

**Antimicrobial Activities and phytochemical properties of Saudi *Olea europaea subsp. cuspidata***

Nehad M. Gumgumjee\*and Abdulrahaman S. Hajar

Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Kingdom of Saudi Arabia.  
Corresponding author: Nehad M. Gumgumjee

**Abstract:** *Olea europaea subsp. cuspidata* had been used in traditional medicine for centuries. It was used in hemorrhages treatment and fevers as a metabolism inducer and bile flow stimulator. It was also used as astringent, antiseptic and a general tonic. In the present study, phytochemical of leaves and stem extract and antimicrobial properties against 6 bacterial strains: *Bacillus subtilis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Micrococcus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* using agar well diffusion method were investigated. Results showed that the effect of leaves extract was higher than that of the stems. Petroleum ether extract of the leaves and stems showed no activity against *B.subtilis*, MRSA and *S. aureus* and low activity against *E. coli*, *K. pneumonia* and *P. aeruginosa*. In addition leaves of *O. europaea* ethanol extract caused high inhibition zones against *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *E.coli*, MRSA and *B.subtilis*. The high performance liquid chromatography (HPLC) analysis indicated the presence of 15 phenolic compounds as major active constituents in the leaves (Aspartic, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine). Analyzing using HPLC for leaves constituents, glutamic acid showed the highest concentration, 121.17 followed by Aspartic and lysine 109.51, 89.08 µg/g, respectively. The concentration of other phenolic compounds ranged from 17.61 to 77.93. Methionine was the lowest phenol compound 17.61 µg/g.

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**Key word:** *Olea europaea*, antibacterial, phytochemical constituents, crude extracts.

**1-Introduction**

The use of both plant extracts and phytochemicals, as antimicrobial can be of great significance in therapeutic treatments. In previous few years, studies have been conducted in this contest to elucidate such efficiency (Almagboul et al., 1985). Many plants have been used due to their antimicrobial effects, which are attributed to compounds synthesized during secondary plant metabolism (Saxena et al., 1994; Santos et al., 1995).

There have been many studies on antimicrobial properties of plants growing in Saudi Arabia (Gumgumje et al., 2012; Gumgumjee & Hajar, 2012; Hajar & Gumgumjee, 2013). However, as far as we know no much antimicrobial studies were conducted on Saudi Arabia native populations of *O. europaea subsp. cuspidata* leaves and stems extract, particularly those grown in Albahah city, southwest Saudi Arabia. Many Olive species and sub-species belonging to *Olea europaea* L. are typically Mediterranean originated, (Aragon and Palancar, 2000). Sudjana et al, (2009) reported on the antimicrobial activity of commercial *Olea europaea* (olive) leaf extracts against *Campylobacter jejuni*, *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA). Phenolic compounds known to inhibit the growth of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Aziz et al., 1998, Paster et

al., 1988). Tassou and Nychas (1991) specified that Oleuropein inhibit sporulation of *Bacillus cereus*. Hydroxytyrosol also reported to be effective against clinical human pathogenic strains of *Haemophilus influenzae*, *Moraxella catarrhalis*, *Salmonella typhi*, *Vibrio parahaemolyticus* and *S. aureus* (Bisignano et al., 1999).

The Present study thereby designed to evaluate phytochemical and antimicrobial properties of the leaves and stems extracts of the native *O. europaea subsp. cuspidata* which grows on the cool summit of Albahah province, southwest Saudi Arabia.

**2-Materials And Methods****2.1. Materials:****2.1.1. Samples:**

Samples of leaves and stems of *O. europaea subsp. Cuspidata* were collected during May, 2013 from Albahah province (19°59'14.12"N, 41°27'53.01"E) southwest Saudi Arabia from cool summit at 2242 M.A.S.L. Species status of this plant was fervid at Faculty of Sciences Herbarium (Serial No. 1597), King Abdul-Aziz University, Jeddah.

**2.1.2. Bacterial strains**

Bacterial cultures were prepared for In-vitro antibacterial assay of the six Bacteria strains, three Gram positive: *Bacillus subtilis* (ATCC11774); *S.aureus* (ATCC29213) and MRSA (ATCC4698) and three Gram negative *Escherichia coli* (ATCC8739);

*Klebsiella pneumonia* (ATCC700603) and *Pseudomonas aeruginosa* (ATCC27853).

Those strains were provided by Microbiologics® USA. Tested organisms were sub cultured on nutrient agar (Oxoid laboratories, UK) slopes. These stock cultures stored in the dark at 4°C until used.

## 2.2. Methods:

### 2.2.1. Sample preparation :

The plant leaves and stems were washed in running tap water to remove debris and dust particles and then rinsed in distilled water for 5 min, then removed and air dried under room temperature until constant weight.

### 2.2.2. Extract preparation

According to Boeru and Derevici (1978). Briefly, ten grams of dried leaves and stems were thoroughly washed in running water prior to cutting into small pieces (1 to 2 mm) by blender. Extraction was done by adding 100 ml of either ethanol, ethyl acetate or petroleum ether. The extracted solutions were collected after filtration, evaporated under reduced pressure at 40°C until dryness; subsequently, diluted by dimethyl sulfoxide (DMSO) and stored at 20°C. until analysis

### 2.2.3. Phytochemical screening

#### *a-Identification of phenolic acids*

Phenolic compound were subsequently checked for purity by high pressure liquid chromatography (HPLC). HPLC grade water and MeOH were used for all analyses. Phosphoric acid buffer was made using HPLC grade  $\text{NH}_4\text{H}_2\text{PO}_4$  and  $\text{H}_3\text{PO}_4$ . Phenolic extraction and hydrolysis

Phenolic compounds in plant were extracted as described by Mattila et al., (2005). Approximately, 15 ml of 4N NaOH was added to 200 ml of each concentration of water extract in 50 ml Pyrex centrifuge tube purged with nitrogen and shaken for 2 h in dark with a wrist - action shaker. After phenolic acids were liberated by alkaline hydrolysis, samples were acidified with ice - cold 6 N HCl to reduce pH to 2. Samples were centrifuged at 3000 g and the supernatant was decanted into 250 ml separator/funnel. The supernatant was extracted with ethyl acetate (3:50 ml) with shaking for 10 s and the mixture was allowed to settle for 5 min between extractions. Ethyl acetate fractions were collected and pooled. The remaining pellet was diluted with 15 ml of distilled  $\text{H}_2\text{O}$ , vortex distributed and re-centrifuged at 3000 g. The second supernatant was re-extracted with ethyl acetate (3: 50 ml) as before and all ethyl acetate fractions were pooled. The phenolic acids-rich ethyl acetate fraction was dried by addition of anhydrous sodium sulfate and concentrated using a rotary vacuum evaporator at 35°C to dryness. The phenolic acids-rich residue was re-solubilized in 2.5

ml of MeOH and stored in a dark prior to separation and quantification by HPLC within 24 h of extraction.

### *b-HPLC analysis*

Phenolic acids were separated by Shimaduz (Kyoto, Japan) HPLC apparatus (model, LC-4A) equipped with visible/ ultraviolet (UV) detector (model, SPD-2AS) at 280 nm and stainless steel column (25.0 cm X 4.6 mm i.d.) (Phenomenex Co., USA) coated with ODS, (RP-18). An aliquot of the sample suspended in MeOH was diluted with 10 mM phosphoric acid buffer (pH 3.5) to the same concentration as initial mobile phase (15% MeOH). Samples were next filtered through a 0.2  $\mu\text{m}$  poly (tetrafluoroethylene) (PTFE) filter prior to injection. The two solvent systems consisted of MeOH (A) and 10 mM phosphoric acid buffer, pH 3.5 (B), operated at following rate of 1.5 ml/min. The phosphoric acid buffer consisted of 10 mM  $\text{NH}_4\text{H}_2\text{PO}_4$  adjusted to pH 3.5 with 10 m M  $\text{H}_3\text{PO}_4$ .

### 2.2.4. Antimicrobial activity

Antimicrobial activity was determined using the agar diffusion assay method as described by Holder and Boyce (1994). DMSO was used as a negative control.

The plates were organized in triplicate. Bacterial cultures were incubated at 37°C for 24h. Antimicrobial activity was determined by measuring the inhibition zone (Agwa et al., **Statistical analysis**).

For each treatment, three replicates and three determinations were conducted. Means of variable, standard error were calculated using SPSS to authenticate the significant differences between both the pathogenic micro-organisms and extract types.

## 3-Results And Discussion

### 3.1. Antibacterial activity

Tables 1 and 2 represent results of the antimicrobial influence of ethanol, ethyl acetate and petroleum ether extracts of leaves and stem of *O. europaea subsp. Cuspidate* which determined by using disc diffusion method against three Gram +ve bacteria (*B.subtilis*, *M.RSA* and *S.aureus*), three Gram - ve bacteria (*E.coli*, *K. pneumonia* and *P. aeruginosa*) at concentrations of 200 mg/ml, the leaves extract showed higher activity against tested organisms than the stems. The antimicrobial influence of leaves and stem extracts obtained by using different solvents were appeared to be very different in terms of effectiveness since some bacterial species were highly resistant and some other were more susceptible to the extracts. In this study, petroleum ether extract of the leaves and stem showed no activity against *B.subtilis*, *M.RSA* and *S. aureus* and low activity against *E.coli*, *K. pneumonia* and *P. aeruginosa* (Table 1and 2). The present results coincided with Muhammed et al., (2012) who reported that The *Olea europaea* leaves petroleum ether extract have no effect on any of the

bacteria tested. Extract obtained with ethanol appeared to be the most effective against all bacteria compared to those obtained for other solvents. In the present study it was observed that the ethanol extract of leaves of *O. europaea* subsp. *Cuspidata* showed large zones of inhibition against *K. pneumonia*, *P. aeruginosa*, *S. aureus*, *E.coli*, *M.RSA* and *B.subtilis*. The main diameter of inhibition zones of the extract against these bacterial strains were 35mm, 34mm, 29.67mm, 26mm, 18.67mm and 17.33mm, respectively (table 1). And the ethanol extract of stem also exhibited appreciable activity against all bacteria tested. The largest inhibition zones produced by stems ethanol extract were against *K. pneumonia*, *S. aureus*, *P. aeruginosa*, *E.coli*, *B.subtilis* and *M.RSA* with zone of inhibition 25.33mm, 22.33mm, 20.33mm, 19.00 mm 16.00mm and 15.67mm respectively (table2). The ethyl acetate extract of leaves and stem of *O. europaea* subsp. *Cuspidata* also exhibited moderate activity against all the bacterial strains used (tables 1, & 2). In present study, extract obtained with ethanol and ethyl acetate showed a better effectiveness against the tested bacteria compared to those obtained with other solvents. The present results were in accordance with (Paudel et al., 2011; Hajar and Gungumjee 2014) results. This is probably because that the ethanol allows better extracting of the phenolic compound from the leaves.

Our study corroborated the earlier claims by (Hannachi et al., 2009). The antibacterial properties of *Olea europaea* subsp. *Cuspidata* leaves in the present study agreed with earlier claims by Akhtar et al., (2006) and Foidl et al., (2001). Different reports were recorded by Markin et al., (2003) and Sudjana et al.,(2009) on the antimicrobial activities of olive leaves against different pathogenic bacteria strains.

However, Reza et al., (2012) reported that *O. europaea* did not present broad-spectrum antibacterial activity, but had appreciable activity on *H. pylori* and *C. jejuni*. Sudjana et al.,(2009) and Faiza et al.,(2011) also reported that the olive extracts showed an unusual combined antibacterial and antifungal action and that ethyl acetate and acetone extracts revealed a wide range of antimicrobial activity. Markin et al., (2003) also reported that water extract of olive leaves with a

concentration of 0.6% (w/v) on one hand killed *E.coli*, *P. aeruginosa*, *S. aureus* and *K.pneumonia* in 3h exposure, and on the other hand inhibited *B. subtilis* only when the concentration was increased to 20% (w/v) which possibly due to spore forming ability of this species. In another study, Korukluoglu et al, (2010) investigated the effect of the extraction solvent on the antimicrobial efficiency of *S. aureus*, *E. coli*, *S. enteritidis*, *S. thypimurium* and some others. They reported that solvent type affected the phenolic distribution and concentration in extracts, and antimicrobial activity against tested bacteria. As ethanol extracted OLE showed the highest antimicrobial efficiency against *E. coli* and *S. enteritidis*, acetone extracted OLE showed the highest antimicrobial efficiency against *S.thypimurium* (Korukluoglu et al, 2010).

Antimicrobial activities of standard antibiotics control positive showed an inhibitory effect against all the tested bacteria and that ciprofloxacin was the more effective than streptomycin. The present results was in accordance with Shital (2010). The mean diameters of inhibition zones against all bacteria tested were mentioned in tables 1&2. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mode of action because of the alarming increase in the incidence of new and re-emerging infectious diseases.

### 3.2. Phytochemical screening of *Olea europaea* subsp. *Cuspidata* leaves

The phytochemical screening of leaves of *Olea europaea* subsp. *Cuspidata* presented in Table 3 showed that the HPLC analysis successfully provided the presence of 15 phenolic compounds. The major phenolic compounds isolated from leaves of *Olea europaea* were Aspartic, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. Glutamic acid has the highest concentration followed by Aspartic and lysine 121.17, 109.51, 89.08 µg/g, respectively. The concentration of other phenolic compounds ranged from (17.61 to 77.93). Methionine compound was the lowest phenol compounds; 17.61 µg/g. The present results was in accordance with Ana et al., (2007).

**Table1. Antimicrobial activity of *O. europaea* subsp. *cuspidata* leaves concentration of crud extracts 10 mg/ml Mean diameter of inhibition ±Standard error mean (SEM)**

Solvent used	<i>B.subtilis</i>	<i>MRSA</i>	<i>S.aureus</i>	<i>K.pneumoniae</i>	<i>E.coli</i>	<i>P.aeruginosa</i>
Ethanol	17.33±0.33	18.67±0.33	25.67±0.33	35.00±0.58	26.00±0.58	34.00±0.58
Ethyl acetate	14.67±0.33	14.33±0.33	23.00±0.58	30.33±0.33	23.33±0.33	22.00±0.58
Petroleum ether	14.33±0.67	14.67±0.33	00.00±00.00	25.33±0.33	22.67±0.33	19.67±0.33
Streptomycin	25.00±00.00	23.67±0.33	19.00±00.00	25.00±00.00	23.00±00.00	22.00±00.00
Ciprofloxacin	34.00±00.00	46.00±0.00	38.00±00.00	42.00±00.00	44.00±00.00	42.00±00.00

**Table2. Antimicrobial activity of *O. europaea subsp. cuspidata* stem concentration of crud extracts 10 mg/ml Mean diameter of inhibition  $\pm$ Standard error mean (SEM)**

Treatments	<i>B.subtilis</i>	<i>MRSA</i>	<i>S.aureus</i>	<i>K.pneumoniae</i>	$\square$ <i>E.coli</i>	<i>P.aeruginosa</i>
Ethanol	16.00 $\pm$ 0.00	15.67 $\pm$ 0.58	22.33 $\pm$ 0.67	25.33 $\pm$ 0.33	19.00 $\pm$ 0.00	20.33 $\pm$ 0.33
Ethyl acetate	13.67 $\pm$ 0.33	13.33 $\pm$ 0.33	20.00 $\pm$ 0.00	23.33 $\pm$ 0.33	18.66 $\pm$ 0.33	17.67 $\pm$ 0.33
Petroleum ether	00.00 $\pm$ 0.00	00.00 $\pm$ 0.00	00.00 $\pm$ 0.00	22.33 $\pm$ 0.33	15.00 $\pm$ 0.00	17.00 $\pm$ 0.00
Streptomycin	25.00 $\pm$ 0.00	23.67 $\pm$ 0.33	19.00 $\pm$ 0.00	25.00 $\pm$ 0.00	23.00 $\pm$ 0.00	22.00 $\pm$ 0.00
Ciprofloxacin	34.00 $\pm$ 0.00	46.00 $\pm$ 0.00	38.00 $\pm$ 0.00	42.00 $\pm$ 0.00	44.00 $\pm$ 0.00	42.00 $\pm$ 0.00

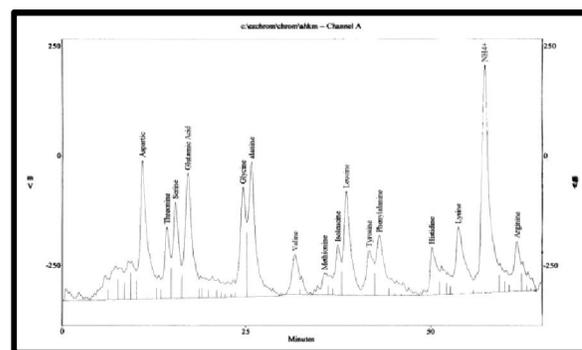
The antimicrobial capacity of phenolic compounds is well known (Rauha, et al., 2000; Puupponen-Pimiä, 2001; Markin et al., 2003; Zhu, et al., 2004; Proestos, et al., 2005; Pereira, et al., 2006). In addition, extracts may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extracts (Borchers et al., 2004).

Phenolic compounds within olive leaves extract have shown antimicrobial activities against several microorganisms including: *E. coli*, *S. aureus*, *K. pneumoniae*, *B. cereus*, *S. typhi* and *V. parahaemolyticu*. Morteza et al., (2012) reported that olive leaves aqueous extract showed good antimicrobial activities and highest inhibition zone (11.5 mm) against *Salmonella typhimurium* PTCC 1639. OLE is a potent source of polyphenols having antioxidant, antimicrobial, antiinflammatory and antiviral properties. Reza et al (2012) reported that their results clearly indicated that using olive leaves had the beneficial effect in controlling the microbial infections. Thus, *O. europaea* leaf extract are considered a more suitable in aspect of antibacterial activity.

This inhibitory effect of the extract on the growth of these microorganisms could be attributed to the presence of some phytochemicals that were found present in the *Olea europaea sub sp. Cuspidate* extract. The demonstration of antibacterial activity against pathogenic bacteria and fungi may be an indicative of the presence of broad spectrum antibiotic compounds (Doughari, 2006). Previously, antimicrobial activities of various plants has been studied by various authors (Nisar et al., 2010 a, b; Qayum et al., 2011; Zia-Ul-Haq et al., 2011; Gumgumje et al., 2012; Gumgumjee & Hajar, 2012; Haja r& Gumgumjee, 2013). The optimal effectiveness of the medicinal plant may not be due to one main active constituent, but may be due to the combine action of different compounds originally present in the plant (Bai, 1990).

**Table3: Chemical composition analysis of *O. europaea subsp. cuspidata* leaves extracts.**

Phenol compound	Retention time(min)	Conc( $\mu$ g/ml)
Aspartic	11.07	109.51
Threonine	14.35	35.45
Serine	15.48	42.92
glutamic acid	17.20	121.17
Glycine	24.65	22.47
Alanine	25.83	67.58
Valine	31.70	19.95
Methionine	35.70	17.61
Isoleucine	37.50	32.16
Leucine	38.63	86.84
,tyrosine,	41.73	57.40
Phenylalanine	34.80	73.85
Histidine	50.17	63.09
Lysine	53.75	89.08
Arginine	61.68	77.93



Retention time (min)

**Figure 1. Phenol compound of *Olea europaea subsp. cuspidata* leaves extracts.**

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