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ORIGINAL ARTICLE

Evaluation of antibacterial and antifungal activities of olive *(Olea europaea)* essential oil

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Context: Essential oil *Olea europaea* was investigated for its antibacterial and antifungal activities. **Aim:** To evaluate antimicrobial activity of *O. europaea* essential oil against infectious microbial pathogens. **Settings and Design:** Seeds of *O. europaea* were grounded by using domestic mixer and powdered material was hydro-distilled in Clevenger apparatus continuously for 5 hrs to yield essential oil. Essential oil was analysed on Gas-Chromatography-Mass spectrometry (GC-MS) from which 24 components were identified, representing total 99.98% of the oil. Extracted oil was evaluated for their antibacterial and antifungal activities. **Materials and Methods:** Paper disc diffusion and serial micro-dilution assays were performed for the determination of inhibition zone diameters and minimal inhibitory concentration, respectively. **Results:** The *O. europaea* essential oil showed the diameter of inhibition zone (DIZ) ranging from 19.4 ± 0.07-26.4 ± 0.09 mm at a concentration level of 28 µg/disc (W/V) separately in all the ten strains tested. The minimum inhibitory concentration of essential oil against bacterial strains was obtained in a range of 7.0-56.0 µg/ml while in and fungal strains it was in a range of 7.0-28 µg/ml. **Statistical analysis:** All statistical calculations are expressed as mean ± SE of three replicates. Data were analyzed by one-way Analysis of Variance (ANOVA) to locate significant variations in oil activity in various bacterial and fungal strains followed by the Duncan's multiple range tests. **Conclusions:** Antibacterial and antifungal activities of *O. europaea* essential oil are due to the presence of certain secondary plant metabolites such as terpenoids, steroids and flavonoids, esters, and acids, which were identified in the essential oil. The oil components can be further investigated for their biological activities and study to overcome the problem of drug resistance in microbes.

Key words: Antimicrobial activity, essential oil, gas-chromatography-mass spectrometry analysis, inhibition zone diameters, minimum bacterial concentration, minimum fungicidal concentration, minimum inhibitory concentration, *Olea europaea*

INTRODUCTION

Plant essential oils are aromatic oily liquids, which possess broad-spectrum antimicrobial activity against diverse groups of pathogens.[1] Essential oils possess specific volatile odour or flavours and are obtained from various plant parts such as flowers, buds, seeds, leaves, twigs, barks, woods and roots.[2] Plant species of more than 60 families of angiosperms mainly Lamiaceae, Rutaceae, Geraniaceae, Apiaceae, Aspetaceae, Lauraceae, Fabaceae, and Poaceae possess essential oils with various chemical constituents such as lectins, polypeptides, alkaloids, phenols, quinines, flavones, flavonoids, terpenes, tannins, coumarins, benzene derivatives, various hydrocarbons and straight chain compounds, which showed antibacterial,[3] antifungal,[4] anti-cancer,[5,6] and anti-oxidant activities.[7] Essential oils are isolated as single or in combination without making any change in their chemical composition.[8]

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These are used for a wide variety of purposes,[9] such as flavoring, perfuming[10] and food preservation.[11-13] These are obtained by fractionation or rectification and steam distillation of various plant parts in Clevenger apparatus.[14] Essential oils possess mixed functional groups, too complex in their structure and are highly volatile at a very low temperature. Due to high volatility, essential oils easily spread in the environment and medium. These act more efficiently against drug-resistant microbes and show least residual effect in the body, inhibit growth and metabolism of a variety of infectious pathogens mainly microbes. These are also used as alternative medicine in aromatherapy for treatment of cancer.[15,16]

O. euproaea is a small tree, which is most commonly grown in European countries. Since last one decade, its plantation has been growing in Gujarat and Rajasthan. In India *O. euproaea* is known as Jaitoon. Olive oil is highly rich in monounsaturated fats, antioxidants and vitamin E. Olive oil is claimed to have a significant effect on cholesterol level and decrease the risks of cardiovascular diseases and diabetes. However, in the present study, *O. europaea* essential oil was screened for its antimicrobial activity *in vitro* by applying various bioassays. Hence, various antimicrobial susceptibility tests and growth inhibitory bioassays were conducted

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by setting controls and treatments done in seven bacterial strains i. e. *Escherichia coli, Bacillus cereus, Klebsiella pneumoniae, Lactobacillus acidophilus, Staphylococcus aureus and Streptococcus pneumoniae and Micrococcus luteus* and three isolates of fungi i.e. *Candida albicans, Aspergillus niger, Rhizopus stolonifer*. Minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC), minimum fungicidal concentration (MFC), inhibition zone diameters were determined in bacterial and fungal strains were determined in presence of each varying concentrations of essential oil. Results were compared with broad-spectrum antibiotics.

MATERIAL AND METHODS

Plant Material

The seeds of *O. europaea* were collected from Bikaner and Nagaur District of Rajasthan. Olive farming experts identified the plant.

Extraction and Isolation of Essential Oil

Seeds of *O. europaea* were grounded by using domestic mixer and powdered material was hydro-distilled in Clevenger apparatus continuously for 5 hrs to yield essential oil.[13] The course powder was extracted with pure methanol thrice and dried residue was dissolved in known volume of fresh solvent (W/V) before testing the antimicrobial activity.

Antimicrobial Activity

Source of Microorganisms

Cultures of seven pathogenic bacterial strains each of *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778), *Lactobacillus acidophilus* (ATCC 53103), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (ATCC 15380) and *Streptococcus pneumoniae* (ATCC 12755) were maintained in the laboratory in Luria Broth (2% w/v) regularly for four days at 37°C before being used in experiments. For experiments, a portion (100 µl) of the overnight culture was mixed in the tests and control for inoculation. For activity, testing bacterial cultures were stored at 4°C and sub cultured after every 8th day in solid agar plates. For determination of antifungal activity of plant latex, fungal strains of *Aspergillus niger MTCC 1344*, *Candida albicans MTCC 227*, *Rhizopus stolonifer* MTCC 3789 were grown in the laboratory. Moreover, each test fungi was maintained in strain specific agar medium mainly Sabouraud's agar and potato dextrose agar and its pure cultures were established by using single spore isolation technique.

Disc Diffusion Assay

The *in vitro* antimicrobial activity of *O. europaea* was evaluated in agar-disc diffusion assay and the inhibition zone diameters were measured in the presence and absence of essential oil. Serial dilutions of essential oils were made by taking 5.6 gm (5.6 ml) of essential oil and to it 5.6 ml of Luria broth media was added and mixed and then half of it was transferred in the next tube containing 5.6 ml of media. It was followed for 10 regular steps for making all down series dilutions. From all dilutions, 1.4 gm/ml (1.4 µg/µl) concentration was found suitable for study because of activity found in microbial cultures. From this solution, further dilutions were made as 0.7, 1.4, 2.8, 5.6 and 11.2 gm/ml concentrations, and this gives a final concentration of 0.7 µg/ml-11.2 µg/µl (W/V). Six different concentrations of essential oils ranging from 0.7 µg/µl-11.2 µg/µl (W/V) were used for agar-disc diffusion assay. However, 10 µ1 of each concentration coated on sterile filter paper discs (Whatmann No. 1) of 6-mm size to have above mentioned concentrations. These oil-impregnated discs were dried under laminar flow cabinet. Before starting the experiments, the inoculum size was determined and adjusted to prepare a final colony number as to 10^8 colony forming units (CFU/ml) in sterile agar plates. Bacterial inocula were spread evenly on to the surface of agar plate by using a sterile rubber pad spreader. After which essential oil coated discs were positioned on the inoculated agar surface in the centre. Each essential oil was assayed in triplicate for antibacterial activity testing. All treated and untreated plates were incubated for 24 hrs at 37°C. Dimethyl sulfoxide (DMSO) was used as negative control, whereas ampicillin was used as standard (positive control) to compare the bacterial growth and Griseofulvin was used to compare fungal growth in negative control. The radial growth of fungi was measured after 12 hrs interval up to 36 hrs of initial inoculation. The average percentage inhibition of growth in presence of various essential oils was calculated by using following formula,

Inhibition (%) =100 (C-T)/C

Where C = diameter of fungus colony in control plates, T = diameter of fungus colony in tested plates.

Minimum Inhibitory Concentration (MIC)

Bacterial growth inhibition was accessed in the presence of different increasing concentrations of essential oil in Luria broth culture medium and MIC values were determined for each bacterial and fungal strain. For this purpose, essential oils were diluted in a concentration range from 32 µg/ml to 0.0078 µg/ml by using serial micro dilution method. The tested essential oil was added to fresh suspension after following the serial dilutions up to 10^{-10}. Each oil was assayed in triplicate. Before conducting experiments all the conditions for *in vitro* anti-microbial activity were standardised to determine MIC and MBC values. The MIC values were considered as the lowest concentration of essential oil, in which no turbidity in the culture flask was visualised after 24 hrs of incubation at 37°C. The turbidity in the culture flasks was considered as visible growth of microorganisms. Further,

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it was standardised in terms of absorbance at 600 nm in a visible spectrophotometer. For determination of MBC growth inhibitory assays were performed. For this purpose, size of inoculum was adjusted to prepare a final colony number as to 10^8 CFU/ml in a sterile agar plates. The incubation of test and control cultures was also performed at 37°C for 24 hrs. For comparison, both negative and positive controls were set and bacterial colony number was counted in all test and control discs. For comparison three broad-spectrum antibiotics i.e. ampicillin was used as standard to compare the bacterial growth while Griseofulvin for comparison of fungal growth. Results were interpreted by using a standard table that relates to the degree of microbial resistance prescribed by NCCLS (National Committee for Clinical Laboratory Standards). A plot of MIC on a logarithmic scale *versus* zone inhibition diameters (arithmetic scale) was prepared for each essential oil and antibiotic to know the susceptibility level. These plots were used to find the zone inhibition diameters corresponding to the drug concentrations and that of the essential oils. The low MIC value was considered as susceptibility of essential oils/drugs to the pathogen, whereas high MIC value (with a small zone inhibition diameter) was considered as resistant.

Gas-Chromatography-Mass Spectrometry

GC/MS analyses of *O. europaea* essential oil were carried out on a Shimadzu GC-MS-QP2010 apparatus equipped with a -5 column (60 m × 0.25 mm i. d., film thickness of 0.25 µm). Helium was used as carrier gas at a constant column flow 1.2 ml/min at 173 kpa inlet pressure and injector volume was 1.0 µL. The test was performed according to a set temperature programming, which was maintained from 100°C to 200°C with constant rise of 5°C/min and then held isothermal at 200°C for 6 min. Further, the temperature was increased by 10°C/min up to 290°C and again held isothermal at 290°C for 10 min. The injector and ion source temperatures were 270°C and 250°C, respectively. The crude and active bands (AB-1) and (AB-2) (2 mg/ml) were dissolved in methanol (HPLC grade, Merck, India) and are injected with a split ratio of 1:10. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 950 Dalton. The identification of the essential oil components was based on the comparison of their relative retention time (tR) and mass spectra with those of commercial standards (for the main components) and on the gas chromatography (GC) retention indices (RI) determined relative to n-alkanes (C8-C32). The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the Wiley and National Institute Standard and Technology (NIST) libraries mass-spectral database. The relative percentages of the essential oil constituents were calculated from the GC peak areas. Further, quantitative analysis was performed by means direct peak area intention technique based on the TIC.

Statistical Analysis

All statistical calculations are expressed as mean ± SE of three replicates. Data were analysed by one-way ANOVA to locate significant variations in oil activity in various bacterial and fungal strains followed by the Duncan's multiple range tests.

RESULTS

Plant origin natural products are known to have more antimicrobial activity against drug resistant microbes. This activity could act as chemical defence against pathogenic diseases. However, in the present time, both the traditional and folk medicines have been considered as alternatives of synthetic drugs for healthcare to the patients. However, for screening pharmaceutical and therapeutic potential of these natural products various bioassays are developed to detect and confirm the anti-pathogenic effects and establish a good correlation with disease pathogens. For obtaining broad-spectrum drugs, essential oils are found to be good therapeutic-targeting molecules. The present study emphasises the composition of essential oil isolated from *O. europaea* and its effect on inhibition of bacterial and fungal growths.

The chemical constituents of essential oil from *O. europaea* are listed in Table 1. Twenty-four components were identified by using GC representing 99.98% of the oil. The main constituents of essential oil were identified 2-propanone (8.80%),2,4-imidazolidinedione (14.92%), Z-(13,14-Epoxy) tetradec-11-en-1-ol acetate (7.77%), 1-methyl-3-beta-phenylethyl-2,4,5- trioxoimidazolidine (1.00%), 1-(Hydroxymethyl)-1,2-ethanediyl ester (9.41%), 2-Hydroxy-1-3-propanediyl ester (1.26%), 9-Hexadecenal (44. 10%), 6,11-Hexadecadien-1-ol (5.02%). The dominant components are 2-Heptadecanon and n-Octadecanoic acids but in different ratios [Table 1] together with few compounds found in minor concentrations [Figure 1]. These results are in accordance with the previously published data on *O. europaea*.

Disc diffusion assays were conducted with *O. europaea* essential oil to measure growth inhibition zone diameter and screen anti-microbial potential. The essential oil of *O. europaea* has shown higher range of inhibition zone diameter 19.4 ± 0.07-26.4 ± 0.09 mm at a concentration level of 28 µg/disc. The positive control has shown diameter of inhibition zone (DIZ) ranging from 16.2 ± 0.07 - 20.0 ± 0.09 mm at concentration of 30 µg/disc. All DIZ corresponding to test organisms are mentioned in Table 2.

The results of MIC obtained against all the bacterial strains have been given in Table 3. Lower MIC values presented have shown very high antimicrobial susceptibility in *O. europaea*. *E coli, Bacillus cereus, L. acidophilus* and *S. pneumoniae* are

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Table 1: Chemical composition of essential oil isolated from *O. europaea*

Peak no.	RT (minutes)	Area (m²)	Composition %	Name of the compound
1	7.635	46770304	8.80	2-Propanone
2	16.917	3928993	0.74	2-hydroxy ethylbenzene
3	18.460	1003300	0.19	Monohydroxy benzene
4	19.752	921565	0.17	p-cresol
5	22.406	1491206	0.28 2-	Methyl-6-(4-methyl-1, 3-cyclohexadien-1-yl)-2- hepten-4-one
6	23.036	3490638	0.66	2-Heptadecanon
7	24.416	4115653	0.77	3,7-cyclodecandien-1-one
8	24.811	79292408	14.92	2,4-Imidazolidinedione
9	25.758	3395006	0.64	11-hexadecenal
10	26.738	1170784	0.22	cis-o-coumarinic acid lactone
11	28.235	41314283	7.77	Z-(13,14-Epoxy) tetradec-11-en-1-ol acetate
12	28.704	3837276	0.72	9-octadecenoic acid ethyl ester
13	29.224	2800122	0.53	9, 12 octadecedien-1 ol
14	29.809	3089352	0.58	citronellyl acetate
15	30.984	3512859	0.66	4-flourophenyl ester
16	31.315	1171461	0.22	3, 7 dimethyl-2-6-octadienylester
17	31.917	1156967	0.22	2-methylphenyl ester
18	33.149	5291723	1.00	1-Methyl-3-.beta.-phenylethyl-2,4,5- trioxoimidazolidine
19	34.416	50009751	9.41	1-(Hydroxymethyl)-1,2-ethanediyl ester
20	35.031	4225575	0.80	n-Octadecanoic acid
21	38.947	1729184	0.33	1-4 ethanobenzocyclodecene
22	39.503	6709710	1.26	2-Hydroxy-1-3-propanediylester
23	40.119	234337943	44. 10	9-Hexadecenal
24	41.537	26656611	5.02	6,11-Hexadecadien-1-ol
Total			99.98	

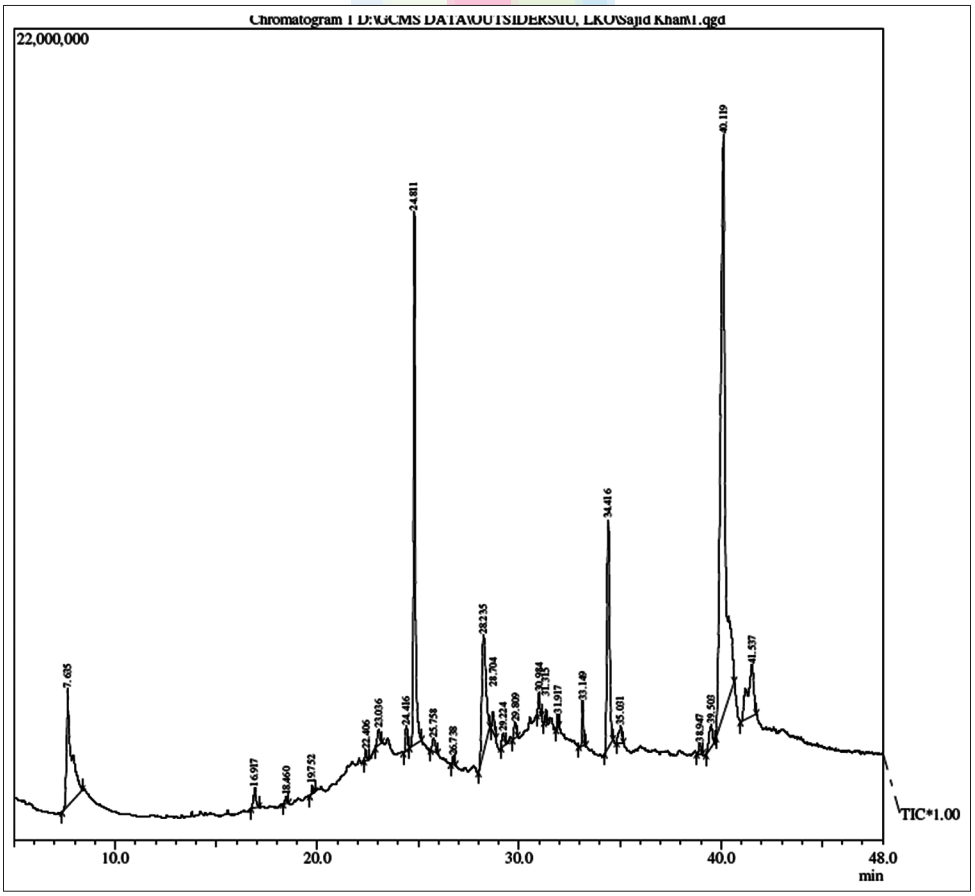

Figure 1: GC-MS spectrum of olive essential oil obtained from *Olea europaea*

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most sensitive to essential oil with an MIC value of value of 7.0-28.0 µg/ml. [Table 3]. The MIC value of broad-spectrum antibiotics ranged from 7.5-60 µg/ml [Table 3].

The anti-microbial activity of the tested essential oil against the three examined fungal strains is shown in Table 3. The *O. europaea* essential oil examined exhibited notable fungi static and fungicidal activity. The lowest MFC was expressed against *Candida albicans* as compared to flucanozole indicate its extra-ordinary antifungal effects.

DISCUSSION

In the present investigation, *O. europaea* essential oil GC-MS analysis showed presence of 24 compounds representing more than 99.99% of the essential oil [Table 1]. The inhibition zones of the essential oils on tested organism show a significant correlation with MIC values ($P < 0.05$). Based on growth inhibition zone diameters obtained in tests, results were divided in to three categories i. e. resistant (>7 mm), intermediate (>12 mm), and susceptible (>18 mm). Maximum growth inhibition diameter was obtained 26.4 ± 0.09 mm against *Micrococcus luteus* followed by24.2 ± 0.07 mm against *Lactobacillus acidophilus*. Olive oil has shown significantly higher growth inhibition zone diameters in *Aspergillus niger* 21.6 ± 0.054 mm, *Candida albicans* 25.4 ± 0.04 mm and 22.3 ± 0.11 mm in *Rhizopus stolonifer* than the broad spectrum antifungal drug Griseofulvin 19.3 ± 0.09 mm [Table 2]. Similar growth-inhibition zone diameters were reported in *P. aeruginosa* 33.3 mm, *B. subtilis* 29.9 mm *P. vulgaris* 29.4 mm, *K. pneumoniae* 20.8 mm and *S. aureus* each,[16] *Clostridium ferfringens, E. coli* and *Lactobacillus acidophilous*,[17] *Bacillus species*,[18] *Staphylococcus aureus*[19,20] and *Salmonella enteritidis* in presence of different essential oils.[21]

Table 2: Zone of inhibition of essential oil from methanolic extract of *O. europaea*

Name of the organism (mm)	Essential oil (IZD in mm)	Control (mm)	
		Negative	Positive
Escherichia coli	23.3±0.5	-	19.3±0.09
Bacillus cereus	22.2±0.04	-	18.1±0.07
Lactobacilus acidophilus	24.2±0.07	-	17.7±0.05
Micrococcus luteus	26.4±0.09	-	20.0±0.09
Staphylococcus aureus	20.5±0.15	-	17.3±0.11
Klebsiella pneumoniae	21.3±0.02	-	18.6±0.20
Streptococcus pneumoniae	19.4±0.07	-	16.2±0.07
Aspergillus niger	21.6±0.054	-	19.2±0.04
Candida albicans	25.4±0.04	-	18.6±0.09
Rhizopus stolonifer	22.3±0.11	-	19.3±0.09

Values are expressed as mean±SD (*N*=3) and values followed by same letter are not significantly different at the *P*<0.05. Determined by Duncan's Multiple Range test. Positive control – Ampicillin/Griseofulvin, negative control – DMSO

Table 3: Antimicrobial activities of essential oil from methanolic extract of *O. europaea* on different microbes and their corresponding MIC

Name of the organism	MIC (µg/ml)	
	Essential oil	Positive control
Escherichia coli	7.0	60
Bacillus cereus	14.0	30
Lactobacilus acidophilus	28.0	30
Micrococcus luteus	56.0	60
Staphylococcus aureus	56	60
Klebsiella pneumoniae	56	60
Streptococcus pneumoniae	28	30
Asper gillusniger	28	30
Candida albicans	7.0	7.5
Rhizopus stolonifer	14.0	15.0

Positive control is ampicillin/Griseofulvin, MIC – Minimum inhibitory concentration

Olive oil (*O. europaea)* has shown 7.0 µg/ml MIC value against *E. coli,* 14.0 µg/ml against, *Bacillus cereus*, 28.0 µg/ml *Lactobacillus acidophilus* [Table 3]. Similarly, it has shown MIC value in a range of 7-28 µg against *Aspergillus niger, Candida albicans, Rhizopus stolonifer* [Table 3]. Similar MIC 6 µg/ml was reported in allicin and diallyl sulfur compounds against *Helicobacter pylori*.[22] Similarly luteolin,[23] thymus,[24] phenolics[17] 2006) and Cavacrol,[3] di-terpenoids[25] isolated from various essential oils have also shown stronger antimicrobial activity against few bacteria mainly against oral pathogens[26] and *Escherichia coli* O157:H7[3]. Similarly, juniper oil extracted from *Juniperus communis* (L) has shown strong bactericidal activity against both Gram-positive and Gram-negative bacteria with MIC values between 8 and 70% v/v.[27] Same oil has also shown stronger fungicidal activity against *Candida* sp. (MIC from 0.78 to 2% v/v). *Sardinian juniperus* essential oil was found active against foodborne pathogens and spoilage microorganism.[7] Similar, antimicrobial activity was exhibited by different *Mentha* species i. e. *Mentha longifolia*. L, *Mentha aquatica* and *Mentha piperita.* L against *E. coli* with very low MIC value (4 µL/mL)[4] and *Hypericum* species such as *Hypericum scabrum, Hypericum scabroides* and *Hypericum triquetrifolium* essential oils.[28] Moreover, di-terpenoids isolated from *Sagittaria pygmaea* has shown antibacterial activity against *Streptococcus mutans* (ATCC 25175) with MIC value of 15.6 µl/ml.[25] Essential oils from *Coriandrum sativum* (L) *Foeniculum vulgare* Miller Var. *vulgare* (Miller),[28] *Cinnamomum osmophloeum*.[29] Besides this, *Dracocephalum foetidum* essential oil also exhibited strong antibacterial activity against methicilin-resistant *Staphylococcus aureus* (MRSA).[30] For comparison of antimicrobial activity of essential oils certain broad-spectrum antibiotics were also tested against same bacterial strains, which have shown marginal activity or intermediate effect.

In various bioassays, *O. europea* essential oil has shown very high anti-bacterial and anti-fungal activities *in vitro.* Due to presence of volatile components, i. e. phenolic compounds in higher concentration[17] and very diffusion at room

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temperature olive essential oil displayed high susceptibility against both Gram-negative and Gram-positive bacteria. In addition, it may also increase the plasma membrane permeability that results in higher leakage of fluid material from bacterial cells[31] and inhibit microbial respiration.[32] Therefore, major antimicrobial activity seems to be post diffusion action of essential oils on growth and metabolism of both the bacterial and fungal strains.[33,34] No doubt, *O. europaea* essential oil contains so many promising molecules, which can be used for therapeutic purposes mainly pharmacological potential.[35] Like other plant, natural products essential oils possess broad-spectrum antimicrobial activity against pathogenic microbial strains.[36] As high antimicrobial susceptibility obtained in tests in comparison to drugs, olive essential oil and its components can be used for formulation of highly active non-antibiotic drug that may be less toxic and show lesser side effects.

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