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In vitro cytotoxicity and antimicrobial activities of some common essential oils

Ishrak Khafagi*, Ahmed Dewedar and Shaimaa Farouk

Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

ABSTRACT

Cytotoxicity, antibacterial and anticandidal activities of seven common essential oils including anise *Pimpinella anisium* L., black cumin *Nigella sativa* L., caraway *Carum carve* L., clove *Syzgium aromaticum* L., cumin *Cuminum cyminum* L., fennel *Foeniculum graveolens* Mill, and rosemary *Rosmarinus officinalis* L. were investigated against *Artemia salina* (Brine shrimp), two Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis,* four Gram-negative bacterial strains *Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa* and two yeasts, *Candida albicans* and *Candida tropicalis*. The oils of clove and rosemary showed strong cytotoxic activity against the nauplii of brine shrimps. The oils of black cumin, clove, fennel and rosemary exhibit antimicrobial activity. Clove oils gave broad spectrum antimicrobial activity. The sensitivity of minimal inhibitory concentration assay of clove oil against the tested yeast were comparable to that of the cytotoxicity assay. The biological activity of those steam-distilled oils were heat-resistant as they did not lose activity after autoclaving.

KEYWORDS: essential oils, antimicrobial activity; cytotoxicity

INTRODUCTION

The antimicrobial activity of higher plants and essential oils derived from them have been well known for centuries (Mitscher *et al.* 1987; Hepper 1990). Volatile or essential oils are volatile in steam. They differ entirely in both chemical and physical properties from fixed oils. They are secreted in oil cells, in secretion ducts, cavities or in glandular hairs. Herbs and spices are antimicrobially active themselves (Akgul & Kivanc 1986-1989; Khafagi & Abdel-Kareem1998) and are distinguished by having particular essential oils.

Large quantities of essential oils are produced annually all over the world for flavouring (e.g. oil of lemon), in perfumery (e.g. oil of rose) or as starting materials for the synthesis of other compounds (e.g. oil of turpentine). For therapeutic purposes they are administered as inhalations (e.g. eucalyptus oil), orally (e.g. clove) and mouthwashes (e.g. thymol). Many essential oils including those of anise, caraway, cumin, clove, rosemary and black cumin are employed in the practice of aromatherapy (Davidson & Parish 1989). There is a tradition of using the essential oils of herbs and spices in medicine as flavouring component, as for example anise, clove, fennel and mint. Their role is to mask the essential odour of the active medicinal ingredient and hence render the preparation acceptable for consumption (Helliwell & Ransom 1981).

Biologically active compounds from plant sources have had a dramatic impact in medicine including: quinine for treatment of malaria; reserpine for controlling hypertension; cocaine as a muscle relaxant; and vincristine for treating children with leukemia. Tropical plants have an enormous but as yet untapped potential to yield novel drugs and medicine (Deans & Svoboda 1990).

Usually, the essential oils produced from medicinal plants can be used for the production of fine pharmaceutical drugs, flavouring foods and drinks and for flavouring the rudimentary odours of prepared pharmaceutical products (Fransworth *et al.* 1985). As essential oils are

*Address for Correspondence

essential additives to food or drug products, the biological activities of those oils should be fully explored. The antimicrobial activity of most essential oils produced world wide is frequently reported: including antibacterial (Deans & Ritchi 1987), antifungal (Garg & Dengre 1988), amoebicidal (Blasi *et al.* 1990), etc. Antimicrobial activity of essential oils against human, animal, plant (Panizzi *et al.* 1993) and food-borne (Smith-Palmer *et al.* 1998) pathogens have been investigated. *In vitro* activity of common constituents of essential oils (Hinou *et al.* 1989; Shapiro *et al.* 1994) have also been recorded.

Cytotoxic activity of most common essential oils is important to be assayed too in order to elucidate completely the biological response of those volatile oils. There are only scarce reports of cytostatic activity of some essential oils (Saenz *et al.* 1996). The present study aims to investigate the cytotoxicity and antimicrobial activities of some common essential oils and evaluate the heat resistant ability of the active oils in order to estimate the biological potential of those oils.

MATERIALS AND METHODS

Essential oils: Seven common essential oils (Table 1) were prepared by steam distillation (Evans 1996) from seven different herbs and spices. The major volatile constituents of each oil is shown in Table 1.

Table 1. Main essential oil constituents of the selected oils

Botanical name	Common name	Oil source	Essential oil	Oil %	Chemical class
Labiatae					
Rosmarinus officinalis L.	Rosemary	Leaves	Borneol Cineole	60 2-5	Ester Terpene
Myrtaceae					
Syzgium aromaticum L.	Clove	Flower buds	Eugenol	60	Phenol
Ranunculaceae					
Nigella sativa L.	Black cumin	Seeds	Nigellone	75	Ketone
Umbelliferae					
Carum carve L.	Caraway	Dried fruits	Carvone Limonene	60 10	Ketone
Cuminum cyminum L.	Cumin	Dried fruits	Cuminaldehyde Limonene	35	Aldehyde Ketone
Foeniculum graveolens Mill.	Fennel	Dried fruits	Anethol Fenchone	60 20	Ether Ketone
Pimpinella anisum L.	Anise	Dried fruits	Anethol	80-90	Ether

Cytotoxicity assay: Brine shrimp larvae (*Artemia salina*) are commonly used for cytotoxicity assays in pharmacology. These larvae are sensitive to toxic substances. The ratio between dead larvae (no motility) and living larvae (high motility) in comparison to a control without any toxic substances is used to estimate the toxicity of the test solutions. The test is not only used for predicting cytotoxicity, but is also used as a predictor of antitumor and pesticidal activity (Sanchez *et al.* 1993).

A micro-well cytotoxicity assay using *Artemia salina* was used (Solis *et al.* 1993). Brine shrimp eggs (obtained from Interpet Ltd. Dorking, England) were hatched in sea water supplemented with 6 mg/l dried yeast, oxygenated with an aquarium pump and incubated in a warm room at 22-29°C for 48 hours. Essential oils were dissolved in 50µl Tween 80, then made up to 1 mg/l in sea water. Serial dilutions in 100 µl sea water were made into the 96

wells of microplates. Control wells with either sea water or Tween 80 were included in each experiment. The experiment was done three times. About 100 µl of the nauplii (containing 10-15 organisms) was added to each well and the covered plate incubated at 22-29°C for 24 hours. Plates were then examined under a binocular microscope and the numbers of dead (non-motile) nauplii in each well were counted. Finally, all shrimps were scarified by adding 100 µl methanol to each well. After 15 minutes, the total numbers of shrimps/ well were counted. Probit analysis (Finney1971) was used for calculating the LC_{50} values.

Antimicrobial bioassays: Both diffusion and dilution methods were used for screening the antimicrobial activity of essential oils. Six bacterial species were used for screening the antibacterial activity of the seven selected essential oils including five bacteria capable of human pathogenicity (Table 2 & 3). Two yeasts were used for investigating the anti-candidal activity of the target essential oils. The microorganisms were obtained from the culture collection of the United States Department of Agriculture, Northern Regional Research laboratory (Peoria, Illinois, USA).

I. Hole-plate diffusion method: The hole-plate diffusion method (Ericsson & Sherris 1971) was used for screening the antimicrobial action of essential oils. Bacterial strains were cultured on Nutrient Agar, while yeast was cultured on Sabouraud Dextrose Agar. An inoculum of each microbial strain was suspended in 20 ml of Nutrient or Sabaroud Broth and shaken overnight at 30°C. The microbial cultures were diluted 1/10 with broth before use. The Petri dishes were pre-seeded with 20 ml of Agar medium and 1 ml of microbial culture. The seeded agar was poured into pre-sterilized Petri dishes and allowed to set completely. Cups were then punched out with sterile cork porer No. 5. Various concentrations 2, 10, 20, 50 and 100 µl of essential oils were added to wells (6 mm diameter) punched out in the agar plates. Five replicates were made for each essential oil and the experiment was repeated three times. The Petri dishes were pre-incubated at 5°C for 1h to permit maximum diffusion of the active principles into the agar. The inoculated plates were then incubated at 30°C for 24-48h. The inhibition zones were then measured for each oil at different concentrations against the Gram-positive and Gram-negative bacteria or yeast.

II. Dilution method

a. Assay in Flask cultures: This assay was performed in order to confirm the diffusion method and to avoid obtaining false positive results from the hole-plate assay. An inoculum of each microbial strain was suspended in 20 ml of Nutrient Broth or Sabarouad Broth and shaken overnight at 30°C. The cultures were diluted 1/10 with broth before use. The flasks were pre-seeded with 50 ml of broth medium and 1 ml of diluted microbial culture (Sharma *et al.* 1984). On the basis of the preliminary information obtained from the standard cup assay, appropriate quantities of the active essential oils were added to the flasks containing one of the microbial cultures and the broth medium. For each active essential oil, 10, 20, 50 and 100 µl concentrations were tested in triplicate. Flasks containing no essential oil were used as control. Flasks were incubated in shaker at 30 °C for 48 h. The flasks that did not show any sign of visual growth were considered as positively containing antimicrobial substance(s).

b. Two-fold tube dilution method: Dilution assays usually give a reliable indication of antimicrobial action of a metabolite(s). Minimal inhibitory concentrations (MIC) were determined for the active essential oils against three microbial strains representing both Gram-positive and Gram-negative bacteria a as well as yeast using the serial tube-dilution method.

Heat sensitivity treatments: Evaluating the heat sensitivity of the active essential oils was tested by autoclaving a fraction of each oil at 121°C for 15 min. Both autoclaved and non-autoclaved fractions were tested for the antimicrobial activity against the six test bacterial strains in the same manner as reported above. The antibacterial and anti-candidal activities of

the autoclaved and non-autoclaved fractions of the essential oils were compared against Gram-positive and Gram-negative bacteria and yeast using both diffusion and dilution methods.

RESULTS

Cytotoxic activity: Table 2 displays the LC_{50} of the seven selected oils. It shown from the table that clove and rosemary oils exhibit strong cytotoxicity against brine shrimp nauplii. Data from Table 2 shows that the LC_{50} of those oils is between 4.2-5.0, which represent high cytotoxicity and may predict antitumor and pesticidal potential of those oils.

Table 2. Biological activity of some common essential oils using in vitro short-term cytotoxicity assay against *Artemia salina* and minimal Inhibitory concentration (MIC) against Gram-positive and Gram-negative bacteria and a yeast.

	LC_{50}[a] (µl/ml)	MIC[b] (µl/ml)		
Essential oil	*Artemia salina* nauplii (microwell test)	*Staphylococcus aureus* NRRL[c] B-767	*Klebsiella pneumoniae* NRRL B-14232	*Candida albicans* NRRL Y-12983
Rosemary	5.0 ± 0.24	12.5	> 100	12.5
Clove	4.2 ± 0.19	6.25	6.25	6.25
Black cumin	> 1000	12.5	> 100	12.5
Caraway	> 1000	> 100	> 100	> 100
Cumin	> 1000	> 100	> 100	> 100
Fennel	> 1000	12.5	> 100	25.0
Anise	> 1000	> 100	> 100	> 100

[a] LC_{50} estimated by probit test using the brine shrimp microwell assay.
[b] MIC= Minimal inhibitory concentration of an essential oil against test micro organisms using two-fold tube dilution method.
[c] NRRL= Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Illinois, USA.

Antimicrobial activity

I. Diffusion assay: In the present investigation, the common steam-distilled essential oils showed varying antimicrobial activities against Gram-positive and gram-negative bacteria and yeast. Results are summarized in Table 3.

Table 3 presents the antibacterial and anti-candidal activities of seven common essential oils. The oils of black seeds, clove, fennel and rosemary were active. Clove oils gave broad spectrum antimicrobial activity, while the other three oils were active against Gram-positive bacteria and yeast (Table 3). The antibacterial activity of black cumin, clove fennel and rosemary were heat resistant since it did not get lost during autoclaving (Table 3). The same activity was recorded from the autoclaved and non-autoclaved fractions. Also the experiment shows that the non-active essential oils were not enhanced to be active through the heat treatment.

Table 3. Antibacterial and anti-candidal activities of autoclaved and non-autoclaved fractions of some steam-distilled oils

Oil plant		Microbial strains							
		B.[a]	*S.*[b]	*K.*[c]	*E.*[d]	*Ps.*[e]	*P.*[f]	*C.*1[g]	*C.*2[h]
Anise	(auto)[i]	-	-	-	-	-	-	-	-
	(non)[j]	-	-	-	-	-	-	-	-
Black cumin	(auto)	+++	+++	-	-	-	-	+++	+++
	(non)	+++	+++	-	-	-	-	+++	+++

Caraway	(auto)	-	-	-	-	-	-	-	-
	(non)	-	-	-	-	-	-	-	-
Cumin	(auto)	-	-	-	-	-	-	-	-
	(non)	-	-	-	-	-	-	-	-
Clove	(auto)	+++	+++	+++	++	++	++	+++	+++
	(non)	+++	+++	+++	++	++	++	+++	+++
Fennel	(auto)	++	+++	-	-	-	-	++	++
	(non)	++	+++	-	-	-	-	++	++
Rosemary	(auto)	++	++	-	-	-	-	++	++
	(non)	++	++	-	-	-	-	++	++

[a] *B= Bacillus subtilis* NRRL NRS-744, [b] *S= Staphylococcus aureus* NRRL B-767,
[c] *K= Klebsiella pneumoniae* NRRL B-14232, [d] *E= Escherichia coli* NRRL B-3704,
[e] *PS Pseudomonas aeroginosa* NRRL B-23, [f] *P= Proteus vulgaris,* NRRL B-123,
[g] *C1= Candida albicans* NRRL Y-12983 and [h] *C2= Candida tropicalis.*
[i] auto= The autoclaved fraction of an essential oil which was subjected to most heat treatment.
[j] non= Non-autoclaved fraction of an essential oil.
- = No zone of inhibition, + = 1-10 mm zone of inhibition, ++ = 11-20 mm zone of inhibition, +++ = 21-30 zone of inhibition.

II. Dilution bioassay

Assays in flasks: The antibiotic sensitivity assays of steam-distilled essential oils in shake Flask cultures were comparable to that of the standard cup assay. The highly active essential oils like that of the clove showed activity from below 10 µl. On the other hand, the moderately active oils like that of black cumin and fennel inhibits microbial growth only from high concentration 50 µl.

Minimal inhibitory concentration (MIC): The Minimal inhibitory concentration (MIC) of the broad spectrum clove oils was about 6.25 and that of black cumin, fennel and rosemary were about 12.5-25 and rosemary essential oil against both Gram-positive bacteria and yeast (Table 2).

DISCUSSION

The present investigation shows the cytotoxicity and antimicrobial potential of seven common essential oils. Generally, these steam-distilled oils are used for seasoning Egyptian meals and drinks and in herbal medicine. Results reveal that more than half of the tested essential oils have antimicrobial activity against at least two of the tested organisms, while about a quarter of the oils shows cytostatic activity.

The cytotoxicity bioassay against *Artemia salina* is a simple and inexpensive method to test cytotoxicity, to biodirect phytochemical fractionation of natural products and as a predictor of antitumor and pesticidal activity (Sanchez *et al.* 1993). It indicates also antiviral, antiplasmodial, antifilarial, antimalarial activities (Solis *et al.* 1993). The *in vitro* assessment of the biological action of essential oils is important to estimate their distinct and accumulative potential, to test their use alone as an antibiotic or as a supplement to edible or pharmaceutical products. The results shows that clove and rosemary oils give strong biological activity including cytotoxicity, antibacterial and anti-candidal actions.

The oils of black cumin, clove, fennel and rosemary were antimicrobially active. Clove oils gave broad spectrum activity. The oils of black cumin, fennel and rosemary were active against Gram-positive bacteria and yeast. It is well understood that the antibacterial activity of higher plants and products derived from them is mainly dependent on the habitat and the ecological source of the derived plant material (Farnsworth 1990). Therefore, sometimes there are reports on the antibacterial activity of the plant tissues and not for the oils. Conner & Beuchat (1984) studied the inhibitory activity of some common essential oils

against yeast. Zaika & Kissinger (1981) and Farag *et al.* (1989) isolated the major volatile components which account for the antimicrobial activities of some essential oils.

The main constitutes of clove oil is eugenol (Evans 1996). The broad spectrum antimicrobial activity as well as the strong cytotoxic potential may be attributed to this phenolic component. The inhibitory components of other oils may be different: the aldehyde cuminaldehyde in cumin, the ether anethol in fennel, the ketone nigellone in black cumin and the ether borneol in rosemary (Evans 1996). Kivanc & Akgul (1988) reported that the most active components of the essential oils were phenols, followed by aldehydes and ketones. The essential oils tested in this study fulfil the same trend.

The antibiotic sensitivity assays of steam-distilled essential oils in dilution assays were comparable to that of the diffusion method. Likewise, Sharma *et al.* (1984) reported the comparable antibacterial activity of both standard cup and assay in flasks methods for the antifungal activity of some herbs and spices.

The comparison between cytotoxicity and antimicrobial activities shows that cytotoxicity assay cannot predict antibacterial action. The same observation was recorded by Sanchez *et al.* (1993). This may be because of the prokaryotic cell structure of a bacterial cell is different than the eukaryotic cell of the brine shrimp. Therefore, although the brine shrimp assay is a simple and reasonable for predicting a vast array of activities, an antimicrobial bioassay (e.g. MIC) should be included also in order to fully understand the potential activity of a natural compound(s) or a crude extract.

The antibacterial activity of black cumin and fennel were heat resistant as it did not lost during autoclaving. The same activity was recorded from both autoclaved and non-autoclaved fractions. Similarly, El-Hissy & Ahmed (1973) found that oil plants produce antimicrobial products, which resist moist heat.

In conclusion, both brine shrimp and antimicrobial bioassays should be used together to entirely perceive the possible activity of plant crude extracts or compounds derived from them. The cytotoxic and antimicrobial activities of essential oils are valuable properties in addition to either pharmaceutical or food products.

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الملخص العربى

النشاط المضاد للخلايا وللميكروبات لبعض الزيوت العطرية

إشراق خفاجى، أحمد دويدار، شيماء فاروق

قسم النبات – كلية العلوم – جامعة قناة السويس – الإسماعيلية – مصر.

تم دراسة النشاط المضاد للخلايا وللكائنات الدقيقة لبعض الزيوت العطرية المعروفة بأهميتها الطبية والعطرية فى الطب الشعبى والتى تعطى نكهة للأطعمة والمشروبات، ضد الأرتيميا، وستة أنواع من البكتيريا الموجبة والسالبة لجرام ونوعين من الخمائر. وهذه الزيوت العطرية لنباتات اليانسون، حبة البركة، الكروية، القرنفل، الكمون، الشمر وحصى البان. ولقد وجد أن بعض أنواع من هذه الزيوت لها نشاط مضاد للخلايا وهى زيت القرنفل وحصى البان وأربعة من الزيوت الطيارة لها نشاط مضاد للبكتيريا والخمائر حيث أن زيت القرنفل له نشاط مضاد للبكتيريا واسع المجال، فى حين أن زيوت حبة البركة، وحصى البان والشمر لهم نشاط مضاد للبكتيريا الموجبة لجرام والخمائر.

كما تم تقييم حساسية المضادات الحيوية المنتجة من بعض الأنواع التى لها نشاط حيوى مقاوم للحرارة الرطبة عند درجة ١٢١ درجة مئوية ولمدة ١٥ دقيقة. وقد ثبت أن موادها الفعالة تقاوم الحرارة ويمكن أن يساهم ذلك فى حفظ الأطعمة التى تعالج بالحرارة ضد البكتريا.