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Phytochemical and anticandidal efficacy of *Olea europaea* leaf extract from different cultivars and seasonal variation

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ABSTRACT

Introduction: Olive (*Olea europaea* L.) is one of the important agricultural and medicinal tree species. This study aimed to assess the antifungal efficacy of olive leaf extract (OLE) obtained from different cultivars and during different seasons against *Candida albicans* strains.
Methods: OLE was prepared using four olive cultivars ('Koroneiki', 'Mission', 'Rowghani', and 'Zard') obtained in two seasons (spring and autumn) from Golestan province, North of Iran. The phenolic and oleuropein contents of vegetative leaves were measured by colorimetric and HPLC techniques, respectively. The antifungal capacities of OLEs were tested by agar well diffusion and the minimal inhibitory concentration (MIC) was evaluated by micro-dilution assay.
Results: The findings of our study showed that the total phenolic (27.45-88.16 mg g^{-1}) and oleuropein (3.64-18.13 mg g^{-1}) contents varied in leaf extracts, respectively. The highest amount was found in 'Koroneiki' and 'Zard' (spring leaves) and the lowest in 'Mission' cultivars (autumn leaves). The inhibition zones and MIC ranged from 1.92 to 15.41 mm and from 6.07 to 27.20 mg mL^{-1} based on *C. albicans* strains, respectively. Relationship between total phenolic content as an independent variable (X) and inhibition zones or MIC as dependent variables (Y) fitted polynomial curves.
Conclusion: The present study highlighted the phytochemical and anti-candidal efficacy of OLE derived from olive cultivars or the seasons of harvested leaves against *C. albicans* strains. It is suggested that 'Koroneiki' and 'Zard' cultivars, especially during the spring season, could be exploited to isolate potential broad-spectrum antifungal drugs.

Implication for health policy/practice/research/medical education:
Our findings showed the significant variations of phytochemical profiles and anti-candidal capacities of olive leaves by harvest time and type of cultivar, and spring leaves were suggested as the best raw materials for pharmaceutical industries. The results also emphasized the broad antifungal sensitivity pattern of *Candida albicans* strains to olive leaf extracts.
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Introduction

The genus *Candida*, belonging to the yeast family of Saccharomycetaceae, with approximately 200 species can inhabit the skin and inside the body (1). Although some species occupy certain body sites, others, including *Candida albicans*, as an opportunistic yeast pathogen, represent the most common causative agents of mucosal infections and spread throughout the body, causing systemic infection in immunocompromised patients (2,3). Currently, the therapeutic options of antifungal agents are limited and included four structural classes of drugs such as azoles, echinocandins, pyrimidine, and polyenes. Among the classes, azoles (clotrimazole, fluconazole, itraconazole, ketoconazole, and miconazole) are the most frequently used drugs in the treatment of Candidiasis (4).

The long-term use of the currently available anti-fungal drugs has resulted in the emergence of *Candida* spp. drug-resistant strains during the last few decades (5). The novel therapeutic approaches to overcome the problem of resistance increased the reevaluation of anti-Candida activities of natural products (6).

Olive (*Olea europaea* L.), the type genus for *Oleaceae,*

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is one of the important agricultural tree species with high nutritive and medicinal values (7). Olive leaf extract (OLE) with high level of phenolic compounds and antioxidants, such as hydroxytyrosol, oleuropein, and tyrosol, could be considered as a source of valuable active compounds for health benefits (8).

Several studies suggest the antimicrobial capacity of leaf extracts and oleuropein (9-12). The highest value may be due to the presence of a high level of polyphenols that have been shown to regulate oxidative balance *in vivo* (13). Some limited research activities have also revealed the antifungal properties of OLEs against *Aspergillus*, *Candida,* and *Fusarium* spp. (8,14,15). However, there are some controversial findings concerning their effects on fungal species in different studies.

Some reports indicated the different content and types of phenolic compounds in olive leaves, fruits, and seeds (16,17). The significant changes in their composition might be related to the maturation period of vegetative or generative structures. However, limited work has been done to evaluate the biochemical properties and antimicrobial activity of harvested leaves in different seasons, especially from different cultivars (18,19). Therefore, the challenges of OLE antimicrobial efficacy may be concerned with different phenolic content of leaf usage. As a consequence, this study aimed to evaluate the variation of OLE bioactive compounds from different olive cultivars for two seasons. Also, the effect of these variations was studied on the anti-candidal activity of the tested leaf extracts.

Materials and Methods

Preparation of test microorganisms

In this study, 5 clinical strains (gau.IRAN.11 as CA 02, gau.IRAN.13 as CA 03, gau.IRAN.41 as CA 04, gau.IRAN.45 as CA 05, and gau.IRAN.78 as CA 06) of *C. albicans* from the culture collection of the Department of Plant Protection, Gorgan University of Agricultural Sciences and Natural Resources, and two strains (ATCC 76615 as CA 01 and ATCC 10231 as CA 07) from the Biotechnology Research Center of Iran were included. Sabouraud dextrose agar (SDA, Merck, Germany) was used for the maintenance of fungal strains at 4-6 °C. Subculture was performed on SDA for 48 hours at 35 °C. McFarland standards were used for the count of yeasts, and adjusted to yield 1.5×10^8 CFU mL^{-1}.

Preparation of olive leaf extracts

OLE was prepared using various olive cultivars ('Koroneiki', 'Mission', 'Rooghany', and 'Zard') in two seasons, spring (25 April) and autumn (15 November), from Goran region, Golestan, Iran. Vegetative leaves were dried in the shade and crushed powder was extracted using aqueous methanol. After concentrating the extracts in a rotary evaporator and drying with a freeze dryer, the results were stored at -20 °C. A membrane filter with a pore size of 0.2 µm was used for sterilizing the solutions (20).

Biochemical analyses

Total phenolic content from all OLEs was determined by the colorimetric method with Folin-Ciocalteu reagent at 765 nm (Sigma-Aldrich, the USA) (21). Briefly, 200 µL of extract solution was mixed with 1.0 mL of reagent. After 5 minutes, 1.0 mL of Na_2CO_3 (75%) was added to the mixture and incubated at 50 °C for 10 minutes with intermittent agitation. The results were presented as gallic acid equivalents in milligrams per gram of samples.

The analysis of oleuropein in OLEs was evaluated using a Knauer HPLC system (19) at a flow rate of 0.8 mL/min (in detection wavelength of 254 nm and column temperature of 30 °C). Water/acetonitrile (74/25, v/v), as the mobile phase, was considered for 10 µL of the injection. The oleuropein content was presented as mg per g dry matter.

Determination of inhibition zone

The agar well diffusion method on SDA medium was used for *in vitro* antimicrobial activity of OLEs in 5-mm diameter wells. 100 µL of yeast suspension was spread onto SDA plates and then OLEs (20 µg) were added to the wells. The sealing of Petri dishes with parafilm was performed to avoid contamination. After 48 hours incubation at 35 °C, the zone of inhibition (mm) around the wells was measured for the efficacy of antimicrobial activity. Amphotericin-B (Sigma-Aldrich, the USA) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, the USA) were considered as positive and negative controls, respectively (22).

Determination of minimal inhibitory concentration

The evaluation of minimal inhibitory concentration (MIC) was performed by micro-dilution broth susceptibility assay in Sabouraud dextrose broth (Merck, Germany) as described by Clinical & Laboratory Standards Institute (23). Serial dilutions of OLE were added with inoculums medium following 48 hours incubation at 35 °C. The lowest concentration for complete inhibition of the growth of fungi as compared with the control was determined as MIC. DMSO and amphotericin B were considered as negative and positive controls, respectively (24).

Statistical analysis

All experiments were analyzed using a randomized complete block with a factorial design. Each experiment was performed in three replicates. The data were tested by two-way analysis of variance (ANOVA) and the differences between means were compared using Tukey's HSD tests at $P \leq 0.05$. UPGMA (unweighted pair group method with arithmetic mean) algorithm was used to determine the clusters. All statistical analyses were performed using R 4.2.1 statistical software.

Results

Biochemical analyses

Mean data values of the total phenolic content of all OLE varied from 27.45 to 88.16 mg g^{-1} leaf dry weight on the basis of gallic acid equivalents. Analysis of variance revealed that the differences in total phenolic content in different samples were significant ($P \leq 0.05$, Table 1). It was found that the phenolic contents of OLE were different between cultivar samples. The highest amount was detected in the 'Koroneiki' and 'Zard' cultivars (spring leaves) and the lowest was founded in the 'Mission' cultivar for autumn leaves (Figure 1A). The results also showed a significant difference in phenolic components between spring and autumn leaves. The total phenolic content of OLE from spring leaves was 37.26% higher than the total phenolic content in autumn leaves (Figure 1B).

Figure 2 illustrates the chromatographic analysis of representative samples from the 'Koroneiki' cultivar with different seasons of harvested leaves. The results for oleuropein were similar, and a remarkably higher level was seen in the spring leaves for different olive cultivars (Table 1). The positive correlation and quadratic equations between TPC and oleuropein amount were estimated from spring and autumn leaves (Figure 3).

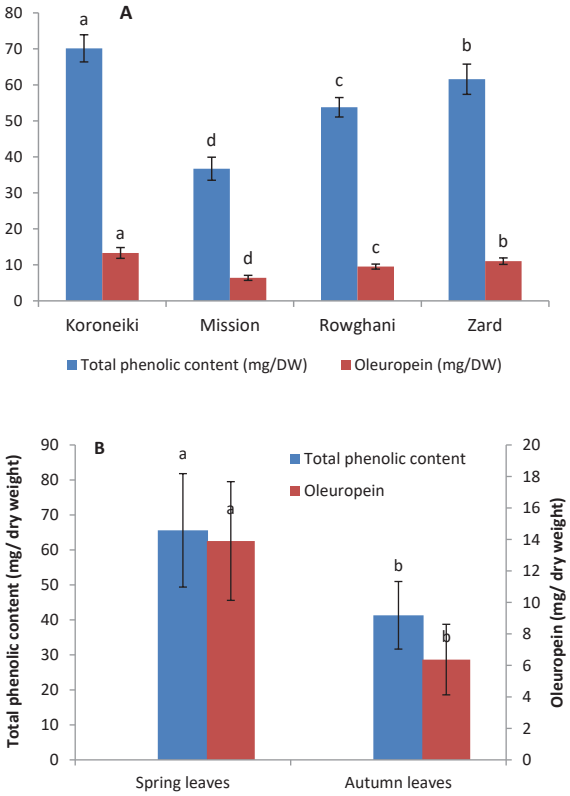

Figure 1. Single-factor effects on the total phenolic and oleuropein contents in different olive cultivars (A) and seasons (B) of the harvested leaves. Different letters in each column indicate statistically significant differences between different treatments (Tukey test, $P \leq 0.05$).

Sensitivity of strains to OLE from different cultivars and seasons

An ANOVA test was performed for testing the significance of the sensitivity of fungal strains to OLE, including olive cultivars, seasons of samples, fungal strains, and their interactions. The significant differences ($P \leq 0.05$) were evaluated between all comparisons for the traits measured. As shown in Table 1, the inhibition zone diameters ranged from 1.92 to 15.41 mm based on different olive cultivars and leaf seasons. Among the tested cultivars, 'Koroneiki' and 'Mission' showed high and less inhibition zones, respectively. In all cases, the inhibition zones from spring leaves were significantly different ($P \leq 0.05$, Table 1). The findings revealed varying degrees of sensitivity for *C. albicans* strains to OLEs. Comparing the differences in

Table 1. Quantification of phytochemical screening extracts in both spring and autumn leaves of the examined olive cultivars

Olive cultivars/Leaf age	Total phenolic content (mg/g dry weight)	Oleuropein (mg/g dry weight)	Inhibition zone (mm)	MIC (mg/mL)
Koroneiki				
Spring leaves	88.66 a	18.13 a	15.41 a	6.07 f
Autumn leaves	53.13 d	8.62 c	10.66 b	10.25 e
Mission				
Spring leaves	46.23 e	9.15 c	5.28 c	18.87 c
Autumn leaves	27.45 f	3.64 e	1.92 d	27.20 a
Rowghani				
Spring leaves	64.53 c	13.57 b	9.42 b	13.57 d
Autumn leaves	43.56 e	6.16 cd	4.87 c	20.54 b
Zard				
Spring leaves	75.32 b	15.26 ab	10.50 b	12.08 d
Autumn leaves	48.52 de	7.15 cd	4.64 c	20.35 b

MIC: Minimum inhibitory concentration.
The data marked by different letters in each column indicate a statistically significant difference (Tukey test, $P \leq 0.05$).

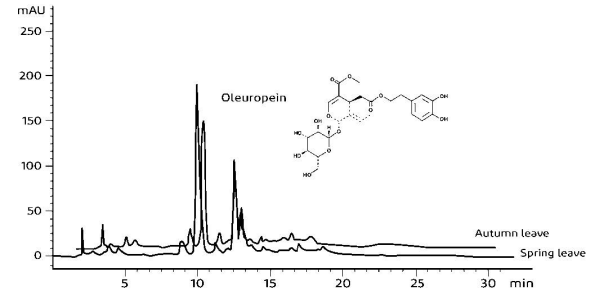

Figure 2. High-performance liquid chromatography (HPLC) chromatogram of oleuropein from spring and autumn leaves in the Koroneiki cultivar.

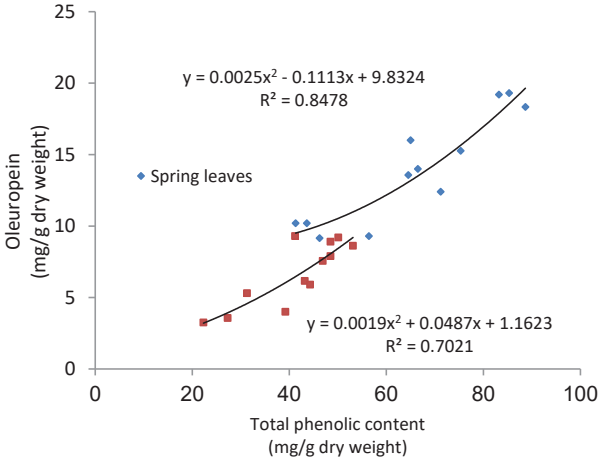

Figure 3. The relationship between the total phenolic or oleuropein contents and antioxidant activity of olive leaf extracts in the seasons of harvested olive leaves.

the sensitivity of the fungi against OLE, *C. albicans* strain 07 was the most sensitive strain to the OLE with a higher inhibition zone than others (Figure 4).

Relationship between total phenolic content as an independent variable (X) and inhibition zones as dependent variables (Y) fitted polynomial curves with $Y=0.0025X^2-0.3844X-12.762$ ($R^2=0.7622$, $P\leq 0.01$, Figure 5). The dendrogram constructed with the UPGMA clustering method on the basis of zones of inhibition revealed 3 and 2 groups with subgroups in each cluster for *C. albicans* samples and OLEs from different olive cultivars, respectively (Figure 6)

Minimum inhibitory concentrations

The MICs of OLE against *C. albicans* strains varied based on the olive cultivars and leaf season, ranging from 6.07 to 27.20 mg mL^{-1} (Table 1). The results showed positive correlations between MICs (Y) and the total phenolic content of samples (X, Figure 5). Relationship between variables fitted polynomial curves with $Y=-0.00497X^2+0.328X-13.279$ ($R^2=0.7407$, $P\leq 0.01$).

Discussion

Medicinal plant extracts as an alternative to chemotherapeutics can be effective against invasive fungal infections (25). These extracts can decrease opportunistic infections with major resistance problems during prolonged use of antibiotics (7,24). Natural extracts can also be used as effective strategies for the production of functional foods to achieve a significant increase in social and environmental sustainability (26).

Polyphenolic compounds, widely present in plants, are secondary metabolites with potent antimicrobial, antioxidant, and anti-cancer activities. Within the past few decades, there is a growing interest in utilizing phenol extract from medicinal plants in the prevention of diseases related to oxidative stress. Olive leaves, rich in polyphenolic compounds, have long been known as herbal remedies with recognized benefits for both human health and industrial purposes. The extracts have traditionally been known for their antihypertensive, antimicrobial, and antioxidative activities. Neutral taste and odor, easily prepared, low-cost extracts, and water solubility of OLE might be the advantages of using OLE in various industrial applications (27).

OLEs are rich in phenolic and flavonoid compounds, therefore they present great potential as natural

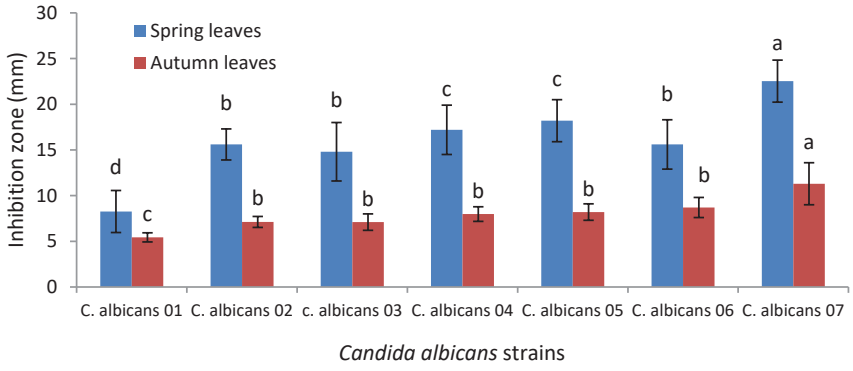

Figure 4. Zone of inhibition (mm) of olive leaf extracts of different olive cultivars from spring and autumn seasons against *Candida albicans* strains (01-07). The columns with different superscript letters are significantly different ($P\leq 0.05$).

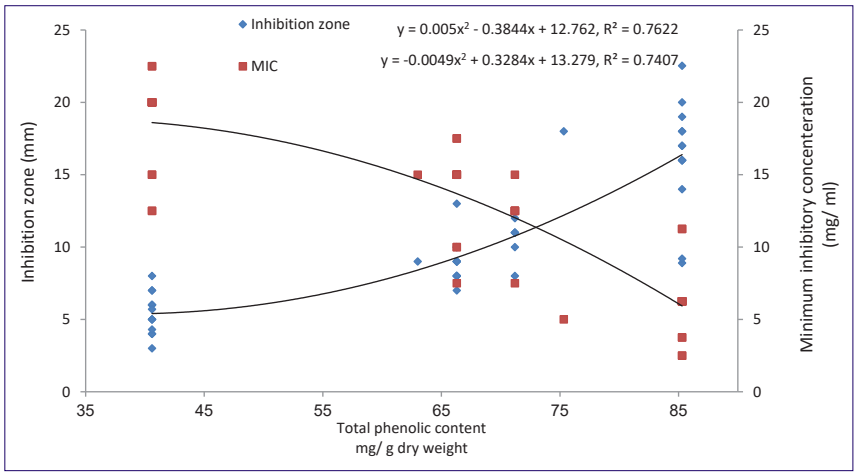

Figure 5. Inhibition zone (mm) and minimum inhibitory concentration (mg/mL) as affected by total phenolic content (mg/g dry weight) of different olive cultivars of olive leaf extract.

antioxidants for functional food, nutraceutical, and pharmaceutical applications (28). However, significant differences in phenolic compounds have been found in the biochemical or genetic characteristics of olive leaves and fruits (19). Our results emphasize the significant effect of cultivar and sampling time on total phenolic content, especially Oleuropein as a primary compound of OLEs polyphenols.

The oleuropein values in OLEs of the selected cultivars in this study were similar in the range of the same cultivars reported for Syrian (29), Algerian (30), Iranian (31), and Portuguese (32) cultivars, although substantially lower for 'Arbequina' reported by Talhaoui et al (33) and 'Koroneiki' and 'Arbequina' reported by Wang et al (19). The range of oleuropein value detected in this study was significantly higher compared to the results of Ben Salah et al (30-52 mg kg^{-1}) (34).

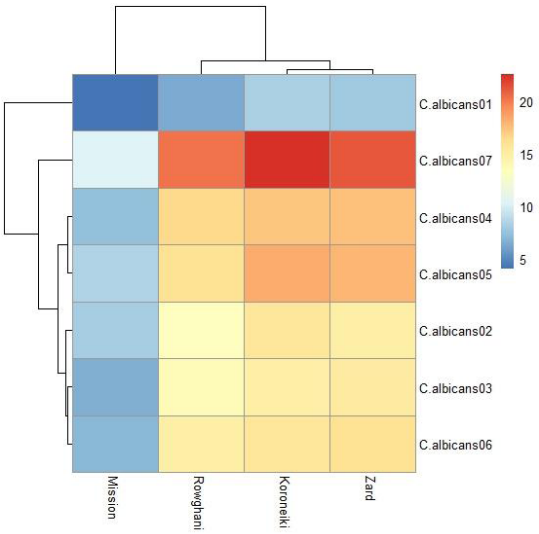

Figure 6. Heatmap distribution of methanolic extract of olive cultivar leaves against *Candida albicans* strains. *C. albicans* strains are arranged in rows and are clustered on the vertical axis (y-axis). Olive cultivars are arranged vertically and are on the horizontal axis (x-axis). Clustering was done using the UPGMA algorithms.

The phenolic content of olive fruit and leaf extracts could inhibit or delay the growth rate of a range of microorganisms (35,36). It is likely that the antimicrobial properties of these compounds are also related to inhibiting the membrane sterol synthesis pathway (19). Although OLE has been mainly tested for antibacterial activity (9,11,12,17,37), the results of this experiment revealed the antifungal activity of OLE in line with other experiments on *Candida* spp. (8,19,37-40). Our results showed the effectiveness of the methanolic extract of OLE in inhibiting the growth of *C. albicans*, providing support for the potential of OLE alone or in combination with other antimicrobials for the purpose of fungal control. In the present study, varied levels of antifungal activities of OLEs accompanied by different inhibitory actions against the strains of *C. albicans* could explain the controversy of findings in other studies about OLE efficacy (41,42). With our results, the observed differences in the antifungal effects are likely to be associated with OLE derived from different olive cultivars or the seasons of harvested leaves

Our findings also showed various degrees of antifungal sensitivity for the test yeasts. Several other studies have been carried out to find the antifungal effects of this plant against *Candida* yeast, among which Nasrollahi and Abolhasannezhad (20) studied the inhibitory effect of aqueous extract of Olive leaves on the growth of a fluconazole-resistant strain of *C. albicans* and reported MIC for the aqueous extract of this plant to be 24 µg mL^{-1}. The MIC for aqueous extract of Olive leaves against *C. albicans* was found to be 46.875 mg mL^{-1} (8) Another study reported that OLE extracted by acetone showed the MIC value of 10 mg mL^{-1} (41). Also, ethanolic OLE showed MIC of 15 mg mL^{-1} against a *C. albicans* isolate in the study by Halawi et al (42). In our results, considerably

higher amounts of methanolic extract of this plant (2.50-22.50 mg mL^{-1}) were concluded as MIC for *C. albicans* strains.

Conclusion

According to the present study and the results of other studies, OLE, as a natural anti-candida substance, may be considered an appropriate drug supplement in the treatment of *C. albicans* diseases. The findings highlighted the sensitivity differences of tested strain OLE. The observed differences in OLE antifungal effects might be due to the varying levels of phenolic capacity in different olive cultivars and seasons of harvested leaves. The results indicated that OLE, especially extracted from the spring leaves with higher antifungal activity, can be used as a good source of antifungal agents. They might be recommended as natural antifungal additives for food or pharmacological industries. Our findings could add fruitful information considering the effects of cultivars and seasonal variations to obtain potential antifungal compounds from olives. The leaves from 'Koroneiki' and 'Zard' cultivars in the spring season could be quite effective to isolate broad-spectrum antifungal drug candidates.

Authors' contributions

MSAH supervised the study. The first draft was prepared by PA and SJS. All authors read the final version and confirmed it for publication.

Conflict of interests

The authors have no conflicts of interest to declare.

Ethical considerations

Authors have carefully monitored ethical issues such as text plagiarism, duplicated publication, misconduct, data fabrication, and falsification

Funding/Support

None.

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