Antimicrobial Activities and Phytochemical Analysis of the Essential Oil of *Lavandula dentata* and *Plectranthus tenuiflorus*, Collected From Al Baha Region, Saudi Arabia

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Abstract: Many outbreaks of bacterial infections due to multi drug resistant acteria have been reported worldwide which may be attributed to contamination of inanimate objects in the hospital setting and facilitated by healthcare workers. Today, fully active antibiotic options available to treat the previous infections are very limited. The present study describes the antimicrobial activities of two plants collected from Al Baha region, Saudi Arabia which used traditionally to treat many microbial diseases. The essential oil of *Lavandula dentata* and *Plectranthus tenuiflorus* were extracted using Soxhlet and the oil extract were active against different pathogenic bacteria including *Acinetobacter* spp. and *Pseudomonas aeruginosa* with minimal inhibitory concentration (MIC) ranging from 50 to150 µl/ml. No toxicity was detected using *Artemia salina* as the test organisms (LD_{50}≥ 600 µl/ml). Moreover, *L. dentata* showed antitumor activity against Erlish cell line at 300 µl/ml. Phytochemical analysis of the plant extracts were determined using gas chromatography–mass spectrometry (GC/MS) and different components were determined. Five compounds were detected in *L. dentata* including Fenchone, Camphor, α- Linolenic acid, trimethylsilyl ester and Tarragon. The antimicrobial activity may due to one or more of the detected materials and more detail studies are needed.

[Aly MM, Al-Ghamdi M, Bafeel SO and Khedr AM. **Antimicrobial Activities and Phytochemical Analysis of the Essential Oil of *Lavandula dentata* and *Plectranthus tenuiflorus*, Collected From Al Baha Region, Saudi Arabia** . *Life Sci J* 2013;10(4):3302-3309] (ISSN:1097-8135). http://www.lifesciencesite.com. 439

Keywords: *Lavandula dentata*; *Plectranthus*, Essential oil; Antimicrobial activity; GC-MS, MIC

1. Introduction

Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases (Al Masoudi *el al*., 2013). However, pathogenic microbes that were once thought to have been controlled by antibiotics are returning in new forms, resistant to antibiotic. The global emergence of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections (Rangan and Barceloux, 2009). The discovery and development of new compounds that either block or circumvent resistance mechanisms could improve the containment, treatment, and eradication of these strains (Fu *et al*., 2007). Plant extracts can act as antibiotics against strains of MDR *Staphylococcus aureus* and other pathogens. The search for antimicrobial agents from plants has a growing interest in the last few decades but the results generated from many of these studies cannot be directly compared (Aly and Bafeel, 2010)

Imelouane *et al.* (2009) and Hui *et al*. (2010) reported that high antibacterial activities of essential oil of *Lavandula dentata* against *L. monocytogenes* and against four rhinitis-related bacteria including *S. aureus*, *Micrococcus ascoformans*, *P. vulgaris* and *E. coli*, respectively due to disruption the permeability barrier and loss of cell content. The essential oil of lavender contained borneol which has been reported to have significant antimicrobial activity (Tabanca *et al*., 2001; Vardar *et al*., 2003), α-pinene, reported to possess antifungal activity (Magiatis *et al.,* 1999), α-pinene and β-pinene are having antimicrobial potentials (Dorman and Deans, 2000), 1,8- cineole (Sivropoulou *et al*., 1997). Using GC-MS, Gamez *et al*. (1990) identified 1, 8- Cineole (50.6%) in the French *L. dentata* which was totally absent in the Saudi lavender which contained camphor. The essential oil of *L. dentata* from Algeria content 1, 8-Cineole, cic-verbenol and p-cymene-8-ol (Dob *et al.*, 2005). Moreover, D-limonene, geraniol, linalool and linalyl acetate were the potentially toxic compounds in lavender (Coulson, 1999; Hooser, 1990). D-limonene and linalool developed signs of acute toxicosis It is known that lavender oil is known to affect normal brain activity (Yamada , 1994) and α-humulene showed activity against MCF-7, PC3, A-549, DLD-1, M4BEU and CT-26 cell lines (Legault *et al*., 2003).

Al-Garni and Kabli (2005) detected an antimicrobial activity for the aqueous and organic extracts of *Plectranthus tenuiflorus* on *S. aureus*, *E. coli* and *Ps. Aeruginosa, C. albicans, Aspergillus*

fumigatus and *A. niger* and showed that essential oil of the plant contained 85% thymol. Using agar diffusion method described by Alsufyani (2007), *P. tenuiflorus* showed inhibitory effect on *S. pyogenes* and *Ps. aeruginosa*. Al-Yahya *et al*. (1985) determined the Minimum Inhibitory Concentration of *P. tenuiflorus* essential oil using broth dilution method and the oil was active against *S. aureus*, *B. subtilis* and *C. albicans*. The essential oils were rich in phenolic compounds and thymol that are widely reported to possess high levels of antimicrobial activity (Bagamboula *et al*., 2004, Al-Garni and Kabli, 2005).

Literature review showed that the main phytochemical constituents of the genus *Plectranthus* are diterpenoids, phenolics and essential oils (Abdel-Mogib, 2002, Grayer *et al*., 2003). β–Caryophyllene, Epiperitenone oxide, Carvacrol and 6,7-dehydroroyleanone were the common major compounds found (Ascensao *et al*., 1998). Oxygenated monoterpenes were detected by Ngassoum *et al*. (2001). Al-Yahya *et al*., (1985) found that Δ^3-Carene (52.8%) was the major component of essential oil obtained from plant grown in Abha, Saudi Arabia but α-terpinene (10.2%), p-cymene (10.9%) and carvacrol (14.3%) were the major components in essential oil from plant cultivated in Kenya (Mwangi *et al*., 1993), while plant grown in Taif, Saudi Arabia contained Thymol (85.3%) as the principle component of the oil (Smith *et al*., 1996). Species of the genus *Plectranthus* have cytotoxic and antitumor promoting activity and can be used in the treatment of cancer. The essential oils of *Plectranthus were* screened for cytotoxic activity against P388 mammalian cell line with IC50 value of 32-61 µg/ml and cytotoxic activity of *P. nitidus* was attributed to its diterpene content (Pasoski, 2009).

The aim of the present study was determination of the antimicrobial activity, toxicity and phytochemical analysis of *L. dentata* and *P. tenuiflorus,* collected from Al-Baha region and used in Saudi traditional medicine.

2. Material and Methods

Plant material

Aerial parts of *Lavandula dentata* and leaves of *P. tenuiflorus* were collected from Al Baha region in Saudi Arabia during summer 2009. Plants were identified at Faculty of Science, King Abdulaziz University.

Extraction of the essential oil

Aerial parts of *L. dentata* and fresh leaves of *P. tenuiflorus* were used for the analysis of essential oil composition (Hakkim *et al*., 2008) with few modified: about 30g of dried powder aerial parts of *L. dentate* and leave of *P. tenuiflorus* (25g) were extracted in soxhlet (Electromantle ME) with 450 ml of methanol for 12 h at 90˚C. The organic layer was dried over sodium carbonate and filtered through Whatman filter paper No.1. The organic solvents were evaporated under reduced pressure in a rotary evaporator (Heidolph, Germany) at 40ºC. The produced oil was dissolved in 10% dimethyl sulfoxide (DMSO) and kept in small closed vials at low temperature 4°C (Koba *et al*., 2009). The compounds and structures of major components were analyzed by GC-MS (Perkin Elmer).

GC–MS analysis conditions

A weight of about 5 mg of the dried sample extract was dissolved in dichloromethane (100 ng/µl). Into a reaction 1ml vial, 100 µl of the extract solution was mixed with 50 µl MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide), capped and heated at 80 ºC for 5 min into block heater. A volume of 0.5 µl was injected for GC-MS analysis.

Bacterial Isolates

Seven bacteria were obtained from King Fahd Hospital, Jeddah, Saudi Arabia; they were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus epidermidis*, *Klebsiella pneumonia*, *Streptococcus pneumonia* and *Acinetobacter* spp. Moreover, Methicillin resistant *Staphylococcus aureus* (MRSA) and *Micrococcus* spp were obtained from King Abdulaziz Hospital, Jeddah, Saudi Arabia.

Antimicrobial activity

Agar well diffusion method was used, each bacterium was suspended in sterile saline and diluted at ≈ 0.5 McFarland ($1.5x10^8$ $CFU^{/ml}$) and 0.1ml was spread over the surface of Mueller Hinton agar. Agar wells (6 mm a diameter) were filled with 50 µl of the tested essential oil. All plates were left for one hour at 4ºC and then incubated for 24 h at 37ºC. Inhibition zones diameter were measured the obtained results were compared with DMSO as a negative control and Cefixime as positive control. Minimum inhibitory concentrations (MICs) were determined as described by Ter-Laak *et al*. (1991).

Toxicity of the plant extracts

The brine shrimp lethality test was used to predict the cytotoxic of the plant extracts (Meyer *et al*., 1982). Plant extracts in DMSO, at varying concentrations were incubated with the brine shrimp larvae in sea water and control brine shrimp larvae were incubated in a mixture of sea water and DMSO only. After 24hr., the average number of larvae that survived in each vial was determined. The mean % mortality was plotted against the logarithm of concentrations, the concentration killing fifty percent of the larvae (LC_{50}) was determined from the graph (Meyer *et al*., 1982).

Antitumor of plant extracts

The antitumor activity against Ehrlich carcinoma and Lymphoma cell line were determined. The cells were grown in RPMI 1640 medium (Sigma, USA) with 10% fetal calf serum (FCS) (Gibco, USA) at 37˚C under a humidified atmosphere consisting of 95% air and 5% CO_2 for 48 hr. Cells were treated with different doses of the plant extract (200-1000 µl/ml) for 24 hours, centrifuged for 2 min at 1500 g and counted after removing the supernatant using hemacytometer and trypan blue (Sigma, USA) in normal saline (1:1 v/v). The percentage of cell viability was assessed to determine the 50 % lethal dose by which 50% of cells are killed (LD_{50}).

Morphological change by scanning electron microscopy

The tested specimens were coated with gold palladium and then examined under a scanning electron microscope (Quanta FEG 450).

Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Science (SPSS for windows, version 16). The variability degree of the result is expressed as mean ± standard deviation (Mean ± SD). The significance of the difference between samples was determined using Tukey HSD test. The difference was regarded significant when $P < 0.05$ and non significant when $P > 0.05$, where P is a level of significant.

3. Results

Antimicrobial activity and MIC of the essential oil

Lavandula dentata essential oil showed great antimicrobial activity against all tested bacteria with inhibition zone from 6.7 to 22.7 mm and no activity on *E. coli*. In case of *Plectranthus tenuiflorus*, the extract showed antimicrobial effect on all tested bacteria. The diameter of inhibition zone was ranged from 6.7-18 mm and no activity was found on *K. pneumonia* (table 1). The results in table 2 showed that MIC calculated for *L. dentata* extract was 50-150 µl/ml for all tested bacteria except *E. coli* (MIC > 150 µl/ml), while MIC of *P. tenuiflorus* extract was 50-150 µl/ml for all tested bacteria except *K. pneumonia* (MIC >150 µl/ml).

Figure 1: (A) Leaves of *Plectranthus tenuiflorus*, (B) Aerial parts of *Lavandula dentate*, collected from Al Baha region, Saudi Arabia

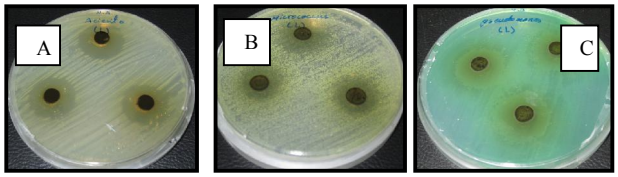

Figure 2. The antimicrobial activity of *Lavandula dentata* extracts against *Acinetobacter* spp. (A), *Micrococcus* spp (B) and .*Pseudomonas aeruginosa* (C) *Micrococcus* spp.

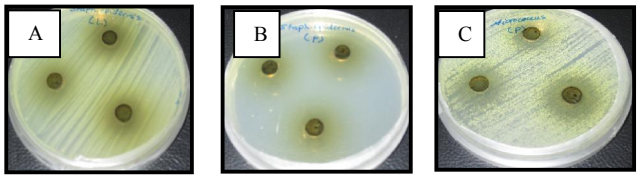

Figure 3. The antimicrobial activity *Plectranthus tenuiflorus* extract against, *Streptococcus pneumonia* (A), *Staphylococcus epidermidis* (B) and *Micrococcus* (C).

Table 2. Antimicrobial activity of *L. dentate* and *P. tenuiflorus* essential oil using agar well diffusion method against some bacterial pathogens

Tested bacteria	Diameter of the inhibition zone (mm) ± SD		
	Lavandula dentata	*Plectranthus tenuiflorus*	Positive control (Cefixime)
Staphylococcus aureus	6.7±6.5	8.6±8.1	28.00
Staphylococcus epidermidis	16±1.7	16±0.0	24.00
MRSA	11.7±10	6.7±5.8	17.00
Micrococcus spp.	15±0.0	13±1.7	28.00
Streptococcus pneumonia	21±1.7	18±2.0	28.00
Acinetobacter spp.	22.7±1.2	12.7±0.5	20.00
Klebsiella Pneumonia	10±8.7	ND	24.00
Pseudomonas aeruginosa	21±1.7	8.3±7.7	27.00
Escherichia coli	ND	10±0.0	25.00

Table 3. MICs (µl/ml) of *L. dentata and P. tenuiflorus* using serial broth dilution method against some bacterial pathogens

Tested bacteria	*Lavandula dentata*	*Plectranthus tenuiflorus*	Positive control (Cefixime)
Staphylococcus aureus	50±1.1	50±11.0	5±0.31
Staphylococcus epidermis	50±1.51	50±8.9	5±0.4
MRSA	50±1.77	50±5.9	5±1.1
Micrococcus spp.	50±1.8	50±3.95	5±1.0
Streptococcus pneumonia	50±1.0	50±5.0	5±1.1
Acinetobacter	50±1.41	50±5.0	25±2.2
Pseudomonas aeruginosa	50±1.81	150±5.0	15±1.4
Klebsiella pneumonia	150±1.9	>150	25±4.4
Escherichia coli	>150	150±10.0	25±4.0

MRSA: Methicillin resistant *Staphylococcus aureus*

Table 4: Toxicity against *Artimia salina and* antitumor activities of the two tested plant extracts

Tested plant	Toxicity against *Artimia salina* (LD_{50}, µl/ml)	Antitumor activity (LD_{50}, µg/ml)	
		Lymphoma cell line	Erlish cell line
Lavandula dentata	600 µl/ml	200	400
Plectranthus tenuiflorus	600 µl/ml	600≥	600≥

Toxicity of plant extracts using brine shrimp lethally test:

The brine shrimp (*Artimia salina*) bioassay was used to test the toxicity of the two studied plant extracts. The commonest stage used is the one 24-48 hours after hatching. Identification of the lethal concentration for 50% mortality after 6 hours of exposure (the acute LD_{50}) makes the test rapid and simple. No toxicity was found using the extract of *L. dentate* and *P. tenuiflorus* up to 400 µl/ml but moderate toxicity was found at 600 µl/ml for the two tested oils

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Antitumor of plant extracts

Antitumor activity (LD_{50}) against Lymphoma cell line and cell line was calculated for the two plant extracts in Table 3. *L. dentata* extract showed antitumor activity against Lymphoma and Erlish cell lines at 200 and 400µl/ml, respectively.

Effect of *L. dentata* extract on cell morphology of *Ps. aeruginosa*

Effect of *L. dentata* oil on *Ps. aeruginosa* morphology was determined after exposure of the cells to sub-inhibitory dose of the tested oil in liquid medium. The cell walls of *Ps. aeruginosa* were crenate and induced nipple formation (Figure 4A, B and C). The treated cells almost seen like a shrunken cells and had many indentations as compared to control. Moreover, *L. dentata* extract treated *Ps. aeruginosa* showed alteration in the outer membrane integrity with cell walls being disrupted and damaged resulting in a release of cell contents to outside of the bacterial cells.

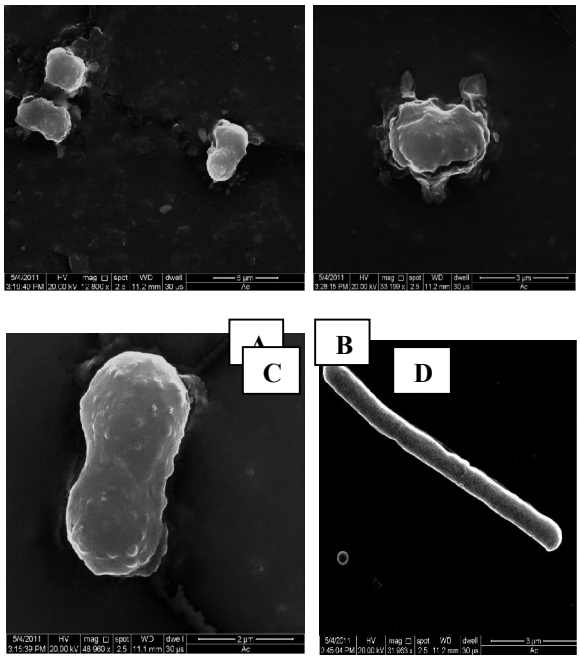

Figure 4. Scanning electron microscopy of treated *Pseudomonas aeruginosa with Lavandula dentata* extract (A, B and C) with different magnification and untreated cells (D)

Chemical composition of the essential oil

The main compounds of the two plants were identified using GC–MS. The results obtained were illustrated in Table 4. The essential oil of *L. dentata* isolated by Soxhlet apparatus were

Bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl-, (±) Fenchone; Bicyclo [2.2.1] heptan-2-one, 1,7,7-trimethyl, (±) Camphor; Trimethylsily ether of glycerol; 9,12,15-Octadecatrienoic acid, methylester, (Z,Z,Z); 11, 14- Eicosadienoic acid, methyl ester and α- Linolenic acid, trimethylsilyl ester (Figure 5). While *P. tenuiflorus* essential oil (extracted by Soxhlet apparatus) contained three compounds, Silane, trimethyl [5-methyl-2- (1-methyl) phenoxy]; Phytol, trimethylsily ether and α- Linolenic acid, trimethylsilyl ester.).

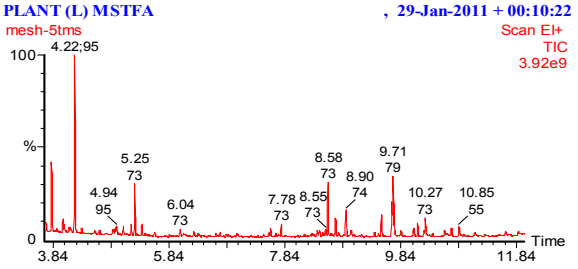

Figure 5. Mass spectra of of *Lavandula dentate* plant extract

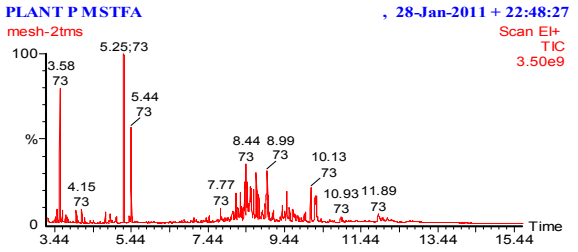

Figure 6. Mass spectra of *Plectranthus tenuiflorus* plant extract

4. Discussions

The extract of *L. dentata* and *P. tenuiflorus* showed excellent antibacterial activity against *Ps. aeruginosa*, *S. pneumonia* and *Acinetobacter* spp. which usually considered to be opportunistic pathogens and of recent have been reported to cause a number of outbreaks of nosocomial infections in hospitalized patients like septicaemia, pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infection (Towner, 1997; Levi and Rubinstein, 1996, Al Masoudi *el al*., 2013). The previous results, suggest potential antibacterial activity of the essential oil of *L. dentata,* which facilitate its application in future research for the pharmaceutical industries. Unfortunately, lower activity of *L. dentata* extract was showed on *S. aureus*. Similarly, Imelouane *et al*. (2009) has indicated that the essential oil of *L. dentate* was active against *K. pneumonia, E. coli, S. aureus* and *S. pneumonia* with high activity on *Listeria monocytogenes* and *Ps. aeruginosa* was the only bacterium that was not susceptible and have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier, highly resistant even to synthetic drugs (Skocibusic *et al*., 2006). Also, *Ps. aeruginosa* was considered resistant to *Tarchonanthus camphorates* essential oil and even to the reference antibiotic chloramphenicol, since no inhibition zone was observed (Matasyoh *et al*., 2007). This bacterium has shown resistance to different antimicrobial agents and diterpenes present in *Salvia* species (Darias *et al*., 1990). These results open the opportunity for the use of Saudi lavender essential oils as an antibacterial agent especially against *Ps. aeruginosa*. On contrast to our result, Hui *et al*. (2010) found that *L. dentata* essential oil was active against *S. aureus* and *E. coli* while in this study, the oil showed no activity against *E. coli.*

In this study, *P. tenuiflorus* essential oil showed *In vitro* antimicrobial activity against all tested bacteria except *K. pneumonia*. The high antibacterial activity of the investigated plant was against *S. pneumonia* followed by *S. epidermidis* with low MIC value while the MIC for *Ps. aeruginosa* and *E. coli* was 150 µl/ml and for *K. pneumonia* was > 150 µl/ml. Alsufyani (2007) reported antimicrobial activities of *P. tenuiflorus* against *Ps. aeruginosa* and *S. pyogenes*, while, Al-Garni and Kabli (2005) demonstrated high activity against *S. aureus, E. coli* and *Ps. aeruginosa*. The essential oil of *P. cylindraceus* showed good activity against *K. pneumonia*, *S. aureus* and *B. subtilis* and was moderately active against *Salmonella choleraesuis*, *Ps. aeruginosa* and *E. coli*.

Brine shrimp (*Artimia salina*) larvae are commonly used for toxicity assays in pharmacology. These larvae are sensitive to toxic substances (Pelka *et al*., 2000). Brine shrimp lethality assay, used to measure the toxicity of plant extract, is a general bioassay, which is an indicative of toxicity, antibacterial activities, pesticidal effects and various pharmacologic actions (McLaughlin *et al.,* 1991). The brine shrimp bioassay considers a useful tool for the isolation of bioactive compounds from plant extracts (Sam, 1993). The method is often used because it is simple, inexpensive and low amount of materials are sufficient to perform the assays on micro scale (Rahmatullah *et al.,* 2010). In our study, the brine shrimp lethality assay was used for the evolution of toxicity of two plant extracts at different concentration .A toxicity was found using the plant extracts of *L. dentate* and *P. tenuiflorus*. Similarly, the methanolic extract of *Plectranthus amboinicus* showed toxicity in albino mice (Female) after ingestions of 2000 mg/Kg of extract during one day (Preeja *et al*., 2011). On contrast to our result, the *L.*

dentata extract showed no toxicity against *Culex pipiens* (Al-Harbi, 2004). Our result indicates that the *L. dentata* extract showed antitumor activity against Lymphoma cell line at 400µl/ml. Similarly to the current result, *L. dentata* showed antitumor activity against MCF-7, PC3, A-549, DLD-1, M4BEU and CT-26 cell lines (Legault *et al*., 2003). On contrast to our result, the essential oils of *Plectranthus nitidus, P. graveolens and P. suaveolens* showed activities against P388 mammalian cell line (Pasoski, 2009).

The activity of the two tested plant extracts may lead to conclude the presence of secondary metabolites responsible for such biological effects. The treated cells of *Ps. aeruginosa* with the oil of *L. dentate* was examined using scanning microscope and compared with control. The oil induced many morphological modifications which may due to one or more of the main components. The phytochemical composition of the two plant extracts *L. dentata* and *P. tenuiflorus* were analyzed by GC-MS and showed various components.Similar to our result, Hassan *et al*. (1976) found that the major constituent in the Saudi *L. dentata* volatile oil was camphor. Another study from Algeria has investigated the main constituents in the oil obtained by steam distillation of *L. dentata* were 1, 8-cineole; Cis-verbenol, P-cymen-8-ol and Fenchone. Moreover, Dob *et al*. (2005) obtained Myrtenal, Pinocarvone, α –terpineol and α-terpinen- 7-al. In addition, Imelouane *et al.* (2009) represent 29 component of essential oil of *L. dentate*: 1, 8 sabinene; bicycle [3.1.0] hexan-3-Ol, 4-methylene-1-(1-methylethyl); myrtenal and α-pinene in addition the oil also contained smaller percentages of borneol; linalool oxide cis; linalool; myrtenol; bicyclo [3.1.1] heptan-2-one, 6; 6-dimethyl-, (1r); and pinocarvone. The antimicrobial activity of the essential oil of *L. dentata* could be attributed to camphor (Carson and Riley, 1995; Pattnaik *et al*., 1997). The differences in the constituents and their ratios among *L. dentata* species from different countries may be attributed to environmental and geographical factors.

The main phytochemical constituents of the genus *Plectranthus* are diterpenoids, phenolics and essential oils β–Caryophyllene, Epiperitenone oxide, Carvacrol and 6,7-dehydroroyleanone oxygenated monoterpenes (Ascensão *et al*., 1998, Ngassoum *et al*. 2001, Abdel-Mogib, 2002, Grayer *et al*., 2003). Compared to other result, Al-Garni and Kabli (2005) showed the main component of the essential oil of *P. tenuiflorus* were obtained by steam distillation was thymol. The majority of phytochemical studies on species of *Plectranthus* have focused on the isolation of a range of diterpenoids which had biological activity (Abdel-Mogib *et al.,* 2002).

Alsufyani (2007) showed that *P. tenuiflorus* contained coumarins, hydrolysable tannins, essential oil, being thymol (62.53%) the major component in the oil and triterpenoids and in the contrast the absence of alkaloids, steroids, anthraquinones, flavonoides, condensed tannins and anthraquinone glycosides. Marwah *et al*. (2007) demonstrated the two most abundant components of the essential oil of *P. cylindraceus* were identified as carvacrol and a-terpinolene.

In conclusion, the results obtained from our screening confirm the therapeutic potency of the *L. dentata* and *P. tenuiflorus* essential oil and thus provide a rationale for their use in traditional medicine. These results also form a good basis for further pharmacological, toxicity and conservation studies. Work must being conducted in a bid to test the efficiency of essential oil in enhancing wound healing process due to its high activity of inhibition pathogenic microbes.

Acknowledgements:
Foundation item: Thanks and appreciation to King Abdulaziz City for Science and Technology, Saudi Arabia, for financial support to carry out this work.

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12/2/2013