like mycelia which were initially white to dirty white (Fig. 1a). After 7–14 d, morphological type of some isolates of *B. cinerea* were conidial type and produced abundant conidia all over the surface of the medium, which caused the colony color seemed darker (Fig. 1b). But, Morphological type of most isolates of *B. cinerea* was conidial-sclerotial type (Fig. 1c–f) and formed black sclerotia with circular or irregular shape and either smooth surface or nodulous, which were firmly attached to the surface of the medium. The type of sclerotia distribution varied in different isolates. Sclerotia scattered in irregular form (Fig. 1c), on concentric rings (Fig. 1d), centrally placed large sclerotia (Fig. 1e) and in the edges of the plates (Fig. 1f). Microscopically, conidiophores were straight, septate and branched at the top (Fig. 1g). Conidia were clustered, single-celled, ovoid or pyriform (Fig. 1h), with average size of 6–15.5 × 5–10.5 µm.

The colony of *B. pelargonii* isolate grew very rapidly on PDA medium, similar to *B. cinerea*. Colony texture was cottony, but the

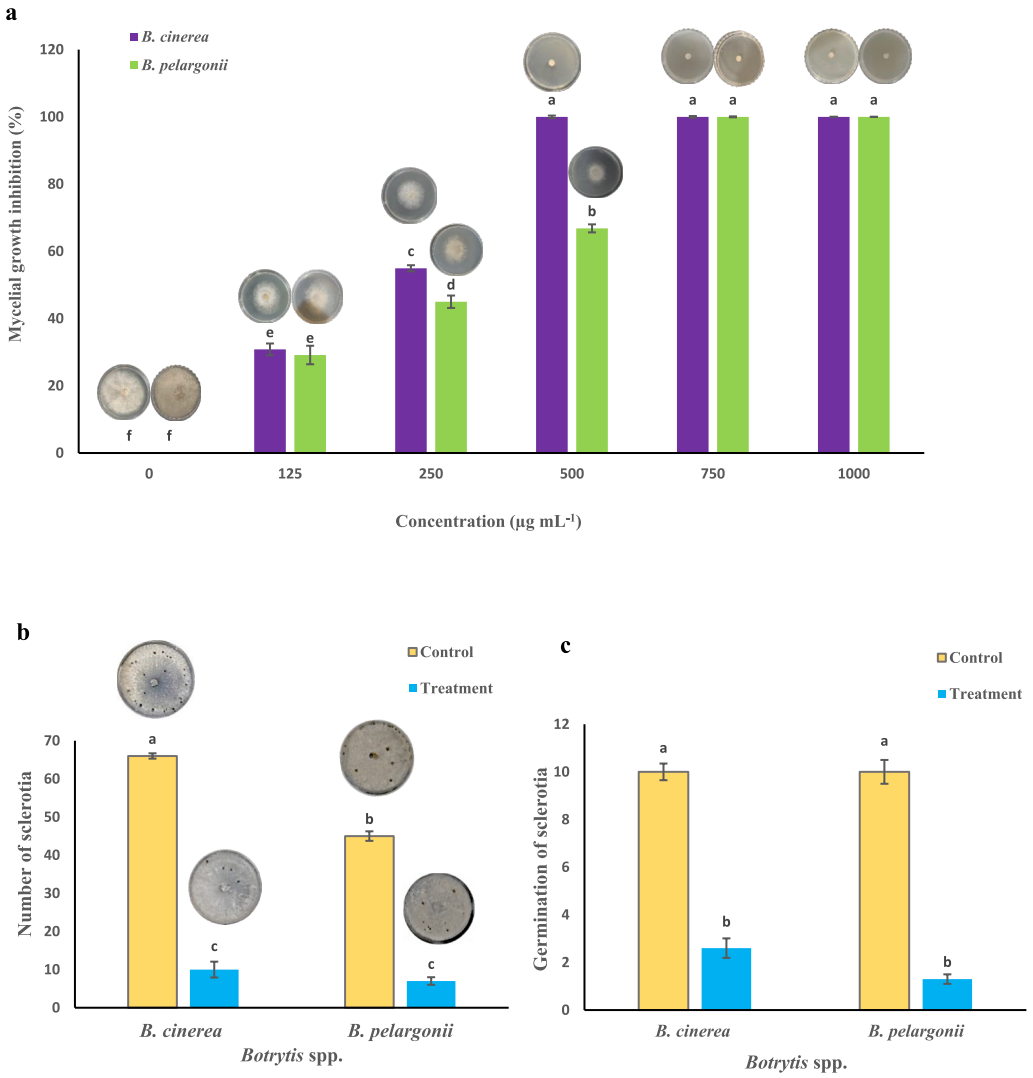

Fig. 3. Antifungal activity of different doses of essential oil (EO) obtained from galbanum of *Ferula gummosa* on mycelial growth of *Botrytis cinerea* and *Botrytis pelargonii* on PDA medium after 3 days of incubation at 20 °C (a), inhibitory effect of *F. gummosa* EO at EC50 concentration on sclerotia production of *B. cinerea* and *B. pelargonii* on PDA medium after 30 days incubation at 8 °C (b) and sclerotia germination of *B. cinerea* and *B. pelargonii* on PDA medium after 3 days incubation at 20 °C (c). The experiments were repeated three times and each treatment had three replications in each repetition. Statistical data analysis was carried out using spss software. Different letters indicate significant differences ($P < 0.05$).

colony color was white to yellowish (Fig. 1i). After 7–14 d, colony of *B. pelargonii* became light brown to gray and generated conidia with black sclerotia all over the medium with age (Fig. 1j). The isolate of *B. pelargonii* was also microscopically similar to *B. cinerea* isolates with direct, and branched conidiophores (Fig. 1k), but the conidia of this species were slightly bigger than *B. cinerea* (Fig. 1l). Average diameter of conidia was in the range of 8.5–22.5 × 5.5–15 μm.

3.2. Molecular identification

The sequences of ITS region and *RPB2* gene of representative isolates were amplified by PCR and expected fragments about 550 bp and 1100 bp in size were detected, respectively (Fig. 2a). The PCR products were purified and sequenced by Macrogen (Seoul, South Korea). The BLAST search of ITS sequences showed that two selected isolates had 99 % similarity with *B. cinerea* and *B. pelargonii* strains of the GenBank. The sequences were deposited in the GenBank database under accession numbers PP889327 (MDS55) and PP889329 (MDS19). Homology BLAST of *RPB2* sequences was also performed and revealed 99.5 % similarity to *B. cinerea* and *B. pelargonii,* respectively. The sequences of *RPB2* were also deposited in the GenBank database under accession numbers PP898304 (MDS55) and PP898305 (MDS19). The dendrogram showed that the isolate MDS55 clustered at the same branch of reference strains of *B. cinerea* and the MDS19 isolate was placed within a clade including reference strains of *B. pelargonii* with high bootstrap support. The tree that resulted from sequence analysis is presented in Fig. 2b, which is rooted using *Sclerotinia sclerotiorum* as an outgroup.

3.3. Pathogenicity assay

Results of pathogenicity assay revealed successful infection by *Botrytis* spp. isolates on strawberry fruits. The symptoms including soft decay with thin layer of gray-brown conidia were observed and strawberry fruits were severely rotten, and the hyphae covered the surface of fruits after 10 d incubation at 20 °C. The same fungus was re-isolated from the inoculated fruit, fulfilling Koch's postulates. In this test, the highest level of pathogenicity was found for the isolates MDS35, MDS29, CHT56 and CHT58 of *B. cinerea* and the isolates SB38 and SB22 of *B. cinerea* had the lowest level of pathogenicity compared to the other isolates of *B. cinerea* and the isolate of *B. pelargonii* (Table 1).

3.4. In vitro antifungal activity of the EO on mycelial growth of Botrytis spp

Inhibitory effects of the EO obtained from *F. gummosa* at 125, 250, 500, 750 and 1000 μg mL^{-1} concentrations were tested against *B. cinerea* and *B. pelargonii* pathogenic on strawberry. The results of antifungal assay are presented in Fig. 3a. According to the obtained results, *in vitro* mycelial growth of the pathogens was prevented by the EO obtained from *F. gummosa* in a dose dependent manner. Complete inhibition of the mycelial growth of *B. cinerea* and *B. pelargonii* occurred at the concentrations 1000 and 750 μg mL^{-1} of the tested EO. But in the presence of 500 μg mL^{-1} concentration of *F. gummosa* EO, only the mycelial growth of *B. cinerea* was completely inhibited. Also, at concentration 250 μg mL^{-1} of this oil, inhibition of mycelial growth of *B. cinerea* species was higher than the data observed for *B. pelargonii*. while at concentration of 125 μg mL^{-1}, no significant difference was observed in the inhibition rate of growth of *Botrytis* spp. The percentage inhibition of *F. gummosa* EO upon mycelial growth of *B. cinerea* was in the range of 30.66–100 % and the percentage of mycelial growth inhibition for *B. pelargonii* was in the range of 22.16–100 %, respectively.

The MIC and MFC of *F. gummosa* EO are presented in Table 2. The results of this test revealed a correlation between the MIC and MFC values and the fungal isolates. The MIC values of *F. gummosa* EO for *B. cinerea* and *B. pelargonii* were 500 and 750 μg mL^{-1}, respectively. The MFC values were higher than the corresponding MIC and their values were 750 and 1000 μg mL^{-1} for *B. cinerea* and *B. pelargonii,* respectively. The EC50 values of EO were affected by the fungus isolates and were 246.8 μg mL^{-1} for *B. cinerea* and 258.5 μg mL^{-1} for *B. pelargonii*. According to the results the EO of *F. gummosa* exhibited a stronger inhibitory effect against *B. cinerea* compared to *B. pelargonii*, resulting in lower MIC and MFC values for *B. cinerea*.

3.5. Effect of the EO on spore germination and germ tube elongation

Effect of the EO of *F. gummosa* on spore germination and germ tube elongation of *B. cinerea* and *B. pelargonii* was assessed. As presented in Table 3, after 12 h incubation, most of the conidia germinated and produced germ tubes in the control. In contrast, treatment with different concentrations of the *F. gummosa* EO significantly suppressed spore germination and germ tube elongation of *B. cinerea* and *B. pelargonii* compared to the untreated control. Non-germinated conidia of *B. cinerea* were found at 500, 750 and 1000 μg mL^{-1} concentrations of the EO of *F. gummosa*. Moreover, the *F. gummosa* EO at 750 and 1000 μg mL^{-1} concentrations showed complete prevention (100 %) of *B. pelargonii* spore germination after 12 h incubation. In lower concentrations of 500 μg mL^{-1} for

Table 2
Minimum inhibitory concentrations (MIC), minimum fungicidal concentration (MFC) and the effective concentration of essential oil (EO) obtained from *Ferula gummosa* for 50 % of maximal inhibitory effect on the fungal growth of *Botrytis* spp.

Fungi	Botrytis cinerea			Botrytis pelargonii		
Treatment	MIC	MFC	EC50	MIC	MFC	EC50
Ferula gummosa EO (μg mL−1)	500	750	246.8	750	1000	258.5

Table 3
Effect of *Ferula gummosa* essential oil (EO) on spore germination and germ tube elongation of *Botrytis cinerea* and *Botrytis pelargonii* on PDA medium after 10–12 h incubation at 20 °C.

Treatment	Concentration (μg mL^{-1})	*Botrytis cinerea*		*Botrytis pelargonii*	
		Germination (%)	Germ tube length (μm)	Germination (%)	Germ tube length (μm)
Ferula gummosa EO	0	100 ± 0 d	424 ± 3.5 d	100 ± 0 e	446 ± 2.02 e
	125	25 ± 1.75 c	120 ± 1.8 c	33 ± 1 d	142.33 ± 0.57 d
	250	8 ± 1.25 b	48 ± 1 b	15 ± 1.04 c	57 ± 1.4 c
	500	0 a	0 a	4 ± 0.5 b	26 ± 2.31 b
	750	0 a	0 a	0 a	0 a
	1000	0 a	0 a	0 a	0 a

The spores (approximately 100) were randomly observed for germination rate and germ tube length measurement in each treatment. The experiment was conducted three times, with each treatment being replicated three times during trial. Statistical data analysis was carried out using spss (ver. 21) software. Different letters indicate significant differences according to Duncan's multiple range test ($P < 0.05$).

B. cinerea and 750 μg mL^{-1} for *B. pelargonii*, a few spores germinated and formed very short germ tubes. In these concentrations, the percentages of spore germination for *B. cinerea* were from 8 % to 25 %, whereas the spore germination of *B. pelargonii* ranged from 15 % to 33 %.

3.6. Effect of the EO on production and germination of Botrytis spp. sclerotia

The effect of *F. gummosa* EO on sclerotia formation by *B. cinerea* and *B. pelargonii* was investigated. The obtained data are presented in Fig. 3b. Results of this test revealed that treatment with the EO at EC50 concentration significantly decreased the number of sclerotia in *B. cinerea* compared to the control. Similarly, the sclerotia of *B. pelargonii* substantially decreased when amended with the EO at mentioned concentrations.

Moreover, at this level, sclerotia germination of *B. cinerea* and *B. pelargonii* were evaluated after 3 d of incubation at 20 °C. As shown in Fig. 3c, complete absence of sclerotia germination was observed in the EO treated Petri plates of *B. cinerea* and *B. pelargonii*.

3.7. Light microscopy

The hyphal structure of 3 d old *B. cinerea* and *B. pelargonii* grown on PDA containing EC50 concentration of the EO of *F. gummosa* and without it as control were observed by light microscope. The observation revealed that controls had normal and regular morphological structures (Fig. 4a and b). While the samples treated with the EO of *F. gummosa* showed degenerative changes in the hyphal morphology, including hyphal deformation, decrease in the diameter of hypha, cytoplasm granulation and vacuolation (Fig. 4c and d).

3.8. Effect of EO on morphology and ultrastructure of Botrytis spp

The SEM analysis was conducted using 3 d old cultures of *B. cinerea* and *B. pelargonii* grown on PDA with or without the EO of *F. gummosa* at the EC50 concentration. The results of SEM observation were shown in Fig. 4e–h. In control, the mycelia revealed a typical morphology, mostly constant diameter and smooth external surface (Fig. 4e and f). But treatment with the EO led to serious damage to the hyphal morphology. The main alterations induced by the EO were hyphal deformation, which appeared folded, swelling and shriveled with rough surface (Fig. 4g and h).

3.9. Effect of the EO on enzymatic activity of Botrytis spp

3.9.1. Qualitative enzyme assays

Inhibitory effects of the EO of *F. gummosa* on the activity of proteas, cellulase, pectinase and amylase produced by the pathogens were investigated using substrate specific media. The fungal isolates showed CWDEs activity by formation of halo around the colony. According to the results presented in Fig. 5 a to 5 d the activity of cellulase (Fig. 5a), proteas (Fig. 5b) and amylase (Fig. 5c) enzymes in both *B. cinerea* and *B. pelargonii* exposed to the EO significantly decreased. The decreased pectinase activity of *B. cinerea* was observed in presence of the EO, while the EO suppressed pectinase activity in *B. pelargonii* (Fig. 5d).

3.9.2. Quantitative enzyme assays

The activities of cellulase and pectinase enzymes produced by *Botrytis* spp. isolates treated with the EO of *F. gummosa* were investigated spectrophotometrically at 550 nm and 560 nm, respectively. According to the obtained data, in the absence of the EO, both tested isolates were able to produce cellulase and pectinase enzymes at various time point after incubation. The highest degree of cellulase activity was observed at 3 d after incubation and the maximum production of pectinase was discovered at 5 and 7 d after incubation. But the activity of these enzymes in presence of the EO remarkably decreased in both *B. cinerea* and *B. pelargonii* (Fig. 6a–d).

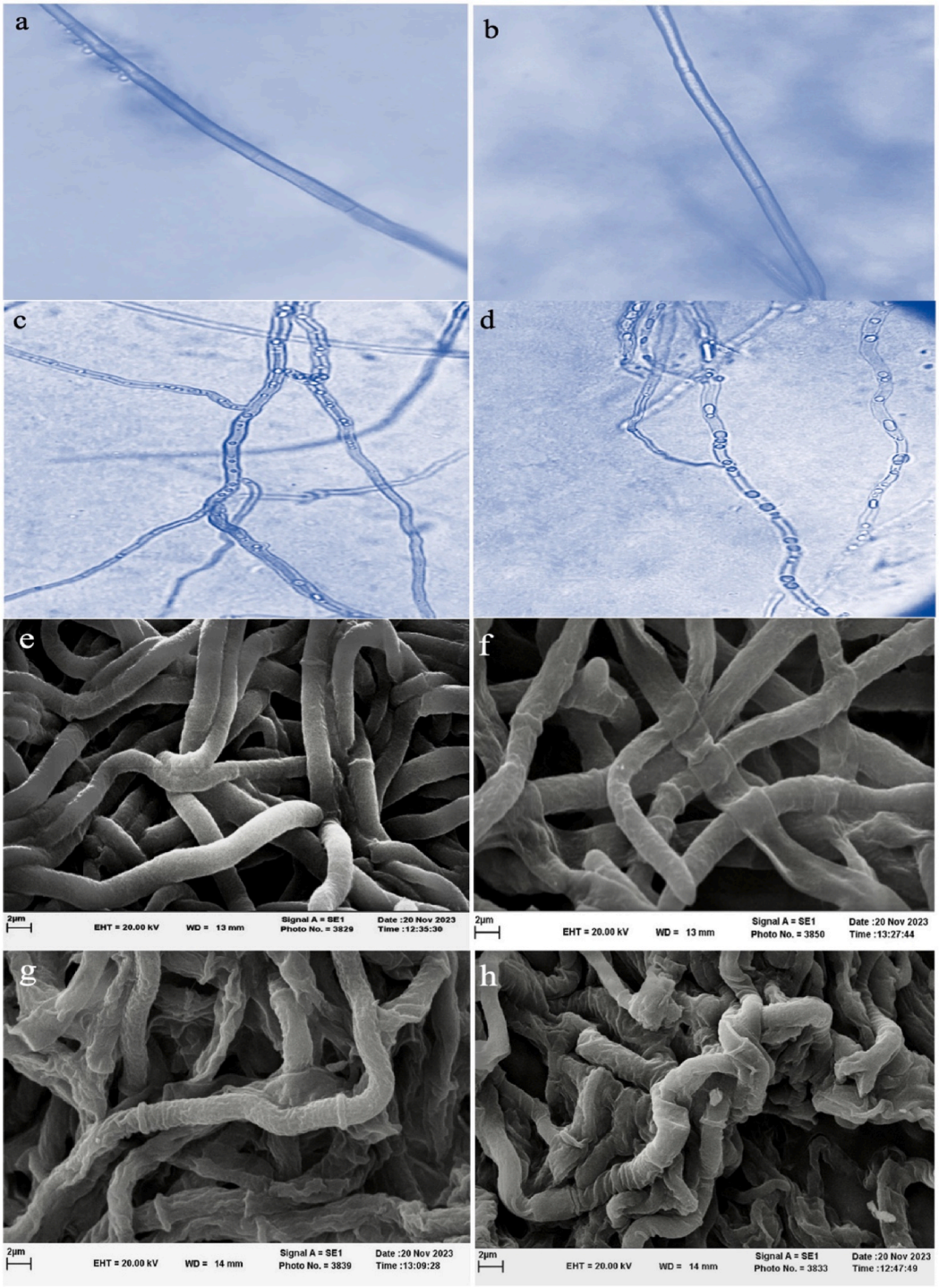

Fig. 4. Effect of *Ferula gummosa* essential oil (EO) at EC50 concentration on hyphal structure of *Botrytis* spp. grown on PDA medium after 3 days incubation at 20 °C. Light microscopy images of *Botrytis cinerea* (a) and *Botrytis pelargonii* (b) hyphae in the control, light microscopy of *B. cinerea*(c) and *B. pelargonii* (d) hyphae treated with the *F. gummosa* EO at EC50 concentration. Scanning electron microscopy (SEM) of *B. cinerea* (e) and *B. pelargonii* (f) mycelium structure in control, the SEM of *B. cinerea* (g) and *B. pelargonii* (h) hyphae containing the FGEO with EC50 concentration.

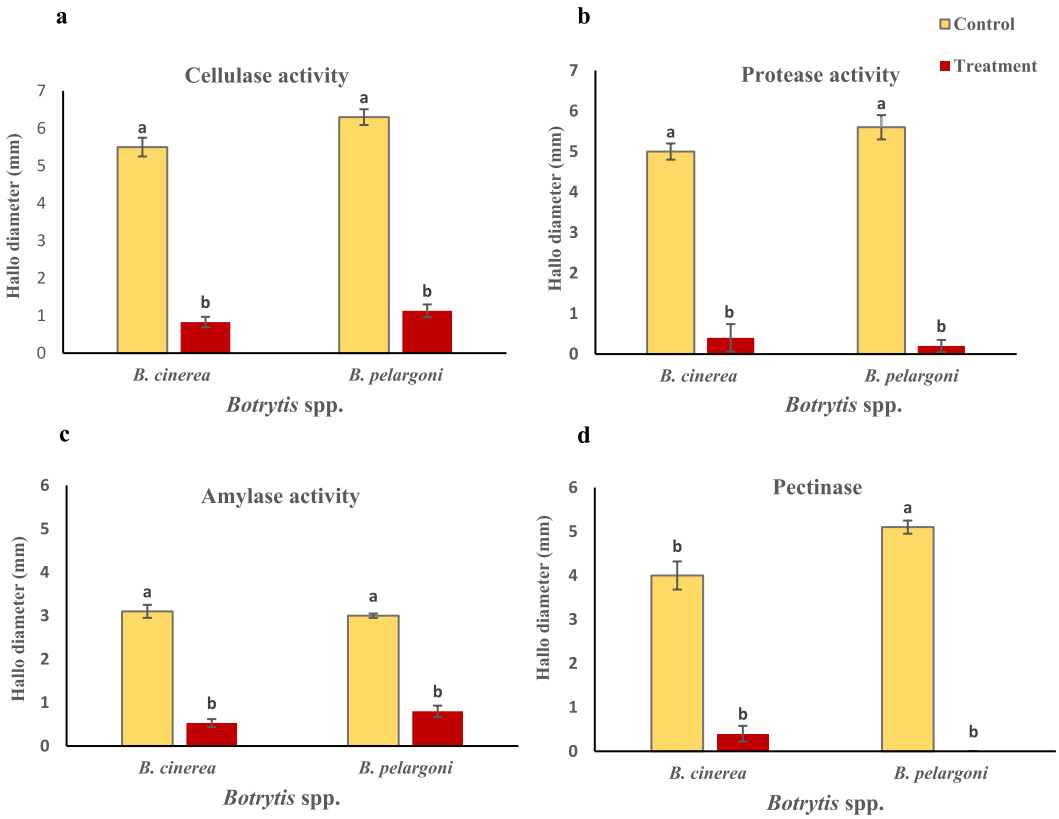

Fig. 5. Effect of essential oil (EO) obtained from *Ferula gummosa* at concentration 0.1 EC50 on cellulase (a), protease (b), amylase(c) and pectinase (d) produced by *Botrytis cinerea* and *Botrytis pelargonii.* The average measurements of halo zones surrounding the fungal colony in three replicates from three independent experiments indicated the secreted enzyme amount. Different letters showed significant differences according to the Duncan test ($P < 0.05$).

3.10. Potential of the EO to reduce gray mold disease on strawberry fruits

Effects of the EO of F. gummosa at 500, 750 and 1000 μg mL^{-1} concentrations were assayed on strawberry gray mold *in vivo*. According to the results presented in Fig. 7a, in inoculated strawberry fruits without the EO as control after 10 d of incubation, the mold grew all over the fruit surface and these fruits showed darker color compared to the treated fruits due to the anthocyanin pigment. In contrast, treatment with different concentrations of the EO obtained from *F. gummosa* showed considerable protective effect and decreased severity of gray mold disease by preventing the growth of *Botrytis* isolates.

In addition, in curative assay a significant decrease in the symptoms of gray mold disease and infection severity occurred in the strawberry fruits coated with the EO compared to the controls. As presented in Fig. 7b and c, in both protective and curative assays, treatment with 1000 μg mL^{-1} concentration of the EO showed the strongest inhibition of symptoms caused by *B. cinerea* and *B. pelargonii.* But, at concentration 500 and 750 μg mL^{-1} the protective effects of this EO were significantly higher than its curative effect. In protective assay, the range of inhibiting gray mold disease caused by *B. cinerea* and *B. pelargonii* were in the range of 38.5–100 % and 43–100 % respectively. And inhibition of postharvest decay of fruits by curative a of different EO concentrations were 27.86–85.14 % for *B. cinerea* and 19.5–78.28 % for *B. pelargonii*, respectively.

3.11. Gas chromatography-mass spectrometry analysis of the EO

The main compounds of the EO obtained from *F. gummosa* galbanum were identified using GC-MS and are presented in Table 4. According to the results, among 22 compounds identified, the major compounds were β-Pinene (37.7), γ-Terpinene (21), a-pinene (12), o-cymene (3.9), α-Phellandrene (3.2) and muurola (2.7). Furthermore, the oil also contained smaller percentage of Carene (1.6), Myrtenol (1.4), isopinocarveol (1), zonarene (0.9), limonen (0.9), ocimen (0.8), sabinol (0.7), and calamenene (0.6).

4. Discussion

In this study, *Botrytis* species were isolated from naturally infected strawberry fruits as causal agents of gray mold disease and identified based on morphological and molecular characteristics. Additionally, the antifungal potency of *F. gummosa* EO was

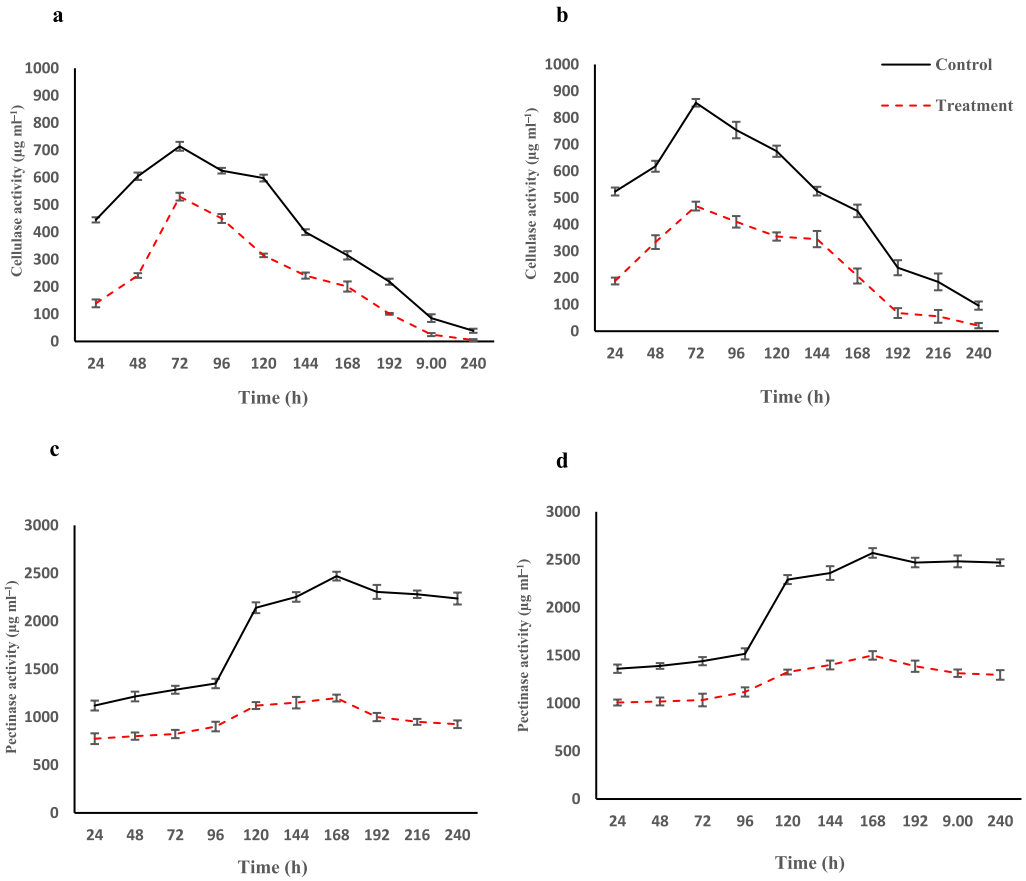

Fig. 6. Quantitative analysis of cellulase activity of *Botrytis cinerea* (a), *Botrytis pelargonii* (b) and pectinase activity of *B. cinerea* (c), *B. pelargonii* (d) with or without *Ferula gummosa* essential oil (EO) at concentration 0.1 EC50. The experiments were conducted three times and each treatment had three replication in each repetition. Different letters indicated significant differences according to Duncan's multiple range test ($P < 0.05$).

investigated to control this postharvest disease. The findings of this study indicate a strong effect of the essential oil of *F. gummosa* in controlling mycelial growth, spore germination, and sclerotia production in *B. cinerea and B. pelargonii*. Additionally, the results showed that this essential oil can significantly reduce the enzymatic activity of the pathogens and prevent the progression of pathogens and spoilage of strawberry fruits. To our knowledge, this is the first report of antifungal activity of *F. gummosa* EO against *Botrytis* spp. *Botrytis cinerea* is one of the most important plant pathogenic fungi globally, that causes gray mold disease in a wide range of plants, with significant economic losses during storage and transportation [58]. In this study, of 54 total *Botrytis* isolated from infected strawberries, 53 isolates were identified as *B. cinerea* and 1 isolate belonged to *B. pelargonii*. The isolates of *B. cinerea* usually produced grayish mycelia and conidia, which became light gray to dark gray later with age as well as black sclerotia. Sporulation was often all over the surface of medium. Conidia were hyaline or pale brown, but in tissue – mass they appeared grayish becoming darker later. Variation in the size of conidia, conidiophores and sclerotia were observed among the isolates. In addition, depending on the isolate, sclerotia were produced in different patterns. These findings were in accordance with previous studies on *B. cinerea* [7,8,37]. The isolate of *B. pelargonii* produced yellow-pigmented colony with numerous sclerotia over the surface. The conidia produced by this species were slightly bigger than those in the *B. cinerea*. This finding was similar to the reports of Mirzaei et al. [7], who isolated this species from different hosts, including strawberry fruits. In addition, our finding was in accordance with the studies of Lu et al. [59], who reported postharvest gray mold by *B. pelargonii* on stored fresh ginseng roots. The morphological observation was confirmed using molecular method. Amplification and sequencing of the rDNA- ITS were performed and analysis of the ITS region showed that the isolates corresponded to the genus *Botrytis*. Phylogenetic analysis of the RPB2 gene grouped the isolate MDS55, in one clade with *B. cinerea* and MDS19 clustered in the same group with the reference strains of *B. pelargonii*.

Our results showed that although *B. cinerea* is a general species with the most abundance, there are other *Botrytis* species in Iran. Similarly, Mirzaei et al. [7] reported that eight *Botrytis* species, including *B. cinerea*, *B. pelargonii*, *B. aclada* sensu lato, *B. faba*, *B. convolute*, *B. gladiolorum* and *B. paeonia*. were collected from different hosts, including strawberry in Iran. Additionally, pathogenicity tests on strawberry fruits were performed and the findings indicated virulence of all fungal isolates obtained in their research.

In general, application of fungicides is the most widely used strategy to control the disease throughout the world. Although the

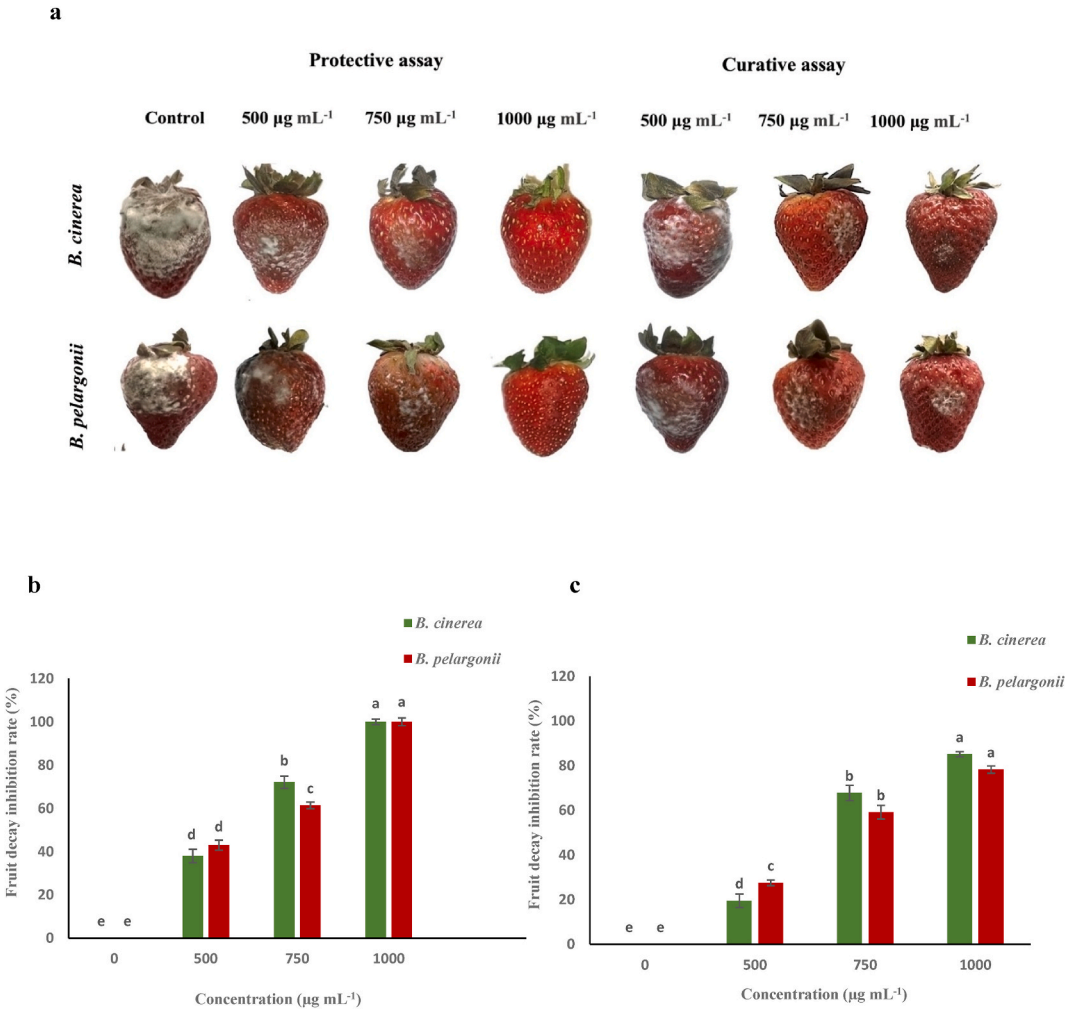

Fig. 7. The effect of treatment with different concentrations of *Ferula gummosa* essential oil (EO) on gray mold disease progression caused by *Botrytis cinerea* and *Botrytis pelargonii* after 10 days incubation at 20 °C (a), The fruits decay inhibition rate by application of *F. gummosa* EO in protective (b) and curative (C) assay after 10 days incubation at 20 °C. The experiments were conducted three times and each treatment had three replication in each repetition. Each concentration, with the same letters represent values that are not significantly different according to the Duncan test ($P < 0.05$).

usage of chemical fungicides is the main tool for controlling phytopathogenic fungi, the widespread application of these chemicals leads to the emergence of new fungicide-resistant pathogenic strains and hazardous residues in food and environment [60]. Consequently, in order to reduce the use of pesticides, extensive research has been done for the possible use of plant natural products as antifungal agents to control fungal pathogens [47]. The EOs are one of the most promising bioactive agents obtained from various aromatic plants [61]. The use of the EOs as edible coatings on fruit surfaces has been shown to be effective in controlling postharvest fungal phytopathogens, as well as in managing of numerous plant diseases [62].

Inhibitory effects of the EO obtained from *F. gummosa* were evaluated against *Botrytis* spp. The *in vitro* antifungal test revealed that different concentrations of the EO significantly inhibited mycelial growth of *B. cinerea* and *B. pelargonii* in a dose-dependent manner. The EO of *F. gummosa* completely inhibited mycelial growth of *B. cinerea* (100 %) at 500, 750 and 1000 µg mL^{-1} concentrations. Additionally, *F. gummosa* EO at concentrations of 750 and 1000 µg mL^{-1} showed similar suppression activity on mycelial growth of *B. pelargonii*. Similar findings were reported by Rguez et al. [19], who found that the EO of *Cupressus sempervirens* inhibited mycelial growth of *B. cinerea* the causal agent of postharvest gray mold disease of tomato, at different phenological stages including vegetative, flowering and fructification. Increasing dose of the oil led to more inhibitory effect against mycelial growth of this pathogen. Also, Wu et al. [63] demonstrated that Juniperus EO inhibited both the mycelial growth and spore germination of *B. cinerea* the causal agent of cherry tomato gray mold in a dose-dependent manner and in concentrations higher than 10 µL mL^{-1} exhibited potent inhibition. In the study conducted by Oliveira et al. [29], the inhibitory effects of essential oils from *Mentha piperita*, *Cymbopogon martinii*, *Cinnamomum camphora*, and *Mentha spicata* were evaluated against *Botrytis* spp., the causal agent of strawberry gray mold, using direct and vapor contact methods under *in vitro* conditions. Their findings indicated that *M. spicata* and *C. martinii* oils had the highest antifungal

Table 4
Gas chromatography-mass spectrometry (GC-MS) analysis of *Ferula gummosa* essential oil (EO). Major chemical composition present in the EO are indicated by bold font.

Number	Component	retention time (min)	Amount (%)
1	α-Phellandrene	5.47	3.2
2	**α-Pinene**	5.66	12
3	Camphene	6.04	0.2
4	**γ-Terpinene**	6.53	21
5	**β-Pinene**	6.68	37.7
6	β-Ocimene	7.37	0.8
7	o-Cymene	7.81	3.9
8	Limonene	7.91	0.9
9	Carene	8.65	1.6
10	Thujone	10.35	0.1
11	Isopinocarveol	11	1
12	Myrtenol	12.55	1.4
13	Sabinone	11.63	0.7
14	Copaene	17.33	0.6
15	Cedrene	18.47	0.2
16	γ-Muurolene	19.6	2.7
17	Guaiol	22.9	0.1
18	β-Guaiene	20.30	0.1
19	Zonarene	20.43	0.8
20	trans-Calamenene	21.07	0.6
21	Globulol	21.3	0.1
22	Cadinol	23.97	0.3

activity, completely inhibiting (100 %) mycelial growth and spore germination of *Botrytis* spp. at high concentrations. In another study, Kahramanoglu et al. [2], demonstrated that the suppression of mycelial growth of three different isolates of *Botrytis cinerea*, the causal agent of gray mold disease in strawberry fruits (*in vitro* tests), was achieved by treating with 0.25, 0.50, 1.00 and 2.00 mL L^{-1} concentrations of *Ziziphora clinopodioides* and *Origanum onites* essential oils. The antifungal effect of these EOs was dose -dependent, with the 0.50 mL L^{-1} and 1.00 mL L^{-1} concentrations of *O. onites* EO and 1.00 mL $L^{-1}$and 2.00 mL L^{-1} doses of *Z. clinopodioides* EO showing more inhibitory effect.

Aminifard and Mohammadi [26], reported the antifungal efficacy of peppermint, fennel and black caraway at different concentrations of 200, 400, 600 and 800 μg mL^{-1}. The result of their study indicated that utilization of fennel and black caraway oils at 400 and 600 μg mL^{-1} concentrations completely suppressed the mycelial growth of *B. cinerea* causal agent of gray mold disease in plum fruits. Moreover, Niu et al. [28] reported the application of ginsenoside CK substance obtained from Ginseng and can be used to control the fruits decay caused by *B. cinerea* and extend the shelf life of cherry tomatoes. Several studies have found the antifungal activity of various compounds obtained from plants against *B. cinerea* but to our knowledge, there are no reports on the inhibitory effect of any EO against *B. pelargonii*, so far. Therefore, our study can be considered as the first report in this case.

Biological activity of the EO depends on its chemical composition, but this factor could be impressed by many factors, such as geographic conditions, climatic and growth stage of the collected plants [19]. According to the results obtained, the EO of *F. gummosa* had more inhibitory effect against *B. cinerea* compared to the *B. pelargonii.* This might be related to the mode of resistance action of fungi against different substances present in various EOs [23]. Similarly, Hong et al. [25] investigated the antifungal activity of vapors emitted from thyme, cinnamon, origanum and fennel oil against mycelial growth and conidial germination of *B.cinerea*, the causal agent of tomato gray mold under *in vitro* condition. The findings showed that Cinnamon oil vapor had the highest effect on suppressing *B. cinerea* conidial germination, whereas all tested EOs showed similar activities in inhibiting *B. cinerea* mycelial growth in dose-dependent manners.

In our research, the results of spore germination assay also showed that the EO of *F. gummosa* strongly inhibited spore germination and germ tube elongation of *Botrytis* spp. And the concentrations of 1000 and 750 μg mL^{-1} of this oil completely prevented spore germination of *B. cinerea* and *B. pelargonii.* Also, the germ tube elongation of *B. cinerea* and *B. pelargonii* significantly reduced by all of concentration in comparison to the control. This is the first report about efficacy of the EO obtained from *F. gummosa* to suppress *in vitro* conidial germination of *Botrytis* spp.

In agreement with finding of this research, He et al. [21] reported that high concentrations of the *Cymbopogon citrates* EOs obtained from different climate conditions completely prevented the spore germination of *B. cinerea* pathogenic on tomato. Another study reported that the EOs of *Mentha pulegium* and *Myrtus communis* inhibited spore germination of *B. cinerea* pathogenic on strawberry compared to untreated control [27]. Infection of *B. cinerea* is predominantly initiated by conidia that attach to surface of the plants, germinate and grow on it [64]. So, suppression of spore germination and mycelial growth by the EO treatments may be limit the pathogens spread by lowering the spore load in the storage atmosphere and on surfaces [23]. The results of this research also revealed that the use of *F. gummosa* EO at the EC50 concentration considerably reduced the number of sclerotia formed in both tested fungal species. This finding is similar to the observation of Cong et al. [44], who reported the number of sclerotia produced by *Botrytis cinerea* on PDA amended with carbendazim at 0.1 μg mL^{-1} was significantly reduced compared to the untreated controls. Moreover, our results showed that the *F. gummosa* EO was able to suppress germination of sclerotia in both tested fungal species. Similar results were

reported by Goussous et al. [45]. They reported reduction in sclerotia formation of *Sclerotinia sclerotiorum* in petri plate containing *Rosmarinus officinalis* and *Salvia fructicosa* ethanolic extract at different concentrations. Also, loss sclerotia germination in *S. sclerotiorum* were observed. To our knowledge, this is the first report about inhibitory effect of the EO obtained from *F. gummosa* on sclerotia production and germination of *Botrytis* spp.

In addition, morphological changes in the hyphae of *Botrytis* isolates were detected by light microscopy. It was found that the EO of *F. gummosa* had severe effect on the mycelial structure of *Botrytis* spp. Hyphal deformation, including twist and thinner hypha, were observed in presence of the EO compared to the normal and smooth hyphae with uniform thickness in the controls. Moreover, morphological degenerations of the fungal hyphae were observed, such as coagulation of cytoplasm and vacuolation. Scanning electron microscopy analysis revealed that the mycelial of *B. cinerea* and *B. pelargonii* were normal and regular in the control. But in the treatment the mycelial surfaces were distorted, shrinked and rough. To our knowledge, this is the first report on the induction of morphological changes in the hyphal structures of *Botrytis* spp. by the EO of *F. gummosa*. Similar observations were reported by Areco et al. [65], who reported that application of the EO obtained from *Aloysia polystachya* caused obvious structural damage, including thinning of the hyphae with rough areas in the surface. But In contrast, the control mycelium maintained the normal structure, with cylindrical hyphae with a smooth surface. Also, Soylu et al. [23], reported that the EOs obtained from aerial parts of *Origanum syriacum*, *Rosmarinus officinalis* and *Lavandula stoechas* make ultrastructural alterations and obvious damages, including folding of the cell, shriveled hyphal aggregates compared with thick and normal mycelial growth of *Botrytis cinerea* in the control. Furthermore, in the research done by Wu et al. [63], SEM observations exhibited that Juniperus EO causing the morphology of *B. cinerea* mycelia to distort, twist and shrivel, which this finding was in accordance with our observations.

Plant pathogenic fungi produce an array of extracellular hydrolytic enzymes, known as CWDEs, which help them to invade, rupture, penetrate and infect the plant tissue. So, a decline in the activities of CWDEs will cause reduction in pathogenic fungi ability to infect their hosts [63]. 118 genes that encode potential Carbohydrate- Active Enzymes (CAZymes) have been identified in the *B. cinerea* genome. These enzymes are associated with plant cell-wall degradation to acquire the source of nutrition from dead tissue. The big enzymatic repertoire of *B. cinerea* may explain its ability to attack more than 200 different plant species [8]. In the current study, effect of the EO obtained from *F. gummosa* on the levels of enzyme production in *Botrytis* isolates were tested by qualitive and quantitative methods. The results of qualitive enzyme assays demonstrated that both of tested *Botrytis* isolates in the control plates were able to secret cellulase, protease, amylase and pectinase. But, varying degrees of enzyme production were detected among *Botrytis* isolates. In the plates amended with the EO of *F. gummosa* at the 0.1 EC50 concentration, significant reduction of enzymatic activities was observed compared to the untreated fungal isolates. Activities of cellulase and pectinase produced by *Botrytis* isolates were also assayed spectrophotometrically. The results of this test showed that activities of these enzymes were significantly lower than those of the controls following treatment with the EO of *F. gummosa*. Similar findings were showed by Wu et al. [63], who reported that treatment by the EO of Juniperus significantly reduced the enzymes produced by *B. cinerea,* including polygalacturonase (PG), pectin lyase and endoglucanase, which are related to pathogenicity.

In the current study the protective and curative effect of the EO obtained from *F. gummosa* were investigated *in vivo* at 500, 750 and 1000 μg mL^{-1} concentrations on strawberry gray mold. The results of this test indicated that untreated strawberries surface covered with a mass of gray mold and the color of the fruits had become relatively darker compared to treated fruits. The red color of strawberries is primarily due to the presence of anthocyanin pigments in the fruit's epidermis and cortex [66]. While, in both protective and curative assays, treatment with *F. gummosa* EO at all concentrations tested significantly decreased the severity of gray mold disease caused by *B. cinerea* and *B. pelargonii* in concentration-dependent manner. Also, at lower concentrations (500 and 750 μg mL^{-1}), the strawberries were more wrinkled and had a darker color. But, at the higher concentration (1000 μg mL^{-1}), the strawberries were less wrinkled and had a lighter color. Coating of strawberry fruits may reduce modification of physiological processes related to the anthocyanin content. Similar to our finding, Duarte and Picone [66], reported that coating with various nanoparticles has antimicrobial properties and is effective in preventing strawberry discoloration during storage period. Soylu et al. [23] reported *in vivo* inhibitory activity of different concentrations of origanum oil against *B. cinerea* as a curative treatment, which as doses of the oil increased, the infection of *B. cinerea* was more suppressed. Oliveira et al. [29] reported that the application of *Cymbopogon martini* and *Mentha spicata* oils on strawberries fruits significantly reduced the incidence and severity of gray mold disease. Moreover, Hong et al. [25] investigated the inhibitory effect of the EOs obtained from origanum, cinnamon and thyme oil against tomato gray mold. They reported curative effect of the used EOs and reduction of gray mold lesion formation on the inoculated tomato leaves, which among these oils, cinnamon oil showed the greatest inhibitory effect to decrease the disease development. Aminifard and Mohammadi [26], showed that fennel, peppermint and black caraway oils at 200, 400, 600 and 800 μg mL^{-1} concentrations prevented *B. cinerea* infection on plum fruits compared with the control and the effect of the three essential oils increased at higher concentrations. In another study, the inhibitory effect of *Origanum onites* and *Ziziphora clinopodioides* EOs has been reported against three different isolates of *B. cinerea* and it can be used to reduce postharvest losses of strawberry due to gray mold [2]. Also, the findings of this study are consistent with the previous study, that showed the protective effect of *Cymbopogon citrates* EO in reduction of decay area of cherry tomatoes infected by *B. cinerea*. In 125 mg L^{-1} concentration of this oil, the rate of rotting of cherry tomatoes was decreased by more than 90 % [21]. Moreover, our results indicated that at all concentrations tested, protective application of the EO had higher inhibitory effect in reducing gray mold disease compared to disease curative control by this EO. In protective assay, inhibition percentage of fruit decay caused by *B. cinerea* and *B. pelargonii* via using different concentrations of the EO were in the range of 38.5 to % 100 and 43 to % 100, respectively. Inhibition rate of the fruit decay caused by *B. cinerea* and *B. pelargonii* in curative assay were 27.86 to % 85.14 and 19.5 to % 78.28. Similar to our findings Hou et al. [67] reported that *Origanum vulgare* EO and its main components including carvacrol and thymol had more inhibitory effect against tomato gray mold disease in protective assay compared to the curative assay. To our knowledge, there are no research about controlling gray mold *in vivo* condition by *F. gummosa* EO and this result is as the first report. In

additions, there are no studies of antifungal effect of natural product against postharvest gray mold disease caused by *B. pelargonii*. So, this is the first report in this area.

In conclusion, using vapors of plant EOs or coating with this EOs can reduce post-harvest disease caused by *Botrytis* spp. during storage and transport. This could be due to suppression of fungal mycelia growth by the EO treatments, which can play an important role in reducing the spread of pathogens by lowering spore load in the storage area, and also on the surface [55].

Chemical composition of the EO of *F. gummosa* was determined by GC-MS analysis. Twenty-two constituents were identified in this oil, including β-Pinene (% 37.7), γ-Terpinene (% 21), a-pinene (% 12), α-Phellandrene (% 3.2), o-cymene (% 3.9) and muurola (% 2.7) comprised the predominant of the detected compounds. The other principal components in *F. gummosa* EO were Carene (% 1.6), Myrtenol (% 1.4), zonarene (% 0.9), isopinocarveol (% 1), limonen (% 0.9), sabinol (% 0.7), ocimen (% 0.8), and calamenene (% 0.6) which these compounds can be responsible for antifungal properties of the EO. The results of this study are in accordance with the finding of Ghannadi & Amree [68], which reported a-pinene (5.7 %), β-pinene (58.8 %), B-myrcene (4.6 %) and o-3-carene (12.1 %), as the main components of *F. gummosa* EO from Kashan, Iran. Also, similar to our findings, Mahboubi [31], reported that β-pinene, α-pinene, σ-3-carene, and limonene are the main substances in *F. gummosa* EO. In another study, β-pinene (40.7 %), -cadinene (7.2 %) and phellandrene (22.7 %) were reported as the major components of this EO [69]. Composition of the EOs could vary greatly depending on many factors, such as geographical location, climate conditions, seasons, plant age, individual genetic variations, developmental stages, growth regulators, diversity of plant parts, harvesting periods, presence of different chemotypes, plant – microbe interactions, methods of drying and oils extracting [70]. Moreover, according to Fukuyama et al. [71] the composition of essential oils can also be influenced by the plant's nutrition.

The present work revealed that the EO obtained from *F. gummosa* inhibited the mycelial growth, conidia germination and sclerotia production of *Botrytis* spp. *in vitro* and it was effective coating to reduce fruits decay and greatly improved the preservation of strawberry quality i*n vivo*. So the EO of *F. gummosa* can be used as an ecofriendly antifungal compound and possible alternative to synthetic fungicides against *Botrytis* spp. These findings should be accredited through large scale trials to determine the coating longevity under storage conditions and it is necessary to find and develop stable formulation of the EO obtained from *F. gummosa* to preserve and reduce its evaporation.

CRediT authorship contribution statement

Fatemeh Maghsoodi: Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Parissa Taheri:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Saeed Tarighi:** Writing – review & editing, Visualization, Resources, Project administration.

Data and code availability statement

Data included in the article/supplementary material is referenced in the article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e42037.

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