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Comprehensive evaluation of pharmacological properties of *Olea europaea* L. for Cosmeceuticals prospects

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Abstract

Background: *Propionibacterium acnes* (anaerobic bacteria) and *Staphylococcus epidermidis* (aerobic bacteria) have been acknowledged as key comedone forming pathological factor, eliciting an inflammation in acne. The present study was conducted to evaluate antibacterial and antioxidant activities of Olea europaea leaves extracts (OLE) of different solvents (methanol, ethanol, deionized water, and acetone) against etiologic pathogens of *acne vulgaris*.

Methods: The antibacterial testing against the selected pathogen *viz*., *P. acnes* and *S. epidermidis* were evaluated using broth micro dilution method recommended by CLSI, in duplicate. Correspondingly the total phenolic content and flavonoid content along with radicals scavenging activity by DPPH assay were also evaluated. The data of antibacterial assay demonstrated that these plant extracts differ quantitatively in their activity against the tested pathogens.

Results: The results (mg/ml) exhibited that *Olea europaea* leaves extracts (MIC:2.263/IC_{50}:1.626, MIC:0.933/IC_{50}:0.636, MIC: 1.054/IC_{50}:1.040, MIC:2.534/IC_{50}:2.500 of aqueous, methanol, ethanol, acetone extracts respectively) are more effective against growth of *P.acnes* as compared to *S. epidermidis* (MIC: Range (Not active at particular concentration), IC_{50}: Range, MIC:1.031, IC_{50}: 0.670, MIC:1.502, IC_{50}:1.234, MIC: Range, IC_{50}:1.890 mg/ml aqueous, methanol, ethanol, acetone extracts respectively). The readings were statically analyzed and also compared with standard drug tetracycline.

Conclusions: The current findings suggested *Olea europaea* L. as a promising source of potential antioxidants and antibacterial activity against *P.acnes* and *S. epidermidis* that may be an efficient therapeutic agent in the pathogenesis of *Acne vulgaris* and proves a potential source of Cosmeceuticals.

Keywords: *Propionibacterium acnes*, *Staphylococcus epidermidis*, CLSI, Antibacterial activities MIC, DPPH

Background

Natural drug resources with their varied biological and pharmacological properties (due to the presence of phenolic acids, flavonoids, tannins, vitamins and terpenoids) represent a treasure for researchers, to combat problem concerning treatment of health disorders or dermal infections. In the last few years, with the increasing doses of conventional drugs, multidrug resistance of pathogens develops. To overcome these persistent dilemmas of conventional treatments an increasing interest in herbal therapy has emerged. The herbal formulations are a viable option that could be useful in reducing the side effects associated with synthetic antibiotic treatment. Emphasis has been mainly on the antibacterial, anti-inflammatory and antioxidant properties of herbal extract [1].

Olea europaea L. leaves are a sort of waste product, this waste product is not profitable; olive leaves are often used as animal feed or simply burned with excess branches gathered [2]. The concern in olive leaves grew in the last few years due to its high pharmacologicalproperties, presence of phenolic compound flavonoids, tannins, vitamins C and terpenoids and high concentration of phenolic compounds [3]. *O. europaea* is the most abundant phenolic compound (up to 14% of the dry weight) with numerous health benefits attributed to it [4]. It has been found to have potent antioxidant and radical scavengers with anti-tumor and anti-inflammatory,

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antimicrobial properties and anti-atherogenic, hypoglycemic, hepatic, cardiac and neuro-protective effects [5, 6]. Oleuropein has a protective effect in counteracting low-density lipoprotein (LDL) oxidation, validated through the estimation of the decreased formation of thiobarbituric acid-reactive substances (TBARS are naturally present in organic specimens, include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress) [7] and malondialdehyde (MDA, compound that results from the decomposition of polyunsaturated fatty acid lipid peroxides) and 4-hydroxynonenal (4-HNE) as lipid peroxides by-products [8]. Its anti-tumour activity has shown to inhibit proliferation and migration of a number of advanced grade human tumors cell lines in a dose dependent manner [9–11]. Its anti-inflammatory activity is also remarkable, demonstrated by decreasingthe production of monocytic inflammatory mediators, decreasing in the production of IL-1β in human whole blood cultures stimulated with monocytes-triggered by LPS [12]. Interestingly, olive oil phenolic compounds reduce the circulating concentrations of IL-6, a pro-inflammatory agent that stimulates inflammation in several pathologies. *Acne vulgaris* is a chronic inflammatory disorder of pilosebaceous unit that affects more than 85% of adolescents and young adults [13]. The increase sebum production, hypercornification of the pilosebaceous follicle, an abnormality of the microbial flora (*P. acnes* and *S. epidermidis*), and the production of inflammation are the main triggering cause of *Acne vulgaris* [14].

The present study intends to evaluate the antimicrobial properties of different solvent extracts of *Olea europaea* L. leaves (olive leaf) against most common but ticklish anaerobic bacteria of human dermal pathogen *i.e. Propionibacterium acnes* and aerobic bacteria *Staphylococcus epidermidis*, causative agent of *acne vulgaris*.

Methods

Olive leaves were received as a gift from Riyadh, Saudi Arabia. All the chemicals and reagents used were of analytical grade, and were either purchased from Himedia.

Preparation of extracts

Dried leaves of *O. europaea* was finely chopped and soaked overnight in 50% of different solvent *i.e.* methanol, ethanol, deionized water, acetone in 1:10 (10 g/100 ml) (Fig. 1). Subsequently, the extracts were filtered with Whatman filter paper no.1. The filtrates were subjected to evaporation under vacuum and moderate temperature in rotavapour (Advance rotatory evaporator).

The test organisms

The test organisms *Propionibacterium acnes* (MTCC 1951) and *Staphylococcus epidermidis* (MTCC 435) were procured from Microbial Type Culture Collection, Chandigarh, India and the media were procured from Hi-Media. The culture of *P. acnes* (anaerobic bacteria) was maintained on Anaerobic Blood Agar Medium supplemented with freshsheep blood. The proper anaerobic environment was provided to the bacterial culture by the Anaxomate advance instrument after which the culture was incubated for 48 h at 37 °C in CO_2 incubator to provide optimum temperature for bacteria growth (Fig. 1). The culture of aerobic bacteria was maintained on Nutrient Agar and incubated in BOD incubator for their appropriate growth (Fig. 1).

Phytochemical analysis

All four solvents were prepared separately for extracting phenolics from leaves: methanol, ethanol, acetone and deionized water (ddH_2O). Leaves (1 g) were ground with a mortar and pestle under liquid nitrogen. The ground leaves were supplemented to a centrifuge tube (eppendrof) containing 10 mL of solvent. The mixture was allowed to

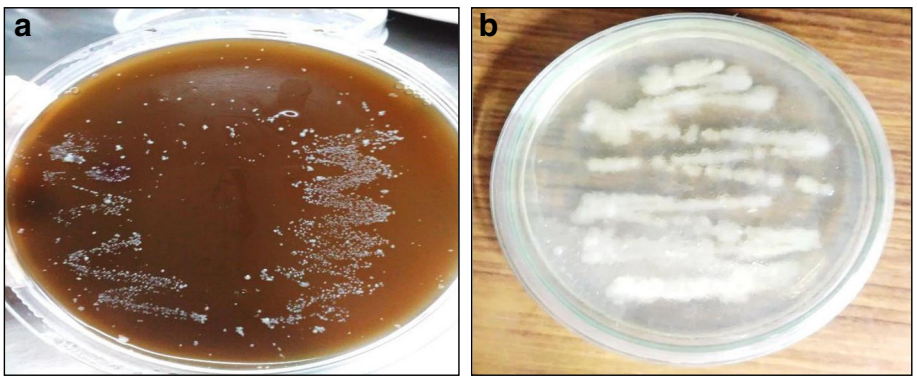

Fig. 1 The pictorial presentation of test pathogen **a** Culture of *P. acne*, an anaerobic bacteria, growth maintained on anaerobioc blood agar supplemented with fresh sheep blood. **b** culture of *S. epidermidis* an aerobic bacteria, growth maintained on Muller Hinton agar

stand in the dark for overnight. The extract was centrifuged 5000 rpm for 10 min, at room temperature, and the supernatants were then filtered using a filter paper (Whatman No. 1). The extracts were subjected to rotary evaporator to make thick slurryunder vacuum at operating temperature below 45 °C. Thereafter, the thick slurry was kept at -20 °C for further usage.

1. **Phenolic content**: 20 μl of each extract solution and standard (tannic acid) were mixed with 1 ml of ddH2O and 100 μl of Folin-Ciocalteu reagent, followed by the addition of 300 μl of 20%Na_2CO3 solution after 1 minutethe resulted mixture now incubated in shaker incubator (temp 40 °C, 30 min). The phenolic content was determined as milligram tannic acid equivalents (TAE)/g of dry weight powder (DW) [15].
2. **Flavonoidscontent**: 4 ml of ddH_2O was mixed with1ml of each olive extract. Subsequently, 5% sodium nitrite solution (0.3 mL) and 10% aluminum chloride solution (0.3 mL) was added and incubated at room temp for 5-10 min. Then 2 mL of 1 M NaOH was added to the mixture, the volume makes upto 10 ml with ddH_2O and subjected to vortex thoroughly. The pink colour developed and show absorbance at 510 nm. Total flavonoids content was determined as mg catechin equivalents per g of dry weight powder (mg CE g-1 DW) [16].
3. **DPPH**: it is a method to measure the antioxidant/ free radical scavenging activity of extracts. The antioxidant activity/radical scavenging of leaf extracts were evaluated using 1, 1-Diphenyl-2-Picryl-hydrazyl. For this purpose 0.1 mM solution of DPPH in methanol was prepared. 30 μL of extracts in different concentration (10, 50, 100, 500, and 1000 μg/mL) were mixed with DPPH solution and thoroughly vortex and incubated for 30 min at 25 °C, decrease in absorbance was measured at A = 517 nm against blank. The standard (butylhydroxyl toluene) was used as synthetic antioxidant positive control. The scavenging ability of the plant on DPPH was calculated using the equation:

$$\text{DPPH scavenging activity } (\%) = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

Where Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample extract or standard [17].

Antibacterial assay-

The susceptibility of the *P. acnes* and *S. epidermidis* was assayed against *Oleo europaea*L. leaf solvents extracts using the broth micro dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) [18]. Freshly prepared Muller Hinton Broth (MHB) medium was used as a base medium for the assay. Stock solutions of all the extracts and standard (Tetracycline) were prepared (50 mg/ml) in Dimethyl Sulfoxide (DMSO) and homogenized by using vortex for 4–5 min. The bacterial inocula suspension was prepared as per 0.5 McFarland standards. The experiment was performed according to CLSI guidelines in flat bottom sterile 96-well microtitre plates. Initial dispensing of 100 μl medium (MHB) in all the wells, followed by the addition of 100 μl, 90 μl and 80 μl of MHB in columns 2, 3 and 4 respectively. Further, 10 μl and 20 μl of drugs (each solvent extract in duplicate wells) were added to each well of columns 3 (sample control) and 4 (dilution well) respectively. Further, serial dilution was done from 4th column wells (2.5 mg/ml) to 11thcolumn wells (0.02 mg/ml) and after dilution, and content dragged from 11th well was discarded so as to maintain 100 μl from 4–11 wells [19] (Fig. 2).

After the serial dilution, 100 μl of bacterial inoculums was added to each well of column 4 to column 12, to make up final volume of 200 μl. Column 1 contained media and formaldehyde to serve as a negative control. Column 12 was taken as the positive control (O. D. control), which contains 100 μl medium and 100 μl inocula (Fig. 3). This is how, set of all extracts were maintained (in triplet). Thesecultured 96 well plate of anaerobic bacteria incubated in CO_2incubator (Galaxy 170 S New Brunswick, USA) for 48 h and aerobic bacterium 96 well plates were placed in BOD incubator for 24 h.

Determination of minimum inhibitory concentrations (MICs) and IC_{50}

For extracts the MIC was determined as the lowest drug concentration showing absence of growth visually or 80% growth inhibition compared with the growth in the drug-free well. IC_{50} defined as the drug concentration that produces 50% of growth inhibition compared to the growth in the drug-free well. Comparative inhibition percent of bacteria inoculum in media treated with extracts were calculated by using formula [20].

$$\%\,\text{Inhibition} = \frac{\text{O.D. Control} - \text{O.D. treatment}}{\text{O.D. control}} \times 100$$

Statistical analysis

All experiments were carried out in duplicate. The data were analyzed using analysis of variance (ANOVA) and significant differences ($p < 0.05$) among means were determined by R commander software.

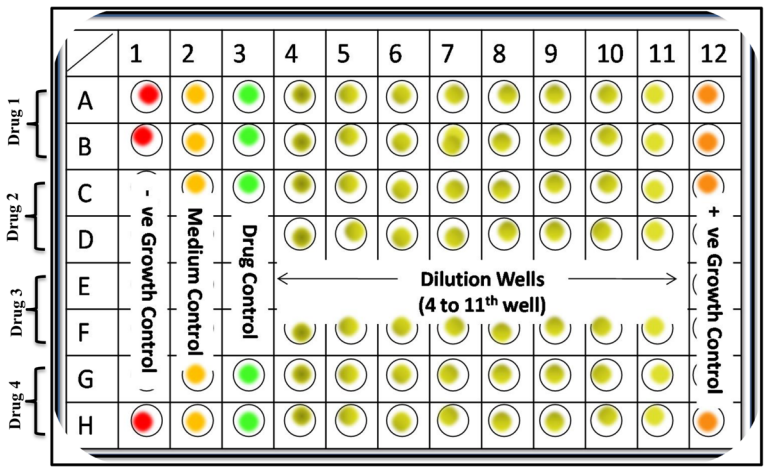

Fig. 2 Pictorial representation of CLSI recommended broth micro-dilution protocol. *a–h* contains drugs (50mg/ml) in duplicated: *a*, *b* contains *Drug 1 i.e. Olea europaea* ethanolic extract, *c*, *d* contains *Drug 2 i.e. O europaea* methanolic extracts, *e*, *f* Drug 3 *i.e.* acetone extract, *g*, *h Drug 4* contain aqueous extracts [36]

Results and discussion

In present study the preliminary qualitative and quantitative analyses of the *Olea europaea* L. leaves extracts (methanol, ethanol, acetone and aqueous) were executed to analyze antibacterial and antioxidant properties against *P. acnes* and *S epidermidis*. The results of our study clearly portray significant antibacterial and antioxidant properties with reference to the MICs as well as IC_{50} (mg/ml) values through 96 well microtitre plates (CLSI recommended broth micro dilution method). The leaves extracts were found to be more effective against anaerobic bacteria *P. acnes* (methanolic extracts MIC: 0.933/IC_{50}:0.636, ethanolic extracts MIC: 1.050/IC_{50}: 1.040, aqueous MIC: 2.263/IC_{50}:1.626 and

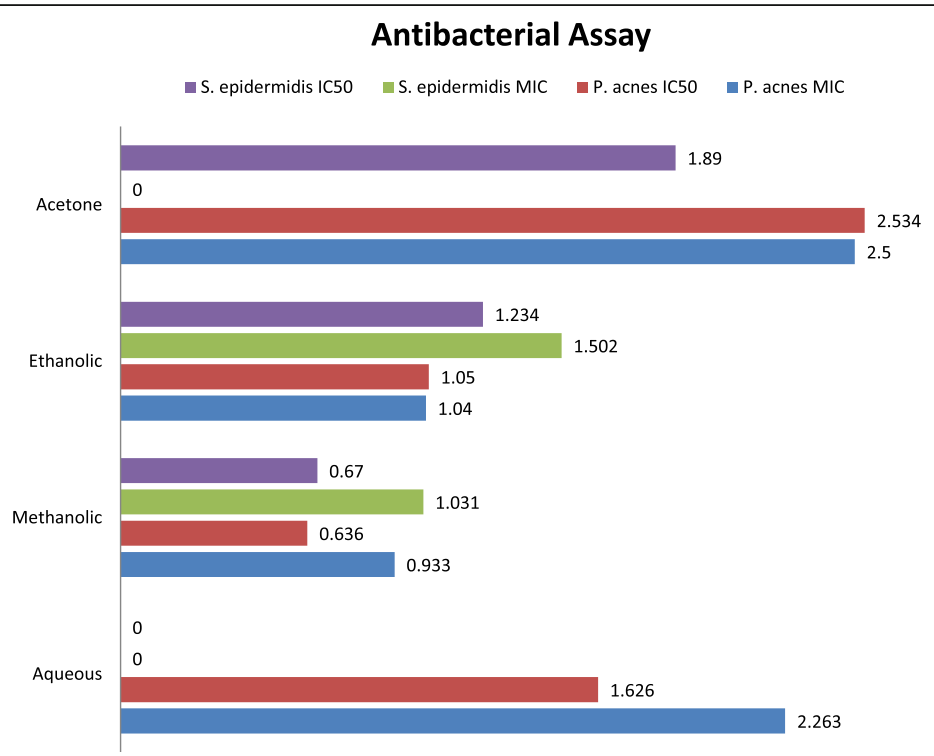

Fig. 3 Graphical presentation of activity of *Olea europaea* leaf extracts (acetone, ethanol, methanol and aqueous) in IC50 and MIC (mg/ml) against *S. epidermidis* and *P. acnes*

Table 1 Antibacterial activity of *Olea europaea* leaf extracts of different solvent along with standard against *P. acnes* and *S. epidermidis*

Pathogenic microbe	Bactericidal activity (IC50 and MIC mg/ml)									
P. acnes	*Olea europaea*								Standard drug (Tetracycline)	
	Aqueous		Methanolic		Ethanolic		Acetone			
	IC50	MIC	IC50	MIC	IC50	MIC	IC50	MIC	IC50	MIC
	1.626	2.263	0.636	0.933	1.040	1.050	2.500	2.534	0.013	0.028
S. epidermidis	Range (NA)	Range (NA)	0.670	1.031	1.234	1.502	1.890	Range (NA)	0.106	0.159

NA not active at particular concentration

acetone MIC: 2.534/IC_{50}:2.500) as compared to aerobic bacteria *S. epidermidis* (methanolic extracts MIC: 1.031, IC_{50}:0.670, ethanolic extracts MIC: 1.502/IC_{50}: 1.234, aqueous extracts MIC: Range/IC_{50}: Range and acetone extracts MIC: Range/IC_{50}:1.890). These quantitative value were compared by standard (tetracycline) against *P.acnes* (MIC: 0.028, IC_{50}: 0.013) and *S. epidermidis* (MIC: 0.159, IC_{50}:0.106) (Table 1).

The *Olea europaea* L. leaves have a tremendous history of traditional therapeutic uses (antimicrobial and antioxidant) [21]. Olive leaves are acknowledged for the secondary metabolites such as oleacein oleuropein [22, 23] Flavonoids and tannins etc. were among the major phenolic contents present in plant extracts [24]. The phenolic radicals were testified to be less reactive and retain lower electron reduction potential than the oxygen radicals [25]. Owing to these properties, the phenolic compounds are reflected as excellent radical scavengers. Thus our study, correspondingly evaluates the total phenolic content and flavonoid content along with radicals scavenging activity by DPPH assay. Our data have validated that the mean phenolic content (PC) in olive leaf extracts in terms of mg dispense from 16.9 to 25.6 mg and flavonoid content (FC) from 9.5 to 24.1 respectively (Table 2). Our results showed that methanolic, ethanolic and acetone extracts had the highest amount of phenolic content as well as flavonoid than aqueous extract [26, 27]. In general, the obtained data showed a statistical significant correlation between leaves extracts (of all the solvents) phytoconstituents analyses, *i.e.* total phenols and total flavonoids.

Higher the antioxidant concentration (in solvent) higher will be the percentage of DPPH scavenging activity of a compound and better will be the antioxidant activity. Further, DPPH radical scavenging activity was found to agree with total phenolics and total flavonoids outcomes. The present data revealed methanolic extracts of olive leaves exhibited appreciative higher free radical scavenging activity, followed by ethanolic ≥ acetone ≥ aqueous respectively, using DPPH. Free radical scavenging DPPH assay data dispensed from 14.7 to 92.5 (Table 2). These values were compared to the value obtained using standard (91.8 μg/ml). These data were also in statistical significant correlation with the data of total phenols and flavonoids. The hight free radical scavenging activity would be due to the high content of phenolic compounds in methanolic extractof *O. europaea* leaf especially oleuropein and hydroxytyrosol [28]. The reducing properties of polyphenols as hydrogen or electron-donating agents relay their potential for free-radical scavengers (antioxidants). Polyphenols holds an ideal chemical structure for free radical-scavenging activities and most of them have been shown to be strong antioxidants in vitro than vitamin E [29]. It has been demonstrated that hydroxytyrosol is empowered with a potent antioxidant activity due to the ortho diphenol function. Thus, the high antioxidant activities of oleuropein can be described by

Table 2 Phenolic content, Flavonoid content and DPPH radical scavenging activity of *Olea europaea* L. leaf extracts along with standard

Extraction solvent	Phenolic content	Total Flavonoid	DPPH radical scavenging activity (μg/ml)				
			10	50	100	500	1000
Methanolic extracts	25.6	24.1	14.7	23.5	66.1	75.2	92.5
Ethanolic extracts	24.0	19.5	14.1	22.9	64.6	70.2	86.8
Acetone extracts	24.8	17.3	11.9	17.6	45.2	60.5	80.8
Aqueous extracts	16.9	9.5	10.5	11.6	24.3	35.2	70.5
Standard							91.2

the presence of hydroxytyrosol unit in its structure [30, 31].

Regarding antimicrobial properties, OLE exhibits high antibacterial activity against anaerobic and aerobic bacteria causing *acne vulgaris*, this activity isalso in correlation with the data of above discussed results. The presence of phenolic content confers *O. europaea* L. natural resistance to microbe (Gram negative, Gram positive) outbreak [32]. Studies have demonstrated that the phenolic compounds may also stimulates anti-inflammatory effects of lipoxygenase activity, leukotriene B4 production [33] and hindering biosynthesis of pro-inflammatory cytokines [34] or tempering inflammatory parameters [35]. Likewise COX-2, an enzyme involved in the generation of some inflammatory mediators (TNF-αand IL-1β mediated enzyme) and the expression of these inflammation inducing enzymes, interleukins and tumor necrosis factors were reported to be attenuated significantly with the treatment of Olive-derived phenolic compounds [36, 37]. All these activities of OLE confer antibacterial activity against pathogens of *Acne vulgaris*.

Conclusions

The developing natural therapies encase naturally derived drugs from active plant extracts, essential oilsand phytomolecules. The antibacterial and antioxidant potential of the olive leaf extracts can be attributed to its high contents of phenols, flavonoids and vitamin C that act synergistically. Although there is an observed significant variation in chemical constituents and biological activities of olive leaf extracts treated with different solvents, the current findings support that this medicinal plant *Olea europaea* L. is a promising source of potential antibacterial and antioxidants that may be efficient therapeutic agent in the pathogenesis of *acne vulgaris* and proves potential source of Cosmeceuticals.

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Authors' contributions

AQ conducted the experiments and design conception and conduction of research. Data interpretation and analysis were done by MP participated in drafting the manuscript revised the manuscript critically for important intellectual content. RK critically reviews the manuscript. AD made the necessary corrections in the write up and gave final approval for the submission of revised version. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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