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ORIGINAL ARTICLE

Spectral characterization of the bioactive principles and antibacterial properties of cold methanolic extract of *Olea europaea* from the Hail region of Saudi Arabia

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Abstract This work aimed to identify the bioactive constituents and antibacterial activity of a cold methanolic extract of *Olea europaea* leaves. GC–MS analysis of the cold methanolic extract of the leaves of *O. europaea* L. revealed several unique bioactive compounds, including 9,12-octadecadienoic acid (Z,Z)-, n-hexadecanoic acid, 9-octadecenamide, (Z)-, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, squalene, 2-(2-Hydroxy-2-phenylethyl)-3,5,6-trimethylpyrazine, Benzoic acid, 4-formyl-, methyl ester, 2-Methoxy-4-vinylphenol, Vitamin E etc. The FT-IR spectrum revealed distinct fingerprint regions at 3313, 2943, 2831, 1662, 1590, 1449, 1300, 1111, and 1020 cm^{-1}. The fingerprinting region is associated with the presence of several bioactive chemicals as determined by GC–MS analyses. The methanolic extract of *O. europaea* leaves revealed a

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broader spectrum of antibacterial activity. On the other hand, the spectrum of activity was substantially narrower than that of standard ciprofloxacin discs.

1. Introduction

Olives, *Olea europaea* L is a slow-growing evergreen treen richly grown, has economic and social impact in the Mediterranean region (Faten et al., 2012). *O. europaea* L leaves extensively use traditional herbal medicine to prevent and treat several diseases, particularly in the Mediterranean region. In 2017, the Mediterranean countries grew over 8 million olive trees, accounting for nearly 98 % of the global crop (Clodoveo et al., 2022; Food and Agriculture Organization, 2017). *Olea europaea* L. is one of the most widely used botanical medications globally. The medicinal benefits of *O. europaea* have long been recognized in the traditional medical literature. It has been shown to lower blood sugar levels, cholesterol levels, and uric acid levels. As a laxative, mouthwash, and as a vasodilator, it has also been used to treat diabetic ketoacidosis, high blood pressure, inflammation, diarrhea, respiratory and urinary tract infections, stomach and intestinal illnesses, asthma, hemorrhoids, and rheumatism, among other things (Hashmi et al., 2015). It also lowers cardiovascular risk factors such as atherosclerosis and blood pressure, helps to maintain type 2 diabetes, and aids in weight loss and maintenance (Debra Rose, 2018). Olive has been extensively researched as a functional food due to its high concentration of biophenols and other bioactive ingredients (Galanakis, 2011; Erbay and Icier, 2010). Olive trees have been cultivated throughout the Mediterranean basin, and olive oil has long been a staple of human nutrition in the Mediterranean area (Al-Ruqaie et al., 2016). Saudi Arabia is a significant consumer of olives and olive oils but contributes only a negligible amount to global olive oil output. Olive farming in Saudi Arabia originated in Al-Jawf, planting olive trees at the beginning of 2007. However, 2009 was the turning point for this kind of agriculture, and it expanded significantly to reach more than 13 million olive trees and later spread to the outskirts of the Tabuk and Hail regions (Al Arabia News, 2018). Hail Province, commonly known as the Hail Region, is well-known for holding the geographically and historically significant twin mountain ranges of Aja and Salma, which are presently protected by the Saudi wildlife authority and are in the northern part of the province. In addition, the province is home to several rocks art sites, two of which have been designated as UNESCO World Cultural Heritage Sites: Jabal Umm Sinman in Jubbah and Jabal al-Manjur (UNESCO, 2015). Hail currently has two million olive trees, half of which produce olive oil continuously. Hail now covers 10% of the agricultural land in the Kingdom. Despite the challenging climate of Hail region, has developed into an essential producer of olive oil. Its continuing production has cemented the island's position as a promising agricultural investment destination in the Mediterranean region (Wafy, 2021).

2. Materials and methods

2.1. Study area, plant collection, and identification

Al Khitah is in the Hail Province of Saudi Arabia and has the following latitude and longitude coordinates: 41.7407394, 27.9850918. (Fig. 1). Fresh *Olea europaea* L leaves with stems were collected from trees in Al Khitah, Saudi Arabia, placed in biohazard polyethylene bags, and transferred to the laboratory right away. Extensive washing with tap water was used to eliminate all adhesive contaminants. A taxonomist registered and deposited a sample of the plant stem with leaves at the University of Hail (UOHCOP 002) as well as Jazan University's Herbarium (JAZUH 1306). *Olea europaea* L leaves were plucked from the collected branches, cleaned thoroughly with Millipore water, and dried in the open air for 30 min. The leaves were dried for ten days in a ventilated room. The dried leaves were collected and powdered, pooled, and stored properly for further analysis. The powdered sample of leaves of *Olea europaea* L was designated as LOP.

2.2. Extraction procedure

The bioactive components of the LOP were extracted by cold methanol maceration. 25 g of LOP was soaked in 50 mL of methanol, stirred with a magnetic stirrer on a hot plate at room temperature for 60 min to get a reaction mixture (RM). The RM was kept in a refrigerator at 4 °C overnight. Then after, the RM was kept in a magnetic stirrer and the process was continued for a week. The macerated RM was centrifuged using a Sigma table-top centrifuge at 2000*g* for 10 min. The supernatant solution was passed through Whatman filter paper, no. 1, and the resulting extract was air-dried at 25 °C. The dried sample was collected and stored in refrigerator at 4 °C for further works.

2.3. FT-IR spectroscopy

The specific functional groups of bioactive molecules in the methanolic extract of LOP were analysed by FT-IR spectroscopy using a Nicolet iS10 FT-IR spectrophotometer (Thermo Scientific, USA). A simple KBr pellet technique was performed, and spectra of the pellet sample were obtained via the FT-IR spectrophotometer against a reference KBr pellet in the range of 400–4000 cm^{-1}, with a resolution of 4 cm^{-1}.

2.4. GC–MS analysis

GC–MS was used to determine the presence of key bioactive components in the methanolic extract of LOP (Thermo Scientific GC–MS-AS 3000 autosampler – IQS detector). Using a TR 5MS capillary column with helium as a carrier gas at a flow rate of 1.2 mL/min, 2 μL of the methanol diluted powdered sample was injected for partial separation of bioactive components. Mass spectrophotometry was conducted, and Xcalibur software was used to perform spectral analysis. The mass spectra were interpreted using the NIST and MAINLIB software libraries (Makeen et al., 2020).

2.5. In vitro antibacterial activity

The bacterial strains used in the study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis*ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella choleraesis* ATCC 10708, *Pseudomonas aeruginosa*

A

B

C

D

E

Fig. 1 (A) The study area, Al Khitah, Hail province (B) Natural habitat of olive trees at collection site (C) Aerial image of olive trees with several branches and cluster of leaves (D) Bunch of olives leaves after collection (E) Stem of olive trees with leaves and fruits during air drying process.

ATCC 27853, *Proteus mirabilis* ATCC 299. 24 h cultures were prepared from the stock culture prior to the antibacterial experimentation. The antibacterial susceptibility test was performed as established by (Moni et al., 2018). To conduct the antibacterial study, Muller Hinton agar plates were created. For sample analytes, the agar well diffusion technique was used, and disc diffusion was used for standard ciprofloxacin discs (5 mcg/disc). Individually, a sterile cotton swab was dipped into the standardized (CFU/ml) culture and streaked on Muller Hinton (MH) agar plate by turning the petri dish to spread the culture equally. The plates were allowed to dry for approximately ten minutes prior to administering the sample analytes. The agar well diffusion technique was carried out by punching holes in inoculated MH agar plates with a sterile stainless-steel borer. After 24 h of incubation at 37 °C, the antibacterial spectrum was determined by establishing inhibitory zones. The spectrum of activity is proportional to the diameter of the inhibitory zones and is summarized in Table 3.

2.6. Statistical analysis

The statistical analysis was done using the Prism 9, Graph Pad Instat software system, USA. Statistical analyses were performed using one-way ANOVA, followed by Tukey's test (post hoc test). Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant for all analyses.

3. Results and discussion

Consumption of vegetables and fruits is beneficial to maintaining a normal, healthy lifestyle because it helps to prevent a variety of ailments. Olives are a rich source of several medicinally valuable substances that can help avoid various ailments (Guasch-Ferré et al., 2022). The present study is designed to explore the medicinal values of the leaves of *Olea europaea* (Olives) trees collected from Hail region, Saudi Arabia. Results from GC–MS analysis of the methanolic extract of LOP revealed the presence of several bioactive compounds, as shown in the chromatogram in Fig. 2. The presence of various bioactive constituents in the methanolic extract of LOP is presented in Table 1, and their structure is represented in Fig. 3.

9,12-Octadecadienoic acid (Z,Z)-, is otherwise called as α-linoleic acid, exhibited retention time 77.07 min, occupying almost 12.65 % in the GC–MS chromatogram. An earlier study suggested the presence of α-linoleic acid in the leaves of olives from southern Brazil (Caroline et al., 2015). Bahloul et al. (2014) observed that the n-hexane extract of olive leaves cultivated in Tunisia contained the highest amount of α-linoleic acid. It is an essential omega-3 fatty acid for normal human growth and development. α-linolenic acid is likely to lower the risk of heart disease by assisting in the maintenance of a normal heart rhythm and pumping action, according to an earlier report (Sina et al., 2021; Watanabe and Tatsuno, 2017; Guasch-Ferré et al., 2014). Consumption of dietary α-linoleic acid is associated with a decreased risk of death from all causes, cardiovascular disease, and coronary heart disease, but with a slightly increased risk of cancer mortality, whereas increased blood levels of α -linoleic acid are associated with a decreased risk of death from all causes and coronary heart disease only (Naghshi et al., 2021). It may also have the additional benefit of reducing blood clots (Yang et al., 2014; Brzosko et al., 2002).

An earlier study, which demonstrated the effect of olive leaf extracts on the oxidation of lipids and proteins in fried pork patties after they had been refrigerated for nine days, revealed that linolenic acid is an effective antioxidant (Botsoglou et al., 2014). n-Hexadecanoic acid, commonly known as palmitic acid, exhibited a RT of 71.52 min with a % occupancy in the chromatogram was 5.18. According to a recent report, palmitic acid significantly inhibited prostate cancer cell growth *in vitro* and *in vivo*. Inhibition of G1 phase arrest was associated with downregulation of cyclin D1 and p-Rb and an increase in p27 expression in the presence of palmitic acid (Zhu et al., 2021). Previous research has shown that palmitic acid has a wide range of antibacterial and antifungal effects (Giancarlo et al., 2021; Chandrasekaran et al., 2011). Palmitic acid has been reported recently in olive oil (Maysa, M., 2020) .

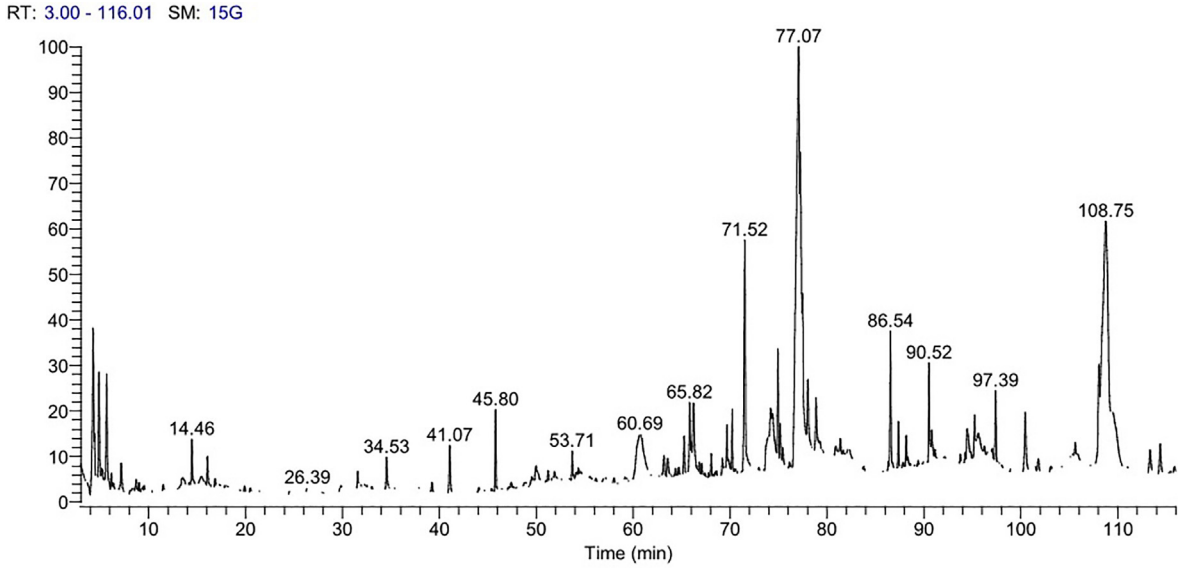

Fig. 2 GC–MS chromatogram of the cold methanolic extract of olive tree leaves.

Table 1 GC–MS detection of possible bioactive compounds in cold methanolic extract of olive tree leaves.

S. No	Compound name	Molecular formula	Molecular weight	Retention time (Min)	Probability Index	Percent area of curve
1	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	77.07	51.24	12.65
2	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	71.52	80	5.18
3	9-Octadecenamide, (Z)-	$C_{18}H_{35}NO$	281	86.54	90.12	2.34
4	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{19}H_{38}O_4$	330	90.52	61.47	1.69
5	Squalene	$C_{30}H_{50}$	410	97.39	45.94	1.18
6	2-(2-Hydroxy-2-phenylethyl)-3,5,6-trimethylpyrazine	$C_{15}H_{18}N_2O$	242	66.23	54.35	1.83
7	Benzoic acid, 4-formyl-, methyl ester	$C_9H_8O_3$	164	45.80	67.28	1.35
8	2-Methoxy-4-vinyl phenol	$C_9H_{10}O_2$	150	41.07	58.82	1.05
9	Benzofuran, 2,3-dihydro-	C_8H_8O	120	34.53	47.02	0.67
10	1,2-Cyclopentanedione	$C_5H_6O_2$	98	14.46	70.88	0.79
11	Vitamin E	$C_{29}H_{50}O_2$	430	108.75	41.72	0.92
12	Methyl salicylate	$C_8H_8O_3$	152	32.26	33.04	0.12

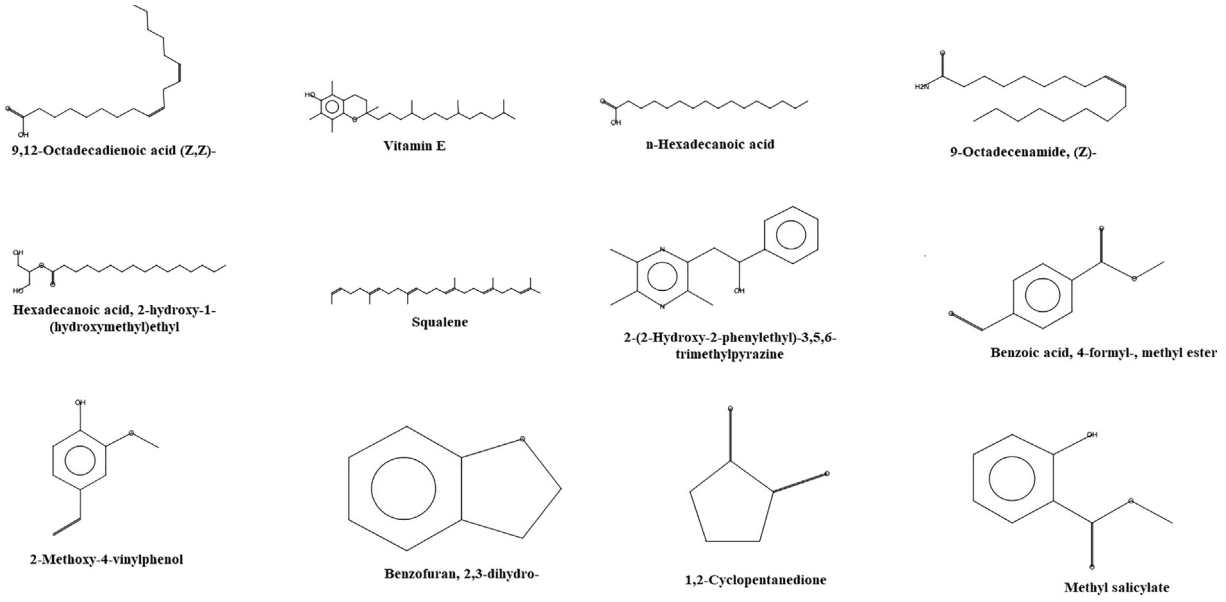

Fig. 3 GC–MS detection of possible bioactive compounds of the cold methanolic extract of olive tree leaves.

9-Octadecenamide, (Z)- is a fatty acid amide known as oleamide. It was identified at 86.54 min RT with a chromatogram area of 2.34%. Oleamide can bind to cannabinoid receptors and produce a variety of biological actions such as sleep induction, immunosuppression, and serotonin and GABA receptor activation (Moses et al., 2007). Oleamide has also been identified as a neurotransmitter that regulates thermoregulation and acts as an antinociceptive (William et al., 2007). Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester is otherwise termed as palmitic acid β -monoglyceride exhibited at 90.52 min RT with 1.69% area in chromatogram. β-palmitic acid is a natural saturated fatty acid found in human milk (Havlicekova et al., 2016).

It influences fatty acid metabolism, increases mineral balance, improves infants' sleep patterns, and reduces crying (Havlicekova et al., 2016 Litmanovitz et al., 2014). Squalene, a polyunsaturated hydrocarbon, was found in the methanolic extract of LOP with the highest RT of 97.39 min. However it only accounts for 1.18% area of the chromatogram in GC–MS analyses.

2-(2-Hydroxy-2-phenylethyl)-3,5,6-trimethylpyrazine has been found with a unique retention time of 66.23 min, a probability index of 54.35, 1.83 % area in the chromatogram. The importance of this chemical, on the other hand, has not been established.

Squalene has been reported for antioxidant, anticancer, drug carrier, skin hydrating, detoxifier, and emollient activities (Se-Kwon Kim and Fatih Karadeniz, 2012). In a study, Squalene has been shown to have antibacterial properties (Tahereh et al., 2021). It was reported in a previous study that the squalene@CPC nanosystems displayed significant efficacy against bacteria and fungi, having the greatest promise for eliminating

Table 2 FT-IR Spectroscopy of the cold methanolic extract of olive tree leaves.

Wavenumber (cm^{-1})	Intensity Estimation	Functional Groups/Class	Compounds
3313	S	O—H str. (hydroxyl)	Vitamin E, Polyphenols, Methyl salicylate, Tocopherols
2943	S	C—H str. (Alkene)	Vitamin E, Squalene
2831	S	CH_2 str. (Symmetrical)	Fatty acids, Vitamin E
1662	W	C═O str (Carboxylic & Ester groups), C═C str. (Alkenes)	Hexadecanoic acid, 9-Octadecenamide Octadecadienoic acid, 1,2-Cyclopentanedione, Amino acids, Oleic acid, Linoleic acid, Fatty acids, Methyl Esters, Squalene, Sterols, Carotenoids, Amino acids
1590	W	—NH str.	Amino acids
1449	M	C—H (Asymmetrical bending)	Squalene
1300	W	CH_2 bend (Alkane, Symmetrical)	Tocopherols
1111	M	C—O—C str.	Polyphenols
1020	S	C—O str.	Tocopherols, Methyl Esters

S: Strong; M: Medium; W: Weak.

Table 3 Antibacterial study of the cold methanolic extract of olive tree leaves.

Organisms	Zone of inhibition (mm)	
	Extract	Ciprofloxacin (5 µg/Disc)
Staphylococcus aureus ATCC 25923	19 ± 1.5	33.66 ± 1.24
Staphylococcus epidermidis ATCC 12228	18 ± 2	33.3 ± 1.24
Enterococcus faecalis ATCC 29212	15.5 ± 1.5	25.33 ± 0.4
Escherichia coli ATCC 25922	15.5 ± 2.5	35.66 ± 0.9
Klebsiella pneumoniae ATCC 700603	15.5 ± 2	26 ± 1.4
Salmonella choleraesis ATCC 10708	14.5 ± 0.5	33.3 ± 1.24
Pseudomonas aeruginosa ATCC 27853	14 ± 2	33.3 ± 0.8
Proteus mirabilis ATCC 299	15 ± 2	23.66 + 1.24

#Each value is the mean of 6 batches with standard deviation. All the values are compared to the standard ciprofloxacin disc by performing Tukey Kramer test (post hoc). All the test values are significantly lesser than the standard ciprofloxacin disc at $p < 0.05$.

Gram-positive bacteria (Fang et al., 2019). An earlier report suggested that squalene is rich in olive oil and has potent antioxidant potent (Owen et al., 2000).

Benzofuran, 2,3-dihydro furan, also termed as coumarins, have been detected at 34.53 min RT with 0.67% area of the chromatogram. Benzofuran is a structural unit found in a wide range of biologically active natural compounds and in a wide range of synthetic materials. Benzofuran derivatives have various biological effects including anti-inflammatory, antibacterial, antifungal, antihyperglycemic, analgesic, antiparasitic, and anticancer qualities (Khodarahmi et al., 2015). It has been reported that coumarins have anti-HIV (Xu et al., 2016) and antitubercular activity (Rama et al., 2010; Efimov et al., 2018). Vitamin E had the maximum RT of 108.75 min, although the chromatogram's percentage area was just 0.92 %. Vitamin E is the most important lipid-soluble component of the cell's antioxidant defense mechanism, and it can only be received through dietary sources. The antioxidant activity of this substance allows it to play a variety of critical roles in the body (Rizvi et al., 2014). Methyl salicylate is a benzoate ester that has been estimated at 32.26 min RT with a probability index of 33.04. However, it occupied a very lesser area 0.12% in the chromatogram curve. Methyl salicylate has analgesic and anti-inflammatory properties (Sánchez-Rodríguez et al., 2019). It is used in several over-the-counter analgesic creams and gels for topical application (Amitava and Amer, 2021; Sarah, 2007).

The results of the FT-IR investigation revealed the existence of distinct patterns of peaks (Fig. 4). The corresponding to major functional groups, were consistent with the presence of bioactive substances, particularly fatty acids, polyphenols, and vitamin E (Table 2). The prominent parabola-shaped peak at 3323 cm^{-1} with stretching vibrations suggests the existence of phenolic O—H groups, which correspond to Vitamin E, Polyphenols, Methyl salicylate, and Tocopherols. The present study also showed unique peaks at 2943 and 2831 cm^{-1} with stretching vibrations corresponding to the presence of fatty acids, vitamin E, and squalene. The stretching vibrations in the fingerprint region at 1662, 1590, 1449, 1300, 1111, and 1020 cm^{-1} also suggest the presence of fatty acids, vitamin E, and squalene. Recent report showed that the macerated methanolic extract olives leaves exhibited unique parabola peak at 3257, 2929, 1687, 1609, 1439, 1260 and 1018 cm^{-1} (Agatonovic-Kustrin et al., 2021). Aqueous extract of olives leaves showed a fingerprint region at 3383 with stretching vibrations, 2935 and 1705 cm^{-1} suggesting oleuropein, apigenin-7-glucoside and/or/or luteolin-7-glucoside (Gulboy et al., 2016).

Table 3 shows the spectrum of antibacterial activity. The readings demonstrate that the antibacterial effect of methanolic extract of LOP was highest against *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228. Interestingly the activity of methanolic extract of LOP was nearby range among Gram-positive bacteria as well Gram-negative bacteria respectively (Saad et al., 2022). However, the efficacy among Gram-positive and Gram-negative bacteria varied (Table 3). Fig. 3 is a self-exemplary suggested that the efficacy of methanolic extract of LOP significantly lesser when

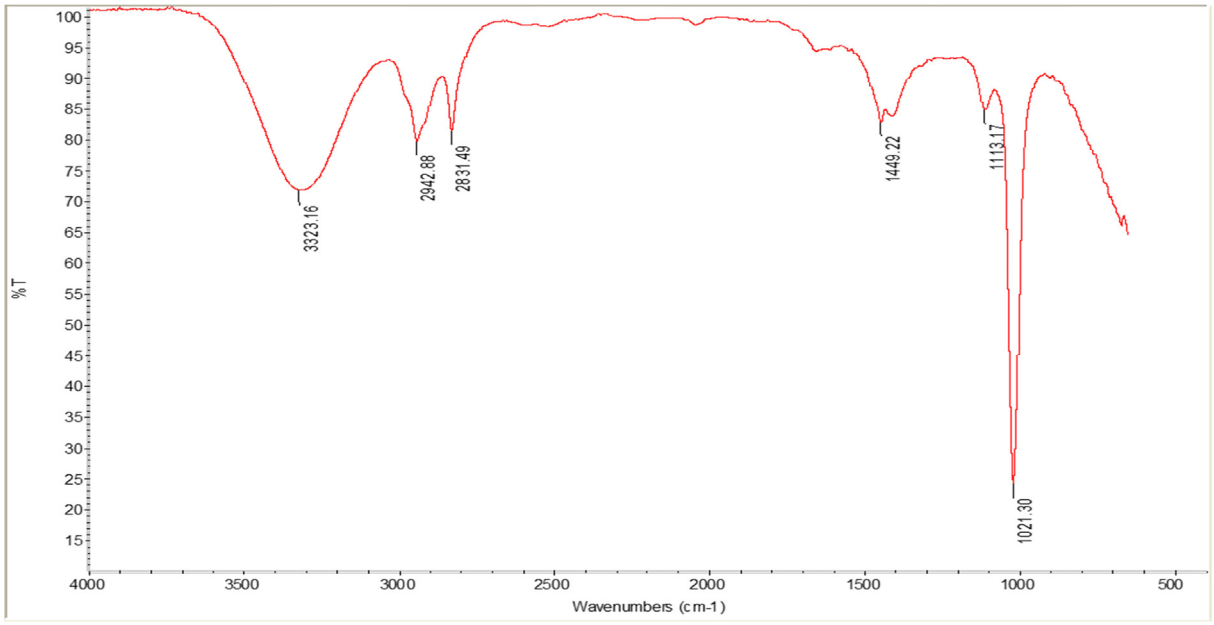

Fig. 4 The FT-IR spectra of compounds of the cold methanolic extract of olive tree leaves.

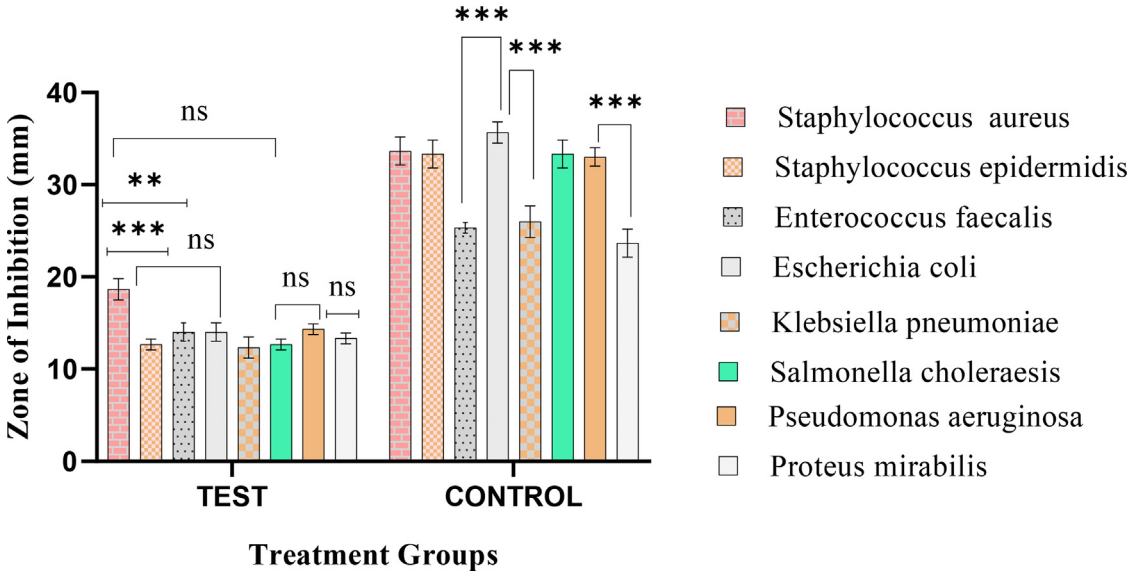

Fig. 5 Comparative antibacterial study of cold methanolic extract of olive tree leaves against standard Ciprofloxacin disc (5 g/Disc). **** The values are very high significant at $p < 0.05$ level. Highly significant at $p < 0.05$ level. *** The values are high significant at $p < 0.05$ level. Highly significant at $p < 0.05$ level, ** significant at $p < 0.05$ level.

compared to standard ciprofloxacin disc. From the analysis, the efficacy of methanolic extract of LOP is extremely significant at $p < 0.05$ level. The efficacy of methanolic extract of LOP was non-significant at $p < 0.05$ level among Gram-negative bacteria. Liu et al., 2017 reported that olives leaf extract inhibited biofilm formation in *L. monocytogenes* and *S. Enteritidis*. An earlier study demonstrated that the spectrum of activity of olive leaf extracts was observed significantly higher against *B. cereus* when compared to the rest of bacteria *S. aureus, E. faecalis, P. Vulgaris, E. coli, S. Typhimurium* (Mukadderat et al., 2014). The current investigation indicated a broader spectrum of action of methanolic extract of LOP despite much lower activity than conventional ciprofloxacin (Fig. 5).

4. Conclusion

Olives of the Hail region showed prominent bioactive substances through GC–MS and FT-IR analyses. According to the present study, the LOP cold methanolic extract contained fatty acids, vitamin E, squalene, polyphenols, and tocopherols. The study suggests that consuming olive leaves is beneficial to human health.

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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Author contribution

RUS: Principal investigator, funding resources; SMS: Conceptualization, experimentation, processing of results, writing and editing; ZUR, MSA, VKB, AAA: Performed experiments; RHA, RHA, NFA, KMW, FNA, AMH, FMA : Collection of plant materials and processing.

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