

ANTIBACTERIAL, ANTIFUNGAL, ANTITUMOR AND TOXICITY OF ESSENTIAL OILS OF *SALVIA OFFICINALIS*, *THYMUS VULGARIS*, *EUGENIA CARYOPHYLLATA* AND *ARTEMISIA ABSINTHIUM*

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ABSTRACT: Essential oils are aromatic and volatile liquids extracted from plants. The chemicals in essential oils are secondary metabolites, which play an important role in plant defense as they often possess antimicrobial properties. In this study, essential oils of four plants were investigated for their antimicrobial activity against eight bacterial pathogens and seven fungal strains. The used plants were *Salvia officinalis*, *Thymus vulgaris*, *Eugenia caryophyllata* and *Artemisia absinthium*.

All of plants essential oils showed antibacterial and antifungal activities. Zones of inhibition against pathogenic bacteria ranged from 10 to 28 mm and MIC from 25 to 150 µg/ml, while zones of inhibition against fungal strains ranged from 10 to 26 mm and MIC from 25 to 150 µg/ml. Cytotoxicity against *Artimia salina* and antitumor activity against *Ehrlich ascites* carcinoma and Lymphoma cell line were investigated. *A. absinthium* showed the best antitumor activity on Lymphoma cell line with moderate cytotoxic effect on *Artimia salina*. Analysis of *A. absinthium* essential oil by GC-MS discovered two major compounds; camphor and fenchone which may be involved in the biological activity of the plant extract.

Key words: *Salvia*, *Thymus*, *Eugenia*, *Artemisia*, essential oil, GC-MS.

INTRODUCTION

Antimicrobial chemotherapy has conferred huge benefits on human health. Many microorganisms that have acquired resistance to drugs through a variety of mechanisms have emerged and continue to plague human beings (Tomoo and Keizo, 2009). Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine (Essawi and Srour, 2000). Since ancient times, herbs and their essential oils have

been known for their varying degrees of antimicrobial activity (Juven *et al.*, 1994). The use of plant compounds to treat infection is an age-old practice in a large part of the world, especially in developing countries, where there is dependence on traditional medicine for a variety of diseases (Gangoue-pieboji *et al.*, 2006). Traditional healers have long used plants to prevent or cure infectious conditions and plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found *in vitro* to have antimicrobial properties (Cowan, 1999).

Sage (*Salvia officinalis* L., Lamiaceae) has antimicrobial potential (Bozin *et al.*, 2007) and is used as a medicinal plant to treat dental diseases in Brazil (Oliveira *et al.*, 2007). The antimicrobial activity may probably present because the essential oils are composed of several substances that can interact jointly in a synergic/potential form, giving them strong antimicrobial activity (Delamare *et al.*, 2007 and Faleiro *et al.*, 2003).

The genus *Artemisia* consists of small herbs and shrubs (Baytop *et al.*, 1984 and Davis, 1982) and has much pharmaceutical interest (Baytop *et al.*, 1984 and Kalemba *et al.*, 2002). *Artemisia* species have been used in folk remedies as an antipyretic, antiseptic, antihelminthic, tonic, diuretic and for the treatment of stomach ache (Baytop *et al.*, 1984).

Eugenia caryophyllata (clove) was found to be effective against egg and adult of *Pediculus capitis* (Yang *et al.*, 2003). It has antiseptic as well as bacteriostatic and bactericidal activity against *E. coli* and *S. aureus* (Burt and Reinders, 2003 and Ernst, 2001). Growth of *Helicobacter pylori* being one of the major causes of peptic ulcer disease has been shown to be inhibited by *E. caryophyllata* (Bae *et al.*, 1998). Several studies have demonstrated potent antifungal activity (Arina and Iqbal, 2002; Giordani *et al.*, 2004; Pawar and Thaker, 2006 and Park *et al.*, 2007), antiviral (Chaieb *et al.*, 2007a) and antibacterial effects of clove (Cai and Wu, 1996; Lopez *et al.*, 2005; Li *et al.*, 2005 and Betoni *et al.*, 2006).

The antimicrobial and fungicidal activities of *Thymus vulgaris* (thyme), were analyzed (Panizzi *et al.*, 1993) against Gram negative and positive bacteria (Marino *et al.*, 1999). Carvacrol was identified to be a predominant compound in thyme oil (Karaman *et al.*, 2001). The composition of essential oils of some *Thymus* species was analyzed and *in vitro* antimicrobial activities of its components were reported and antibacterial activity of methanolic extract of

T. pubescense was detected (Azaz *et al.*, 2004 and Mehrgan *et al.*, 2008).

MATERIALS AND METHODS

Plant materials:

Aerial parts, fruits, roots or leaves of the plants were collected from local markets in Jeddah, Saudi Arabia during summer 2012. The collected plant materials were put in clean plastic bags and transferred directly to the lab. The plants under investigation were *Salvia officinalis*, *Thymus vulgaris*, *Eugenia caryophyllata* and *Artemisia absinthium*.

Tested organisms:

Bacterial and fungal isolates were obtained from Biology Department, Faculty of Science, King Abdul-Aziz University (KAU), Jeddah. The bacterial strains were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* (ESBL) ATCC 700603, *A. baumannii* ATCC 1656-2, *P. mirabilis* ATCC 12453, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* (MRSA) ATCC 33591 and *Enterococcus faecalis* (VRE) ATCC 51299. The tested fungi were *Aspergillus flavus*, *Aspergillus niger*, *Epidermophyton floccosum*, *Trichophyton mentagrophytes* and *Microsporum canis*. Two strains of yeasts were investigated in this study; *Candida albicans* and *Cryptococcus neoformans*.

Preparation of plant extracts:

Dried powder of *Salvia officinalis*, *Thymus vulgaris*, *Eugenia caryophyllata* and *Artemisia absinthium* (100 g) was placed in Soxhlet apparatus (Electromantle ME) with 450 ml of methanol for 12 h at 90°C. The extract was dried over sodium carbonate anhydrous (BDH, England) and filtered through Whatman filter paper No.1. The organic solvents were evaporated under reduced pressure in a rotary evaporator. The produced oil was dissolved in dimethyl sulfoxide (DMSO) and kept in sealed dark glass bottles at low temperature (4°C.).

Antimicrobial Activity:

This test was carried out using agar well diffusion method according to Joshi *et al.* (2009). Bacteria or yeast were taken and shaken in the sterile distilled water corresponding to 10^8 CFU/ml for bacteria and 10^6 CFU/ml for yeast (Mihajilov-Krstev *et al.*, 2010). Fungal inocula were prepared by flooding Petri dish with 8 to 10 ml of distilled water and the conidia were scraped using sterile spatula. The spore density of each fungus was 1.5×10^4 spore/ml (Adigüzel *et al.*, 2005). Minimal inhibitory concentration (MIC) was determined by the method recommended by Ter-Laak *et al.* (1991). Each antimicrobial agent was serially diluted by transferring 100 μ l of the antimicrobial agent through sterilized microtitre plate containing 100 μ l media (nutrient broth for bacteria, Sabouraud dextrose broth for fungi and yeast). Freshly prepared standard number of cells (1.5×10^8 CFU/ml for bacteria or yeast and 4×10^4 spore/ml for fungal isolates) was added to the media that contained some drops of phenol red. Glucose metabolisms were measured by a change of the color of phenol red indicator from red to yellow. MIC was determined at the concentration with no color change and DMSO were used as a control.

Toxicity and antitumor activity of the plant extracts:

Cytotoxicity is performed by Brine Shrimp Test (BST). Different dilutions of each plant extract made in DMSO were prepared (100, 200, 300 and 400 μ g/ml) and 0.5 ml of each dilution was added to 4.5 ml of brine solution and maintained at room temperature for 24 h under the light (Krishnaraju *et al.*, 2005). The surviving larvae were counted by light microscope and the number of dead shrimp in each vial was recorded. The concentration at which 50% of the larvae were killed (LD_{50}) was determined as the toxic concentration (Lachumy *et al.*, 2010). The antitumor activity of the tested plants was determined against Ehrlich carcinoma and Lymphoma cell line. 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% CO₂ for 48 h. The percentage of cell viability was assessed to determine the 50% lethal dose by which 50% of cells are killed (LD_{50}).

Chemical analysis of the crude extracts by GC-MS:

The compounds and structures of major components were analyzed by GC-MS (Perkin Elmer).

RESULTS AND DISCUSSION

Antimicrobial activity of the essential oils of *S. officinalis*, *T. vulgaris*, *E. caryophyllata* and *A. absinthium* was investigated against some Gram positive bacteria, Gram negative bacteria, yeast and filamentous fungi. Table (1) and (2) are showing the effect of plant essential oils on pathogenic bacteria.

All of plant essential oils showed antibacterial activity. The inhibition zones are variable according to the type of the plant. *E. caryophyllata* and *S. officinalis* recorded the same maximum inhibition against *K. pneumonia*. The former showed also the highest inhibition zone against *P. mirabilis*, while the latter showed the highest zone against Methicillin Resistant *S. aureus* 33591. For all other bacteria, *A. absinthium* exhibited the largest inhibition zones than other plants essential oils ranging from 17 mm. to 28 mm. MIC values were 50-150 μ g/ml. However, the moderate activity against *P. mirabilis* and the slight decreasing in the inhibition zones against *K. pneumonia* and MRSA than *S. officinalis* did not exclude *A. absinthium* essential oil as the strongest against most of bacterial strains in this study. In this respect, Sengul *et al.* (2011) obtained broad spectrum antimicrobial activity of *A. absinthium* methanolic extract against many Gram positive and Gram negative bacteria. Also, Erel *et al.* (2012) reported antimicrobial activity of *A. absinthium* essential oil against *E. coli*, *S. aureus* and *E.*

Table 1. Antimicrobial activity of essential oils using agar well diffusion method against bacterial pathogens.

Bacteria	Plants	<i>Salvia officinalis</i>	<i>Artemisia absinthium</i>	<i>Eugenia caryophyllata</i>	<i>Thymus vulgaris</i>	Control
<i>P. aeruginosa</i>		16.7±1.5	28.00	18.6±8.1	18.6±8.1	ND
<i>K. pneumoniae</i>		26±1.1	24.00	26±0.0	16±0.0	ND
<i>P. mirabilis</i>		17.7±1.0	17.00	26.7±5.8	16.7±1.8	ND
<i>A. baumannii</i>		19±0.5	28.00	11±1.7	13±1.2	ND
<i>E. faecalis</i>		24±1.4	28.00	14±2.0	18±1.0	ND
<i>S. aureus</i> 33591		21.7±1.32	20.00	16.7±0.5	12.7±0.15	ND
<i>S. aureus</i> 25923		10±8.7	24.00	15	16	ND
<i>E. coli</i>		21±1.7	27.00	18.3±7.7	18.3±1.0	ND
Bacterial Index*		155.4	196	145.7	129.3	ND

* Bacterial Index: Total Activity (mm).

Table 2. The minimal inhibitory concentrations (µg/ml) of essential oils against bacterial pathogens.

Bacteria	Plants	<i>Salvia officinalis</i>	<i>Artemisia absinthium</i>	<i>Eugenia caryophyllata</i>	<i>Thymus vulgaris</i>	Control
<i>P. aeruginosa</i>		50	50	25	150	>200
<i>K. pneumoniae</i>		100	50	50	50	>200
<i>P. mirabilis</i>		50	50	25	150	>200
<i>A. baumannii</i>		50	50	50	50	>200
<i>E. faecalis</i>		50	100	100	100	>200
<i>S. aureus</i> 33591		50	150	25	100	>200
<i>S. aureus</i> 25923		100	100	50	150	>200
<i>E. coli</i>		150	150	25	100	>200

faecalis. Zanousi *et al.* (2012) reported antimicrobial activity of *A. absinthium* against *E. coli*, *K. pneumoniae*, *E. faecalis* and *S. aureus*. They found that the most effective essential oil of the plant has been extracted from fruits, but there was no antimicrobial activity against *P. aeruginosa* has been detected. *T. vulgaris* oil has the weakest antimicrobial activity against bacterial isolates. Inhibition zone diameter range was 12.7-18.6 mm.

The effect of plant essential oils on yeast and filamentous fungi is represented in Table (3) and (4). The effect of essential oils on the tested fungi has also revealed that the strongest activity was recorded for *A. absinthium* with considerable increasing in inhibition zones. Zone diameter range was 13-25 mm. and MIC was 50-100 µg/ml.

Similarly, Taherkhani *et al.* (2013) found that the leaf essential oil of *A. absinthium* indicated significant activity against *C. albicans*. Also, Juteau *et al.* (2003) recorded the similar result. Only three strains are different in their susceptibility response. One of them is *T. mentagrophytes*, since it was more susceptible to the essential oil of *E. caryophyllata*. Others were *A. flavus* and *M. canis*. They were affected by the oil of *T. vulgaris* with the largest inhibition zone of 21 and 23 mm, respectively and MIC 100, 25 µg/ml, in that order.

Cytotoxicity and antitumor activity of the plants essential oils are shown in Table (5). Investigation of antitumor activity of plant essential oils showed that *A. absinthium* was the third active essential oil against Lymphoma cell line and Erlich cell

Table 3. Antimicrobial activity of essential oils using agar well diffusion method against selected pathogenic yeasts and fungi.

Fungi	Plants	<i>Salvia officinalis</i>	<i>Artemisia absinthium</i>	<i>Eugenia caryophyllata</i>	<i>Thymus vulgaris</i>	Control
<i>A. flavus</i>		19±1.3	16±0.23	17±1.00	23±1.0	ND
<i>A. niger</i>		19±0.18	21±0.08	19.8±1.4	20±0.15	ND
<i>E. floccosum</i>		14±1.0	24±0.22	19±1.0	21±0.18	ND
<i>T. mentagrophytes</i>		17±1.0	13±0.03	19±0.90	11±1.0	ND
<i>M. canis</i>		18±0.10	19±0.14	18±1.00	21±0.18	ND
<i>C. albicans</i>		18±0.11	26±0.16	18±0.10	10±0.15	ND
<i>C. neoformans</i>		19±0.18	25±0.01	19±0.17	17±2.16	ND
Fungal index*		140	165	147.8	140	ND

* Fungal Index: Total Activity (mm).

Table 4. The minimal inhibitory concentration (µg/ml) of essential oils against pathogenic fungi

Fungi	Plants	<i>Salvia officinalis</i>	<i>Artemisia absinthium</i>	<i>Eugenia caryophyllata</i>	<i>Thymus vulgaris</i>	Control
<i>A. flavus</i>		150	100	100	25	>200
<i>A. niger</i>		150	100	100	50	>200
<i>E. floccosum</i>		150	100	100	50	>200
<i>T. mentagrophytes</i>		150	100	50	50	>200
<i>M. canis</i>		150	100	100	100	>200
<i>C. albicans</i>		100	50	100	50	>200
<i>C. neoformans</i>		100	50	100	50	>200

Table 5. Toxicity against *Artimia salina* and antitumor activities of the different concentrations of selected plant essential oils (EOs).

Plants	Toxicity against <i>Artimia salina</i> LD ₅₀ (µg/ml)	Antitumor activity (LD ₅₀ µg/ml)	
		Lymphoma cell line	Erlich cell line
<i>Salvia officinalis</i>	600	400	400
<i>Artemisia absinthium</i>	400	600	400
<i>Eugenia caryophyllata</i>	600	200	400
<i>Thymus vulgaris</i>	≥ 600	≥ 600	≥ 600

line. The highest active essential oil was *E. caryophyllata*, while *T. vulgaris* has no antitumor activity. In accordance, Jeong Seo (2003) reported that the artemisetin isolated from Afsantin (*Artemisia absinthium* Linn.) exhibited marked antitumor activity against melanoma. Taherkhani (2014) also confirmed that the essential oil displayed good cytotoxic action towards the human tumor cell line. Toxicity against *Artimia salina* has been studied, the highest cytotoxic effect was recorded for the essential oil of *A. absinthium* (LD₅₀ 400). However this value means a moderate cytotoxic effect. Taherkhani (2014) reported that cytotoxicity of the oil towards human tumor cell line is much higher than that required for healthy human cells. These results indicate low adverse effects for the oil. Lust (1979) reported that pure *A. absinthium* (wormwood) oil is very poisonous, but with proper dosage, it has little or no danger.

The essential oil of *A. absinthium* was investigated by GC-MS to identify active compounds. Two major compounds were identified, the first one was bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl; common name (fenchone) and the second compound is bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl; common name (camphor). Mass spectra for both compounds are shown in Figures (1) and (2). Fenchone is suggested to be involved in the antimicrobial activity of *A. absinthium*. Similarly, antibacterial

activity of *Foeniculum vulgare* is attributed to fenchone present in its essential oil (Dinesh *et al.*, 2014). In contrast, fenchone was not considered a potent antimicrobial agent as mentioned by Lis-Balchin and Roth (2000). The second compound may also be contributed to the antimicrobial activity of *A. absinthium* is bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl; common name (camphor). Nibret and Wink (2010) recorded that *A. absinthium* essential oil contains 3.7% camphor. Guvenalp *et al.* (1998), reported that camphor is one of the main components of the essential oils of some *Artemisia* species. According to Mukul (2013), camphor and davanone are the major components of *A. absinthium* essential oil. Nibret and Wink (2009) also reported that *A. absinthium* oil containing 38.37% camphor. Camphor is hypothesized to contribute to the antimicrobial activity of *A. absinthium* oil. This is agreed with Mahboubi and Kazempour (2009), they investigated the antimicrobial activity of camphor against Gram positive bacteria, Gram negative bacteria, yeast and filamentous fungi. Camphor is known to be toxic when ingested in large amounts, and may lead to seizures, confusion, irritability and neuromuscular hyperactivity (Liebelt and Shannon, 1993 and Ryno, 2009). Thus the cytotoxic effect of *A. absinthium* essential oil may be attributed to its camphor content.

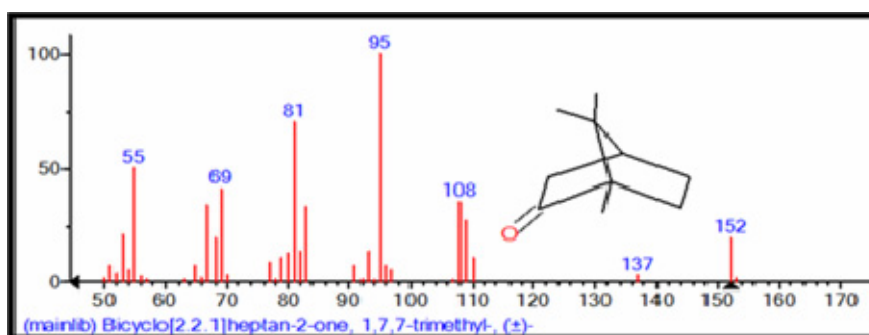


Fig. 1. Mass spectrum of bicyclo [2.2.1] heptan-2-one, 1,7,7-trimethyl, (±).

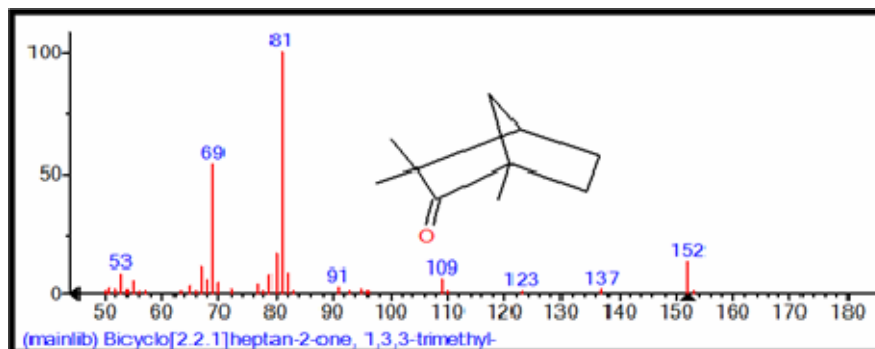


Fig. 2. Mass spectrum of bicyclo [2.2.1]heptan-2-one, 1,3,3-trimethyl, (\pm).

CONCLUSION

Our results pointed out the potential value of *A. absinthium* essential oil against some bacterial and fungal isolates, as it exhibits the strongest antimicrobial activity among all of the tested plant essential oils with moderate cytotoxic effect and antitumor activity. Fenchone and/or Camphor are the major compounds may contribute to the biological activities of the plant essential oil.

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التأثير المضاد للبكتيريا و الفطريات و الأورام وسمية الزيوت الطيارة لنباتات المريمية و الزعتر و القرنفل و الشيح

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الزيوت الأساسية هي السوائل العطرية و الطيارة المستخرجة من النباتات. المواد الكيميائية الموجودة في الزيوت الأساسية عبارة عن مركبات ثانوية تلعب دوراً هاماً في الدفاع عن النبات لأنها غالباً ما تمتلك خصائص مضادة للميكروبات. في هذه الدراسة، تم اختبار نشاط الزيوت الطيارة المضاد للميكروبات لأربع نباتات ضد ثمانية أنواع بكتيرية ممرضة و سبعة سلالات من الفطريات. و كانت النباتات المستخدمة هي المريمية (*Salvia officinalis*) و الزعتر (*Thymus vulgaris*) و القرنفل (*Eugenia caryophyllata*) و الشيح (*Artemisia absinthium*). وقد أظهرت كل الزيوت الطيارة للنباتات نشاطاً مضاداً للبكتيريا و الفطريات. و تراوحت مناطق تثبيط نمو البكتيريا المسببة للأمراض من ١٠ إلى ٢٨ مم و التركيز الأدنى لتثبيط النمو الميكروبي (MIC) من ٢٥ إلى ١٥٠ ميكروجرام/مل، في حين تراوحت مناطق تثبيط نمو السلالات الفطرية من ١٠ إلى ٢٦ مم و التركيز الأدنى لتثبيط النمو الميكروبي (MIC) من ٢٥ إلى ١٥٠ ميكروجرام/مل. تم اختبار التأثير الخلوي السام على الأرتيميا (*Artemia salina*) و النشاط المضاد للأورام على خلايا سرطان إيرلخ الاستسقاءى (*Ehrlich ascites carcinoma cell line*) و خلايا سرطان الغدد الليمفاوية (*Lymphoma cell line*). أظهر نبات الشيح أفضل تأثير مضاد للأورام على خلايا سرطان الغدد الليمفاوية مع تأثير متوسط السمية على خلايا الأرتيميا. و بتحليل الزيت الطيار للشيح بواسطة التحليل الكروماتوجرافي الغازي لطيف الكتلة (GC-MS) تبين وجود مركبين أساسيين هما كامفور (Camphor) و فينشون (Fenchone) المحتمل كونهما أصحاب الدور الرئيسي في النشاط الحيوي للزيت الطيار للنبات.