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Original Article

Identification of Chemical Compounds, Total Phenol and Flavonoid Contents, Antioxidant Potential of *Citrus paradisi* Essential Oil, and Its Effect on Fungi Cause Spoilage Strawberry Fruit

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ABSTRACT

Background and Objectives: *Citrus paradisi* belongs to the Rutaceae family, which is found in the north and south of Iran. The purpose of this study was to extract grapefruit essential oil (GEO) by water distillation and identify its chemical compounds with the help of a gas chromatography device connected to a mass spectrometer (GC-MS). Also, the total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity and antifungal effect were investigated.

Materials and Methods: In this study, GEO was first extracted by water distillation method and its chemical compounds were identified with the help of a GC-MS. The TPC and the TFC of the essential oil were determined using Folin–Ciocalteu method and aluminum chloride colorimetry, respectively. Antioxidant activity of essential oil with 3 methods of DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical inhibition, ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) free radical inhibition and decolorization β-carotene-linoleic acid was determined. Four different methods were used to evaluate the antifungal properties of GEO on *Rhizopus stolonifer* (ATCC 14037), *Botrytis cinerea* (ATCC 28387) and *Aspergillus niger* (PTCC 5010).

Results: The main compound in GEO was limonene (77.50%). The essential oil had TPC equal to 92.75 mg of gallic acid per gram and TFC equal to 61.50 mg of quercetin per gram. The antioxidant activity was of GEO against DPPH radicals (29.90 mg/mL), ABTS (26.60 mg/mL) and β-carotene oxidation (60.25%). According to the results of disk diffusion agar, *Botrytis cinerea* showed the largest inhibition zone (17.80 mm), while *Aspergillus niger* showed the smallest (13.30 mm). The minimum inhibitory concentration values for *Botrytis cinerea* were equal to 4 mg/mL and for *Aspergillus niger* equal to 16 mg/mL.

Conclusions: It is suggested that more research on other pathogenic fungi and also the effect of essential oil on horticultural products should be investigated.

Keywords: Essential oil, Antifungal effect, Gas chromatography, Strawberry

Highlights

- The main compound in grapefruit essential oil (GEO) was limonene (77.50%).
- The essential oil had total phenolic content (TPC) equal to 92.75 mg of gallic acid per gram and total flavonoid content (TFC) equal to 61.50 mg of quercetin per gram.
- The antioxidant activity was of GEO against DPPH radicals (29.90 mg/mL), ABTS (26.60 mg/mL) and β-carotene oxidation (60.25%).
- The minimum inhibitory concentration (MIC) values for *Botrytis cinerea* were equal to 4 mg/mL and for *Aspergillus niger* equal to 16 mg/mL.

Introduction

Grapefruit is a species of the citrus family with the scientific name *Citrus paradisi*, found in the northern and southern regions of Iran. This plant is also cultivated in other parts of the world including the Malka Islands, California,

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Florida, and India. This tree has a branched trunk, simple, green, and fragrant leaves, large almost white, and fragrant flowers, and juicy globular fruits with a yellow or lemon color and a sour and slightly bitter taste. The peel and fruit of grapefruit contain active compounds such as acids, flavonoids, vitamin C, and potassium, and its essential oil consists of hydrocarbon compounds such as citral, limonene, citronal, and geraniol (1-4). The antimicrobial activity of the essential oil of this plant has been investigated and confirmed in the research conducted by Alves et al. in 2015 (5). The antimicrobial activity of grapefruit essential oil was also proven in the research of Zanganeh et al., (2021) (6). Also, the antibacterial and antifungal properties of grapefruit seed and pulp extracts have been reported on 20 types of bacteria and 10 types of yeast (7).

In the study conducted by Mesgarpour et al., (2002), the antioxidant properties of Grapefruit essential oil (GEO) were reported (1). In Yang et al., (2010), the antioxidant effect of GEO was reported along with other plant essential oils such as peppermint, rosemary, lime, lavender, and frankincense (2). The antioxidant effect of grapefruit seed extract, which contains tocopherol, ascorbic acid, and citric acid, has been reported on a mixture of soybean and sunflower oil (8). It has been reported that extracts and essential oils can act as a natural antioxidant and can be a suitable alternative to various synthetic antioxidants (9-19).

Although the use of antimicrobial compounds and chemical preservatives can be effective in preventing and inhibiting the growth of pathogenic agents in food products, the lack of effect of some chemical preservatives on a number of microorganisms and the presence of chemical poison residues in food products have increased the use of natural antimicrobial substances. Natural antimicrobial compounds have the ability to control microbial contamination, reduce the use of antibiotics, increase the shelf life, and strengthen the immunity of cells. Plant essential oils have received considerable attention due to their naturalness and lack of toxic effects compared to chemical compounds. Various researches have shown that plant essential oils contain phenolic compounds such as flavonoids and phenolic acids, which have wide biological effects such as antifungal, antibacterial, antiviral, antioxidant, and anticancer properties. These characteristics have caused the use of these compounds as antimicrobial additives to increase the shelf life of food (9-19).

The purpose of this study was to extract grapefruit essential oil (GEO) by water distillation and identify its chemical compounds with the help of a gas chromatography device connected to a mass spectrometer (GC-MS). Also, the total phenolic content (TPC) and total flavonoid content (TFC) of the essential oil were determined using the Folin–Ciocalteu method and aluminum chloride colorimetry, respectively. The antioxidant activity of the essential oil was determined by 3 methods: DPPH free radical inhibition, ABTS free radical inhibition, and β-carotene-linoleic acid. Four methods of disk diffusion agar, well diffusion agar, minimum inhibitory concentration, and minimum fungicidal concentration were used to evaluate the antifungal properties of GEO in *Rhizopus stolonifer* (ATCC 14037), *Botrytis cinerea* (ATCC 28387), and *Aspergillus niger* (PTCC 5010).

Materials and Methods

Grapefruit essential oil extraction

The grapefruit peel was dried at room temperature and subsequently ground into a powder. 50 grams of the powder were then placed in a Clevenger device along with 750 ml of distilled water for the distillation extraction process, which lasted for 3 h. The GEO was then stored at 4°C until required for use (8).

Gas chromatography/mass spectroscopy (GC/MS)

For the identification and quantification of the primary chemical constituents of the GEO, a gas chromatography system (Agilent 7890A) coupled with a mass spectrometer (Agilent 5975C) was employed. The device was set up with specific conditions: a 0.2 µL injection volume, heating rate of 5°C/min, ionization energy level of 70 eV, helium gas flow rate of 1.1 mL/min, and utilized a DB-5 capillary column measuring 30 m in length, 0.25 mm in diameter, and with a film thickness of 0.25 µm. The retention profiles of the primary compounds were acquired and subsequently compared with those of known samples analyzed using GC/MS under identical operational parameters (23).

Total phenolic and flavonoid contents

The total phenolic content (TPC) of GEO was determined utilizing the Folin–Ciocalteu procedure (24). Initially, 0.5 mL of GEO was mixed with 2.5 mL of Folin-Ciocalteu's reagent (diluted at a 1:10 ratio with distilled water) and 2 mL of 7.5% sodium carbonate solution in a test tube, then shaken well. Subsequently, the mixture was kept at 45°C in a hot water bath for 15 min. The absorbance of the mixture was read at 765 nm using a spectrophotometer, with a blank sample containing water and reagents serving as the standard reference. Gallic acid equivalents (GAE) were employed as the reference standard, and the TPC was expressed as milligrams of GAE per gram of GEO on a dry weight basis.

For the determination of total flavonoids, the aluminum chloride colorimetric method was adapted (24). A volume of 125 µL of GEO was mixed with 75 µL of a 5% $NaNO_2$ solution and left to stand for 6 min before adding 150 µL of $AlCl_3$ (10%) and further incubating for 5 min. Subsequently, 750 µL of NaOH (1 M) was introduced, and the solution volume was adjusted to 2500 µL with distilled water. After 15 minutes of incubation, the mixture turned pink, and the absorbance was measured at 510 nm. The total flavonoid content (TFC) was reported in milligrams of quercetin equivalents (QE) per gram of GEO.

Antioxidant effect

DPPH radical scavenging effect: The hydrogen atom or electron donation ability of the oil was assessed by measuring the bleaching of a purple-colored ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) using a spectrophotometric assay. In this method, a stable radical DPPH is utilized as a reagent. An aliquot of the sample (100 µl) was combined with 1.4 ml of ethanol and then added to 1 ml of 0.004% DPPH in ethanol. The resulting mixture was vigorously shaken and promptly placed in a spectrophotometer to track the decrease in absorbance at 517 nm. The radical-scavenging activities of the samples, represented as the percentage inhibition of DPPH, were calculated using the following formula:

Inhibition percentage (%) = [(AB − AA)/AB] × 100

where AB and AA are the absorbance values of the blank sample and of the tested samples, respectively (25). The antioxidant activity result was reported as IC_{50} (mg/mL).

ABTS radical scavenging effect: The radical scavenging activity of the essential oil against the radical cation ABTS+ was determined following a standard procedure as described in previous studies. To generate $ABTS^+$, a solution of 7 mmol/L $ABTS^+$ was reacted with 2.45 mmol/L potassium persulfate and left to incubate in the dark at room temperature for 16 h. Prior to use, the $ABTS^+$ solution was diluted with ethanol to achieve an absorbance reading of 0.70 ± 0.02 at 734 nm. For the assay, 0.2 mL of the sample was added to 2 mL of the $ABTS^+$ solution and thoroughly mixed. After incubating at room temperature for 6 min, the absorbance at 734 nm was recorded. The ABTS+ scavenging effect was then calculated using the formula:

Scavenging effect (%) = (1−*A*sample/*A*control) × 100%

where $A_{control}$ is the absorbance of control without sample and A_{sample} is the test sample with $ABTS^+$ (26). The antioxidant activity result was reported as IC_{50} (mg/mL).

β- carotene- linoleic acid bleaching test: The spectrophotometric method, as outlined by Dapkevicius et al. (27), was utilized to observe the bleaching of a β-carotene/linoleate solution in the presence of the oil. This involved measuring the absorbance of the solution at 490 nm after 120 min (A) compared to the control sample at time zero (B) and after 120 min (C). The inhibitory effect of the oil on the bleaching of β-carotene/linoleate was determined using the following equation (27):

Inhibitory effect (%) = [(A-C)/(B-C)] × 100

Antimicrobial tests

Four different methods were employed to evaluate the antimicrobial properties of GEO against *Rhizopus stolonifer* (ATCC 14037), *Botrytis cinerea* (ATCC 28387), and *Aspergillus niger* (PTCC 5010) as described in reference (28). These methods included:

- Disk diffusion agar (DDA)
- Well diffusion agar (WDA)
- Minimum inhibitory concentration (MIC)
- Minimum fungicidal concentration (MFC)

DDA: To begin, grapefruit essential oil was prepared at a concentration of 100 mg/ml and sterilized using a 0.22-micrometer membrane filter. Subsequently, sterile discs were immersed in these concentrations for 15 min to ensure full saturation. Each of the tested fungi was then introduced in 100 µl onto sterile sabouraud dextrose agar plates. The discs saturated with essential oil were affixed to the plates and incubated at 27°C for 72 h. Following the incubation period, the diameter of the clear zones devoid of fungal growth (in mm) was recorded as inhibitory zones.

WDA: In this technique, wells were made in the sabouraud dextrose agar plates housing the target fungi. Subsequently, 20 µl of sterile essential oil were dispensed into the wells. The plates were then incubated at 27°C for 72 h. Finally, the diameter of the fungal growth inhibition zone was quantified in millimeters.

MIC: Following the sterilization of the essential oil using 0.45 µm filters, varying concentrations (4-512 mg/ml) of the oil were introduced into test tubes containing 20 µL of microbial suspension. Subsequently, the tubes were incubated in a controlled environment at 27°C for 72 h, and growth was monitored visually by assessing the turbidity observed in the tubes.

MFC: In this approach, tubes exhibiting no turbidity were selected, and 100 µL from these tubes were cultured on the surface of plates with sabouraud dextrose agar culture medium. Following the incubation in the controlled environment (72 h at 27°C), the lowest concentration with no observable growth was recorded as the minimum inhibitory concentration.

Statistical analysis

The experiments were conducted thrice, and data analysis was performed using Minitab software (version 16) employing a completely randomized design in a factorial arrangement. Subsequently, the means were categorized further using the Tukey post-test ($p < 0.05$).

Results

The chemical composition of grapefruit essential oil was assessed using GC/MS, and the findings are outlined in Table 1.

Table 1. Chemical composition of grapefruit essential oil (GEO) determined by GC/MS.

No.	Retention time (min)	Compounds	%
1	9.35	α-Pinene	2.50
2	11.10	β-Pinene	0.50
3	11.33	α-Myrcene	1.10
4	12.70	o-Cymene	3.80
5	12.90	Limonene	77.50
6	13.92	γ-Terpinene	4.80
7	15.66	Linalool	3.10
8	19	3-Cyclohexene-1-methanol, α,α,4- trimethyl-, (R)-	1.10
9	19.44	Decanal	1.30
10	21.10	1,6-Octadien-3-ol, 3,7-dimethyl-, formate	0.90
11	21.66	2,6-Octadienal, 3,7-dimethyl-, (E)-	0.60
12	25.79	cis-Muurola-4(15),5-diene	0.90
			98.10

- Compounds below 0.5% are not mentioned in the table.

The essential oil exhibited a total phenolic content of 92.75 mg GAE/g and a total flavonoid content of 61.50 mg QE/g (Fig. 1).

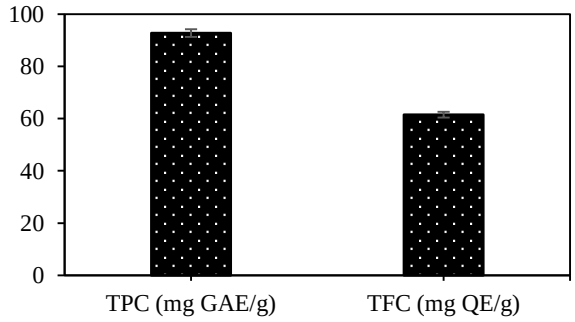

Fig. 1. Total phenol content (TPC) and total flavonoids content (TFC) of grapefruit essential oil (GEO).

Essential oils are renowned for their antioxidant prowess in mitigating cellular damage induced by free radicals. The antioxidant efficacy of GEO is delineated in Table 2, showcasing its significant radical scavenging activity against DPPH (29.90 mg/ml) and ABTS (26.60 mg/ml) radicals, alongside its ability to impede β-carotene oxidation (60.25%).

Table 2. Antioxidant activity of grapefruit essential oil (GEO).

	DPPH-RS test (mg/ml)	ABTS-RS test (mg/ml)	B-carotene bleaching test (%)
Results	29.90 ± 0.21	26.60 ± 0.19	60.25 ± 1.20

The essential oil exhibited notable antifungal properties against various pathogenic fungal species. As per the DDA test results, *Botrytis cinerea* displayed the largest inhibition zone (17.80 mm), while *Aspergillus niger* exhibited the smallest (13.30 mm) (Fig. 2), indicating differing sensitivities to the essential oil. This suggests that *Botrytis cinerea* was the most susceptible, whereas *Aspergillus niger* showed greater resistance to the oil.

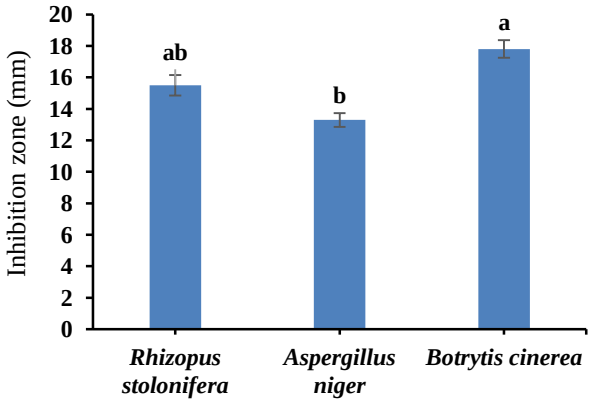

Fig. 2. Antifungal effect of grapefruit essential oil (GEO) based on disk diffusion agar method. The presence of different superscript letters indicates that there are significant differences ($p < 0.05$) between the means being compared.

Similar trends were noted in the WDA test (Fig. 3), with inhibition zones of 18.90 mm for *Botrytis cinerea* and 14.50 mm for *Aspergillus niger*.

The MIC values were determined to be 4 mg/ml for *Botrytis cinerea* and 16 mg/ml for *Aspergillus niger* (Fig. 4).

Furthermore, the MFC values were recorded at 128 mg/ml for *Botrytis cinerea* and 512 mg/ml for *Aspergillus niger* (Fig. 5).

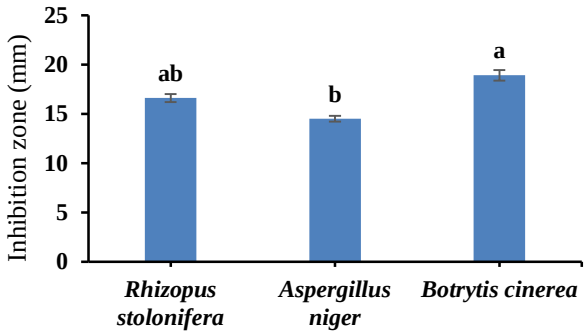

Fig. 3. Antifungal effect of grapefruit essential oil (GEO) based on well diffusion agar method. The presence of different superscript letters indicates that there are significant differences ($p < 0.05$) between the means being compared.

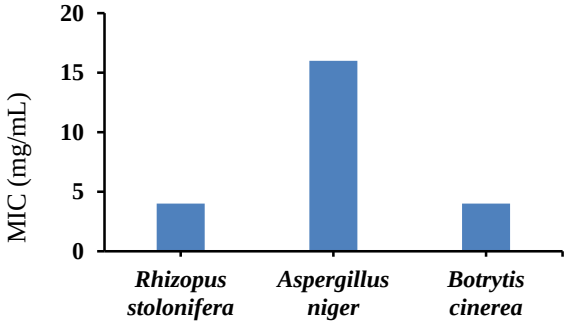

Fig. 4. Antifungal effect of grapefruit essential oil (GEO) based on minimum inhibitory concentration method.

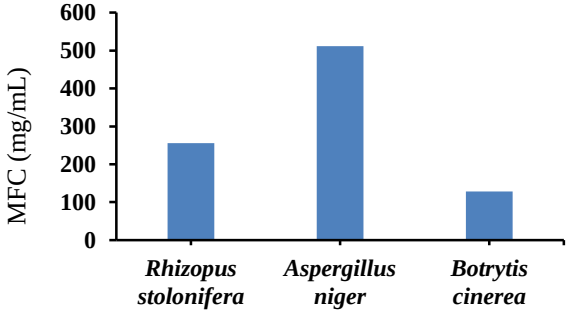

Fig. 5. Antifungal effect of grapefruit essential oil (GEO) based on minimum fungicidal concentration method.

Discussion

The predominant constituent in the oil was Limonene (77.50%), followed by γ-Terpinene (4.80%), o-Cymene (3.80%), linalool (3.10%), and α-Pinene (2.50%). Previous studies have reported limonene content ranging from 70.9% to 93.30% in GEO, corroborating our results (23, 32-34). Variability in the composition of GEO can be attributed to factors such as maturity stages, soil composition, genetic variations, cultivation practices, and climatic conditions.

Amjad et al. (21) reported a total phenolic content of 120.78 mg GAE/g and a total flavonoid content of 75.57 mg catechin equivalent/g in GEO. Similarly, Zanganeh et al. (2020) noted a total phenolic content of 69.57 mg GAE/g and a total flavonoid content of 37.24 mg QE/g in GEO (23). Phenolic compounds are instrumental in conferring antioxidant and antimicrobial properties to essential oils.

Citrus paradisi essential oil has been acknowledged for its remarkable antioxidant potential. Moreover, the antioxidant potential of GEO, denoted by IC_{50} values for DPPH-RS and ABTS-RS assays, was reported to be 22.06 mg/ml and 15.72 mg/ml, respectively (22). Studies have alluded to the exceptional radical scavenging capabilities of *Citrus paradisi* essential oil, indicative of its potent antioxidant attributes (35, 36).

Grapefruit essential oil has been investigated for its antifungal properties, demonstrating inhibitory effects against various fungi, including *Candida albicans* and *Aspergillus niger*. For instance, a study reported an inhibitory rate of 123% against *C. albicans* with a MIC of 2.5 µg/ml (37). Another study affirmed the potent antifungal activity of *Citrus paradisi* against a spectrum of fungi (38). The antifungal efficacy of GEO is attributed to compounds like limonene, sabinene, α-pinene, and β-pinene, known for their antimicrobial properties. These compounds are believed to disrupt the membrane integrity of fungal cells, inhibit ion transport processes, and impede respiration (23).

Conclusions

The results of this study showed that grapefruit essential oil is rich in limonene (77.50%) and its free radical inhibitory activity is significant. The antimicrobial effect of the essential oil against fungi that cause spoilage and contamination post-harvest strawberry fruit was also significant, and grapefruit essential oil inhibited the growth and destroyed the fungi *Rhizopus stolonifer* (ATCC 14037), *Botrytis cinerea* (ATCC 28387) and *Aspergillus niger* (PTCC 5010). In the following, it is suggested that more research on other pathogenic fungi and also the effect of essential oil on horticultural products should be investigated.

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Availability of data and materials

Derived data supporting the findings of this study are available from the corresponding author on request.

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