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Oleuropein determination, sequential phytochemicals analysis, antimicrobial and antioxidant activities of leaf extracts of two varieties *Olea europaea*

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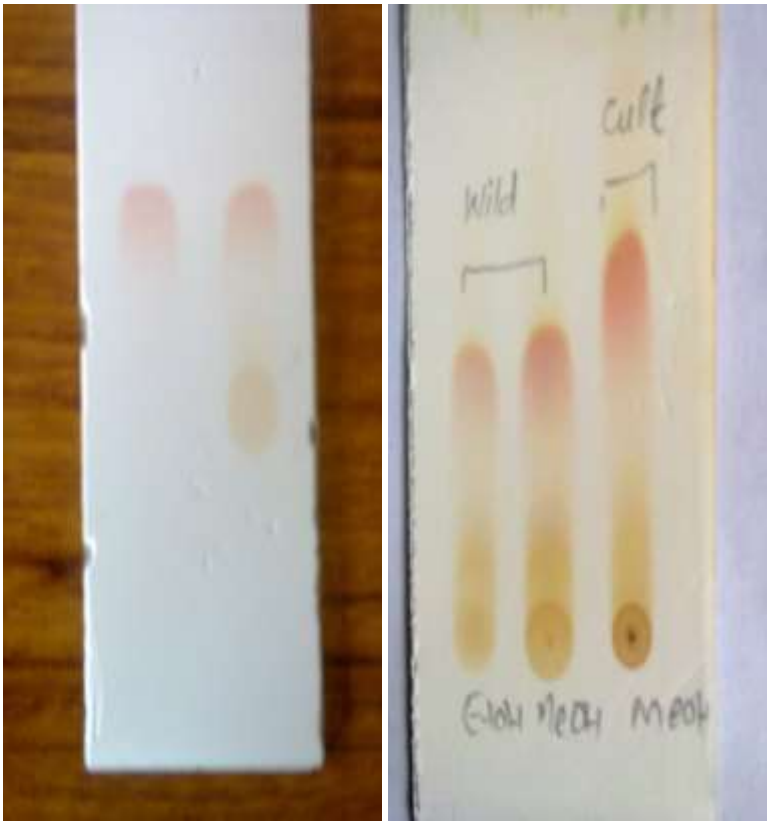
Abstract
In the current study, five different leaf extracts of wild and cultivated varieties of *Olea europaea* were tested for oleuropein, phytochemicals, antimicrobial and antioxidant activities. Thin Layer Chromatography (TLC) showed oleuropein in methanol and ethanol extracts. Phytochemical analysis by Fourier transmission Infra-red Spectroscopy (FT-IR) showed that wild variety contains more quantity bioactive components as compared with that of cultivated variety. The antimicrobial activities determined by disc diffusion method. It was observed that ethyl acetate and methanol extracts of both varieties (wild and cultivated) of olive leaves extracts exhibited maximum activity while minimum antimicrobial activities were observed for hexane extracts. The DPPH free radical scavenging assay of ethanol extract of *O. europaea* showed a linear correlation with phenol contents. Ethanol extract was more effective against most of the pathogenic bacteria while aqueous and ethyl acetate extracts were found more potent against most of the fungal strains. The current study revealed that wild variety of *O. europaea* is as an efficient source of oleuropein and have antioxidant and antimicrobial phytochemicals.

Keywords: *Olea europaea*, Oleuropein, TLC, Phytochemicals, FT-IR, Antioxidant activities

Introduction: Antioxidant activities Antimicrobial activities

Medicinal plants' bioactive components are used for the treatment of severe health problems [1, 3]. *Olea europaea* L., belonging to family *Oleaceae,* well known as olive from pre-history inhibit Mediterranean coastal zones. Food and pharmacological values of its fruits, roots, leaves and oils have gained importance in modern world [4, 7]. Olive reduces hyperlipidaemic complications and atherogenic indexes [8, 9]. The main component of *O. europaea* is Oleuropein possessing polyphenolics which are proven excellent antioxidants because of its scavenging actions preventing oxidation of LDL caused by the free radicals derived oxygen in circulatory systems [10, 13]. Polyphenols in olive oils (i-e carotenoids, phospholipids and tocopherols) possess therapeutic agents. *O. europaea* leaves display antimicrobial and antioxidation potentials. Flavonoids inhibit reduce the potentials of Alzheimer treating mediators [14, 16]. Besides these, numerous phenolic components are being extracted from *O. europaea* chiefly containing oleuropein. Olive oils monounsaturated fatty acids and antioxidants are anti-cancer agents and treat cardiac problems [17, 18]. Keeping in view the above importance of *O. europaea*, advance level research emphasizes on the use herbal plants as pharmaceutical components specifically for controlling microbial resistance to antibiotics. Main objectives of the present work are to investigate oleuropein content of *O. europaea*, its phytochemical components, antimicrobial and antioxidant activities of wild and cultivated varieties of *O. europaea* in Pakistan.

TLC analysis showed that oleuropein was present in *O. europaea* leaves extracts (Fig. 1). Ethanol extract of *O. europaea* confirmed the presence of oleuropein through HPLC, LC/MS analysis in leaves, stems and flowers [4, 19]. Oleuropein is highly potent against various microorganisms and is also important cytoprotective agent against cisplatin induced genotoxicity through the restoration of the antioxidant system of the renal hydroxy-20-deoxyguanosine [20, 21]. The presence of various phytochemicals was further confirmed by FT-IR analysis of the extracts (Fig. 2; A-J). FT-IR spectroscopy is an important tool for identifying the types of chemical bonds (functional groups) present in compounds [4].



(a) (b)

Fig 1: Determination of Oleuropein using TLC plates.

a. isoverbascoside [standard] (b) Methanol and ethanol extract

The results of phytochemical tests of wild and cultivated varieties of olive leaves were recorded in the Tables 1 and 2 and Fig. 2 (A-J) showed the presence of important phytochemicals (i-e alkaloids, phenols, and tannins, flavonoids, saponins, steroids, terpenoids, carbohydrates, and proteins) were present in almost all solvent extracts but wild variety contains more quantity bioactive components as compared with that of cultivated variety. Phenols, tannins, carbohydrates and proteins were absent only from the extracts obtained with n-hexane. Glycosides were absent in the extracts obtained with ethyl acetate whereas phenols were also absent in aqueous extracts. Their further investigative confirmation about phenols, flavonoids, tannins, and saponins etc. The various peaks in the spectra of different phytochemicals are detailed in the Fourier transmission Infra-red Spectroscopy (FT-IR) spectra attached as Fig. 2 (A-J).

Table 1: Phytochemical analysis wild variety of olive leaves extracts

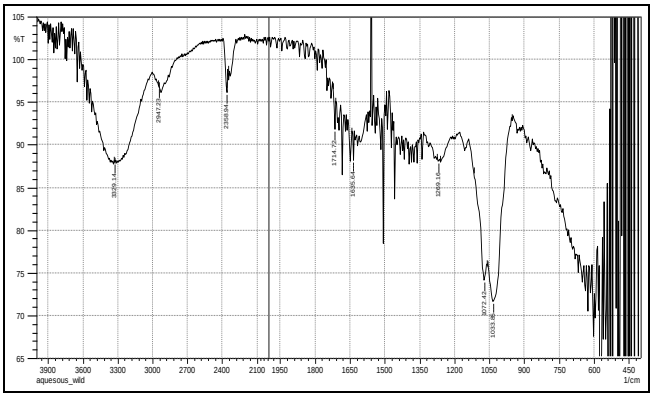
Plant extracts	Alkaloids	Glycosides	Saponins	Flavonoids	Phenols/Tannins	Steroids	Terpenoids	Riboflavin
Aqueous	+	++	+++	++	-	+++	++	+
Methanol	+	+	+++	+++	+	+++	++	++
Ethanol	++	++	+++	+	+	+++	+++	+
Ethyl Acetate	++	-	++	+	++	+++	++	+
n-Hexane	+	+	+	+	-	+++	++	+

- + sign shows the presence of respective phytochemical component.
- − sign shows the absence of a respective phytochemical component.
- ++ and +++ sign means the presence of a respective phytochemical component in high amount.

Table 2: Phytochemical analysis cultivated variety of olive leaves extracts.

Plant extracts	Alkaloids	Glycosides	Saponins	Flavonoids	Phenols/Tannins	Steroids	Terpenoids	Riboflavin
Aqueous	+	++	+++	++	-	++	++	+
Methanol	+	+	+++	++	+	++	++	++
Ethanol	++	++	+++	+	+	+++	+++	+
Ethyl Acetate	++	-	++	+	++	++	++	+
n-Hexane	+	+	+	+	-	++	++	+

- + sign shows the presence of respective phytochemical component.
- − sign shows the absence of a respective phytochemical component.
- ++ and +++ sign means the presence of a respective phytochemical component in high amount.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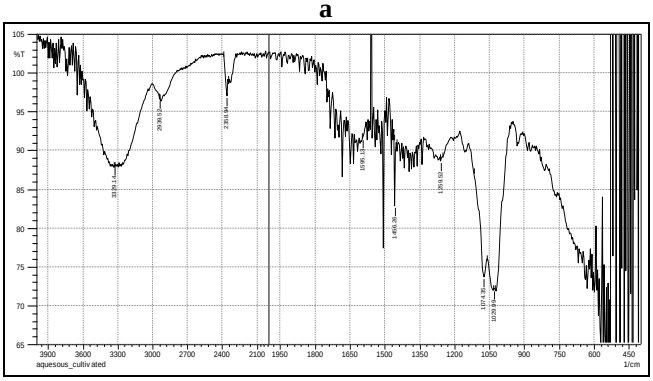
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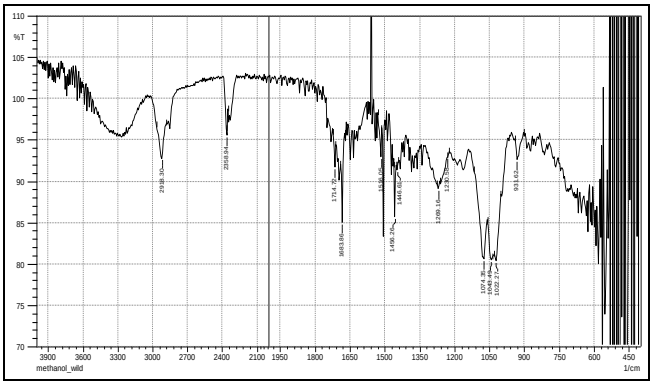
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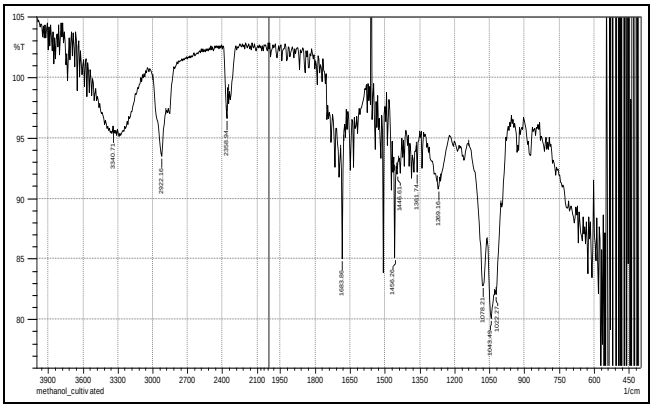
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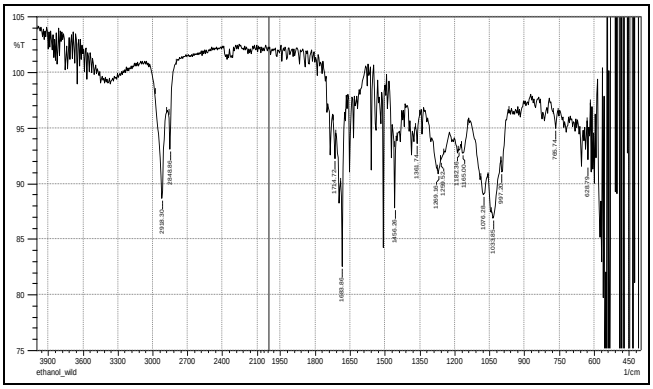
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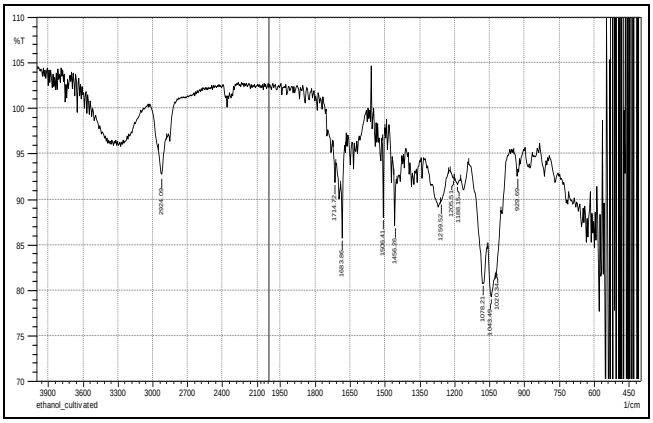
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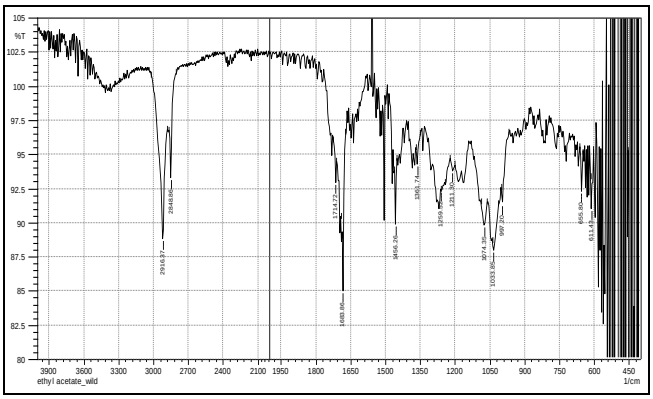
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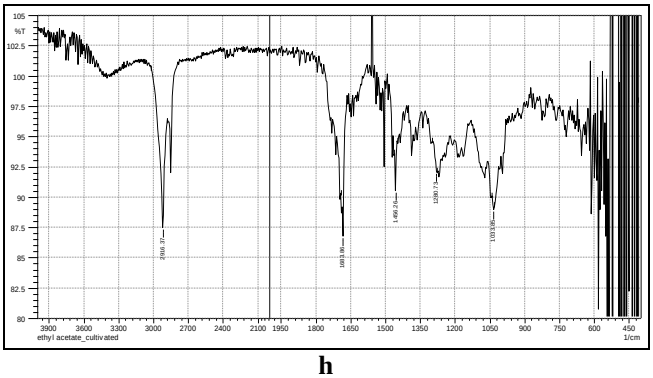
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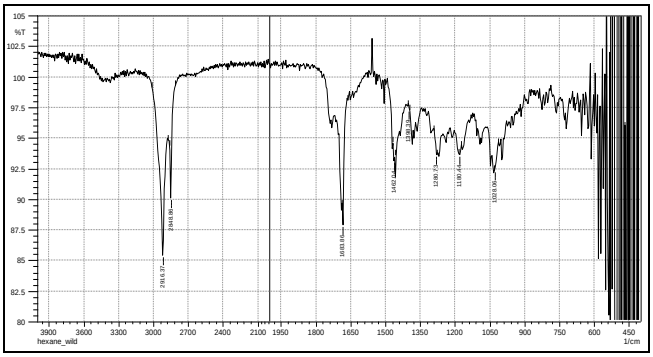

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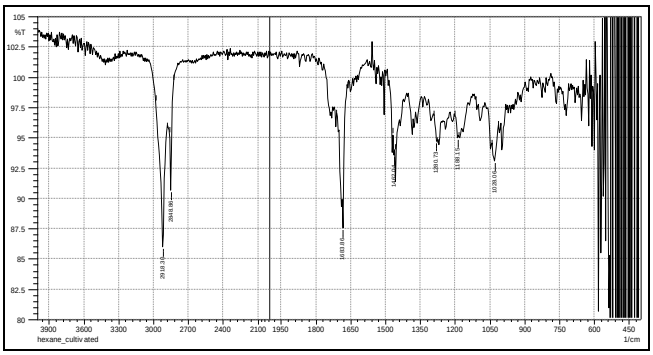

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Fig 2: (A-J) FT-IR spectra of various extracts of wild and cultivated varieties of *O. europaea* A= wild Aqueous, B= cultivated Aqueous, C= wild Methanol, D= cultivated Methanol, E=wild Ethanol, F= cultivated Ethanol, G= wild Ethyl acetate, H= cultivated Ethyl acetate, I= wild n-hexane, J= cultivated n-hexane

Both varieties extracts displayed significant antibacterial activities against all the tested strains as shown in Tables 3and 4. The disc diffusion method resulted from the measurements of the zone of inhibition **(**ZOI**)**, specified that methanol extract exhibited major inhibition activity contrary to *B.atrophus*, with the highest inhibition zones **(**10 mm**)** that were computable with its antibiotic counterpart, clarithromycin with a zone of inhibition **(**16 mm**)**. The lowest ZI value **(**2 mm**)** was observed for n-hexane extract against *P. aurigonosa* and *S. typhi* that was 6 times lower than its antibiotic standard, ciprofloxacin. Antibacterial activities of different plant extracts obtained were seemed to be too much diverse in the sense of efficacy as some of the bacterial strains are found more resistant while some others are found to be highly susceptible to the extracts in comparison with their respective antibiotics used in this study. All the bacterial strains show fewer susceptibilities to both plant extracts as compared to standard antibiotics used which show that both the standard antibiotics and plant crude extracts have higher antibacterial efficacies to Gram-positive bacterial strains as compared to those of Gram-negative bacterial strains.

Both varieties extract also displayed significant antifungal activities against all the tested strains as shown in Tables 5 and 6. The disc diffusion method resulted from the measurements of the zone of inhibition **(**ZI**)**, showed that aqueous extract exhibited major inhibition activity contrary to *A. parasiticus*, with the highest inhibition zones (8 mm**)** that were computable with its antibiotic counterpart, clotrimazole with a zone of inhibition (17 mm**)**. The lowest ZI value **(**4 mm**)** was observed for aqueous extract against *A. nigar* that was four times lower than its antibiotic standard, clotrimazole. Antifungal activities of different plant extracts obtained were seemed to be too much diverse in the sense of efficacy as some of the fungal strains are found more resistant while some others are found to be highly susceptible to the extracts in comparison with their respective antibiotics used in this study. All the fungal strains show fewer susceptibilities to both plant extracts as compared to standard antibiotics used which show that both the standard antibiotics and plant crude extracts have higher antifungal activities to fungal strains.

Due to microbial multi-drugs resistivity, plants are chief sources prior to novel medicines against microorganisms. Saponins exhibit antimicrobial activities [22]. Reports showed the antimicrobial activities of olive leaves extracts against *B. subtilis, K. pneumoniae, S. aureus, P. aeruginosa, S. typhi, P. vulgaris, C. freundii* and *S. pneumonia* [23, 25]. Alkaloids, saponins, and tannins are potent antibiotic agents [26, 28]. Flavonoids, hydroxylated phenol-rich compounds also show antimicrobial activities and possess antioxidant as well as anticancer characteristics [29, 31].

The antimicrobial activity of both varieties olive leaves extracts was determined against gram positive, gram negative bacterial and fungal strain. The plant showed a broad spectrum of antimicrobial activities against both types of bacterial strains. Antifungal activity of olive leaves extracts was evaluated for the first time in this study and results were much more encouraging as expected. Moreover, different types of organic solvents were used in this study to examine the effects of solvents on antimicrobial activity and to determine which solvents are more effective in showing antimicrobial activity. The results supported methanol and ethyl acetate as the best options for antimicrobial olive leaves extracts. The antimicrobial activities of wild varieties methanolic extracts in comparison with the applied antibiotics were due to phenolic compounds and flavonoids present in these extracts

which were further confirmed by the Fourier transmission Infra-red Spectroscopy **(**FT-IR**)** spectra. The antimicrobial activities revealed that methanolic and ethyl Acetate extracts of both varieties (wild and cultivated) of olive showed maximum activity while minimum antimicrobial activities were observed for hexane extracts. It is because alcohol and ethyl acetate extracts give rise to flavonoids and phenolic phytochemicals as compared to hexane which was confirmed from the FT-IR spectra (Fig.2 A-J).

The obtained results of measurement of zone of inhibition (ZI) from the disc diffusion method specified that the methanol extract exhibited strong inhibitory activities against *B. atrophus*, with the highest inhibition zones **(**10±0.40 mm**)** that were computable with its antibiotic counterpart, clarithromycin with inhibition zones **(**16±0.21 mm**)** for both varieties of *O. europea*. The inhibitory activity of these extracts confirmed the antibacterial activity and its potential use in the treatment of microbial diseases.

The lowest ZI value **(**2±1.07 mm**)** was observed for n-hexane extract against *P. aurigonosa* and *S. typhi* that was six times lower than its antibiotic standard, ciprofloxacin. Antibacterial and antifungal activities of different extracts of *O. europea* obtained were seemed to be too different in the sense of efficiency as some of the bacterial and fungal strains are found to extra resistance and others are having more susceptibility towards the plant crude extracts as compared to their respective antibiotics used in this study. The screening of both wild and cultivated varieties of olive leaves extracts especially wild variety proved that they possess high medicinal values and may be effectively used as potential and valuable drugs reservoirs.

Table 3: Antibacterial activity of wild variety of *O. europaea* leaves extracts

Bacterial Strains	Solvent Extracts					Standard Drugs	
	Aqueous	Methanol	Ethanol	Ethyl acetate	n-Hexane	Clarithromycin	Ciprofloxacin
	Zone of Inhibition (mm)						
Bacillus atnophus	7±1.25	10±1.25	9±0.81	9±0.81	7±1.25	16±5.4	-
Salmonella typhi	8±1.25	9±0.81	5±0.8	9±0.81	2±0.81	-	13±0.94
Escherichia coli	3±0.81	8±1.25	5±0.8	9±0.81	3±0.81	-	14±1.02
Agrobacterium tumefaciens	3±0.81	7±1.25	5±0.80	6±1.63	4±1.11	-	13±0.94
Pseudomonas aeruginosa	5±0.8	8±1.25	4±1.11	7±1.25	2±0.82	-	15±0.42
Staphylococcus aureus	7±1.25	9±0.81	5±0.8	7±1.25	3±0.81	14±1.02	-
Klebsiella pneumonia	5±0.8	9±0.81	6±1.63	7±1.25	5±0.8	-	14±1.02
Bacillus subtillus	7±1.25	8±1.25	6±1.63	8±1.25	4±1.11	14±1.02	-
Erwinia carrotovora	7±1.25	10±1.25	7±1.25	7±1.25	7±1.25	-	15±0.42

Table 4: Antibacterial activity of cultivated variety of *O. europaea* leaves extracts

Bacterial Strains	Solvent Extracts					Standard Drugs	
	Aqueous	Methanol	Ethanol	Ethyl acetate	Hexane	Clarithromycin	Ciprofloxacin
	Zone of Inhibition (mm)						
Bacillus atnophus	7±1.25	10±1.25	8±1.37	9±0.81	7±1.25	16±5.4	-
Salmonella typhi	7±1.25	9±0.81	5±0.8	9±0.81	2±0.81	-	12±1.25
Escherichia coli	4±1.11	9±0.81	7±1.25	9±0.81	4±1.11	-	14±1.02
Agrobacterium tumefaciens	7±1.25	7±0.48	5±0.8	8±1.37	3±0.82	-	
Pseudomonas aeruginosa	5±0.8	9±0.81	4±1.11	7±1.25	2±0.82	-	12±1.25
Staphylococcus aureus	7±1.25	6±1.63	5±0.8	7±1.25	3±0.82	15±0.42	-
Klebsiella pneumonia	5±1.25	7±1.25	6±1.63	7±1.25	5±0.8	-	13±0.94
Bacillus subtillus	7±1.25	8±1.37	6±1.63	8±1.37	4±1.11	16±5.40	-
Erwinia carrotovora	9±0.81	6±1.63	8±1.25	7±1.25	6±1.63	-	13±0.94

Table 5: Antifungal activity of wild variety of *O. europaea* leaves extracts.

Fungal Strains	Solvent Extracts					Standard Drugs	
	Aqueous	Methanol	Ethanol	Ethyl acetate	Hexane	Fluconazole	Clotrimazole
	Zone of Inhibition (mm)						
Aspergillus flavus	8±1.01	7±0.62	7±0.21	6±0.58	7±0.54	-	16±2.23
Aspergillus nigar	4±0.36	6±0.59	6±0.38	7±0.45	8±0.71	17±2.86	-
Aspergillus parasiticus	7±0.17	6±0.36	8±0.74	7±0.61	5±0.25	14±1.57	-
Fusarium solani	6±0.56	7±0.32	6±0.83	5±0.33	5±0.49	-	15±0.62
Candida albicans	5±0.82	8±0.55	5±0.71	7±1.03	6±1.63	-	14±1.02
Candida glaberata	6±0.65	7±0.46	6±0.82	5±0.63	5±0.72	-	16±0.81

Table 6: Antifungal activity of cultivated variety of *O. europaea* leaves extracts.

Fungal Strains	Solvent Extracts					Standard Drugs	
	Aqueous	Methanol	Ethanol	Ethyl acetate	Hexane	Fluconazole	Clotrimazole
	Zone of Inhibition (mm)						
Aspergillus flavus	7±0.53	6±0.54	7±0.32	5±0.34	6±0.48	-	16±2.23
Aspergillus nigar	5±0.52	6±0.38	5±0.27	6±0.61	7±0.42	17±2.86	-
Aspergillus parasiticus	6±0.69	7±0.41	7±0.56	6±37	5±0.34	14±1.57	-
Fusarium solani	7±0.47	6±0.44	5±0.31	5±0.29	5±0.70	-	15±0.62

Candida albicans	5±0.71	7±0.38	6±0.55	6±0.34	6±0.52	-	14±1.02
Candida glaberata	7±0.32	6±0.59	7±0.37	6±0.51	5±0.72	-	16±0.81

The 2, 2- Diphenyl Picryl Hydrazyl (DPPH) free radical assay analysis obtained data using standard protocols contrary to various solvents extracts of both varieties *O. europaea* leaves are formulated in the Tables 7 and 8. The results showed that at 100 µg/mL, the n-hexane extracts displayed top scavenging activities (46.1% for wild and 44.6% for cultivated) just tracked by ethyl acetate extract (42.3% for wild and 41% for cultivated), ethanol (41.4% for wild and 38.4 % for cultivated), methanol (36.3% for wild and 43.6 % for cultivated) and aqueous having least activities (28.6% for wild and 31.1 % for cultivated). At 200 µg/mL, the n-hexane extracts showed high scavenging activities (69.3% for wild and 70.1% for cultivated) consecutively followed by ethanol (64.5% for wild and 56.4 % for cultivated), methanol (54.2% for wild and 60.8 % for cultivated), ethyl acetate extract (59.3% for wild and 56.1% for cultivated) and aqueous having least activities (39.4% for wild and 4.6% % for cultivated). At 400 µg/mL, the n-hexane extracts showed high scavenging activities (86.6 % for wild and 82.1% for cultivated) consecutively followed by ethanol (78.3% for wild and 67.3 % for cultivated), methanol (69.1 % for wild and 71.3 % for cultivated), ethyl acetate extract (63.4% for wild and 68.4% for cultivated) and water having least activities (48.3% for wild and 50.1% % for cultivated). At 800 µg/mL, the n-hexane extracts exhibited highest scavenging activities (88.4 % for wild and 83.0% for cultivated) consecutively followed by ethanol (83.1% for wild and 76.6 % for cultivated), methanol (76.5 % for wild and 83.6 % for cultivated), ethyl acetate extract (71.3% for wild and 76.6% for cultivated) and water having least activities (60.4% for wild and 63.2% % for cultivated). Among all the extracts of both varieties *O. europaea* leaves, n-hexane showed highest Inhibition of DPPH (%) of 88.4% compared to that ethanol, methanol and Ethyl acetate activity of 28.6%.

Relative studies on stem ethanolic extract in comparison with flower and leaf for the highest phenols has been done [32]. Phenol components are free radical scavengers to prevent peroxides formation as they show redox abilities for neutralizing free radical groups and are peroxidation decomposers. The polyphenols are effective as anti-cardiovascular, anticancer, anti-inflammation, and antiaging agents [33, 34]. Besides this, lignans, anthocyanins, flavonoids, etc. are significant antioxidants [35]. Recent study on olive proved phenolic alcohols, phenolic acids, flavonoids and oleuropein [36]. Oleuropein is the major polyphenol in olive leaf imparting unpleasant perception to olives and their oils [25]. The phenolic composition of olives is affected by different conditions like cultivars, ecological circumstances and collecting period [37, 39]. The free radical scavenging facts revealed the capabilities of these compounds from olive leaves that compounds to donate electrons/protium for the prevention of free radical facilitated cascades.[40] Olives also cause reduction of unsaturated fatty acids damage in soya oils via hindering lipids peroxides and rise the vitamin E obtainability along with extra phytochemical agents which stop oxidation [40].

The current studies on leaves of *O. europaea* confirmed phytochemicals showing antioxidant antibacterial and antifungal potentials. Further research interests have margins for the pure form of this plant to exploit for its enhanced activities in the course of pharmacological values.

Table 7: Inhibition of DPPH (%) by different extracts obtained from wild variety of *O. europaea*

Concentration (µg/mL)	Solvent Extracts				
	Aqueous	Methanol	Ethanol	Ethyl Acetate	n-Hexane
	Inhibition of DPPH (%)				
100	28.6	36.3	41.4	42.3	46.1
200	39.4	54.2	64.5	56.1	69.3
400	48.3	69.1	78.3	63.4	86.6
800	60.4	76.5	83.1	71.3	88.4

Table 8: Inhibition of DPPH (%) by different extracts obtained from cultivated variety of *O. europaea*

Concentration (µg/mL)	Aqueous	Methanol	Ethanol	Ethyl Acetate	n-Hexane
	Inhibition of DPPH (%)				
100	31.1	43.6	38.4	41.1	44.6
200	40.6	60.8	56.4	59.3	70.1
400	50.1	71.3	67.3	68.4	82.1
800	63.2	83.6	78.1	76.6	83.0

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Conflicts of interest None.

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