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Full Length Research Paper

Effect of plants extracts on the growth of *Candida albicans* and *Staphylococcus aureus*

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The purpose of this study was to estimate the effectiveness of cardamom, cinnamon, ginger, cloves and myrrha extracts on the inhibition of *Candida albicans* and two isolates of *Staphylococcus aureus* (MRSA) and *Staphylococcus aureus (*MSSA) and in different concentrations*.* The results showed that the aqueous extract of spices have no inhibitory effect on the growth of the tested microbes, while, that two types of the Myrrha (*Commiphora myrrha* and *C. molmol*) aqueous extracts inhibited all the tested microbes. Also, the alcoholic extract of four spices had inhibitory effect on the growth of three pathogenic tested isolates. By performing the chemical analysis for the Myrrha, it was noted that it contains three components known for their antimicrobial effect. These components are: 2-fluorodiphenylmethane, Tribenzo-1,2,3,4,5,6anthracene and 2-bromo-1-(4-bromophenyl)-ethanone. In addition, the activity of 8 types of the bacterial antibiotics used pharmaceutically in order to know the sensitivity of the microbes' tested showed that *S. aureus* (MRSA) and *C. albicans* were more resistant, as it not affected by all the tested antibiotics. As a result, it is more effective to use Myrrha instead of industrial antibiotics.

Key words: *Staphylococcus aureus*, *Candida albicans*, *Commiphora,* 2-fluorodiphenylmethane, 2-bromo-1-(4-bromophenyl)-ethanone, cardamom, cinnamon, ginger, cloves.

INTRODUCTION

Many medicinal plants produce antioxidant, antimicrobial and anti-inflammation properties which protect the host from cellular oxidation reactions and other pathogens highlighting the importance of looking for natural antimicrobial drugs (Farzaei et al., 2014). In addition, adding the spices to foodstuffs is not limited to improving the flavor and test, but this also implies preservative and antibacterial effects on the plant and the human pathogens. While the antimicrobial drugs have been discovered and the effectiveness thereof has been proved remarkably in controlling the bacterial infections, but the absolute effectiveness thereof without restriction or condition cannot be admitted, due to the ability of some pathogens to adapt quickly and to resist many of the discovered effective medicines (Cowan, 1999). Many studies have been performed on the natural ingredients

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in the same, their antimicrobial and antifungal effect has been tested and their effect has been compared with the standard antibiotics (Sakagami et al., 2001; Velickovic et al., 2003). Many researchers in the field of natural antibiotics for microbiology have indicated the importance of using the plant extracts distinguished with the ease of obtaining the same and its low side-effects in comparison with the synthetic antibiotics (Adel and Mahasneh, 1999; De-Boer et al., 2005; Shabana and El-Adly, 2016).

Egyimann et al. (2003) have stated that during the last ten years, the infections resulted from *C. albicans* and systemic infections resulting from the same especially among the immunodeficiency patients. The studies have shown that the resistance of yeast is increasing continuously for the artificial antibiotics that are represented in Azoles Group such as the fluconazole (Rex et al., 1995).

The cardamom, scientific name *Electaria cardamom*, is affiliated with Zingiberaceae Family. Cardamom is used in many and various ways in different foods whether solely or mixed with other spices and it is distinguished with many health benefits; it repels the gases, comfortable for the digestive system, catalysts for the digestion, has beneficial effect on the liver and in resisting the cold, fever and mouth inflammations (Bekel, 2007).

In addition, the cinnamon, scientific name *Cinnamon zeylanicum* is affiliated with Lauraceae, its oil is used as gases repellant, eliminates the spasm, general stimulant, antiseptic, anti-diarrhea, treats diabetes, reduces cholesterol, fat and gum diseases, stimulates blood circulation and it has activity against inflammations (Giday, 2001). Also, it has a potential medical use with regards to its antimicrobial properties, especially antibacterial activity (Nabavi et al., 2015).

Ginger, scientific name *Zingiber officinale*, affiliated with Zingiberaceae, common name ginger is known with its uses in the medical field from antiquity (Mascolo et al., 1998; Akoachere et al., 2002). It has been listed in the medical plants, due to its wide extent of medical uses (Ficker et al., 2003; Habsah et al., 2000). Chemical analysis indicate that the ginger contain effective compounds against certain micro biologics; these compounds are shogaols, zingerone and gingerols (Ernst and Pittler, 2000); while Rehman et al., 2011 stated that it contains essential oils consisting of the compounds sesqiterpenoids, B-sesquiphellandrene, phenylpropanoid, cineol, farnesene, bisabolene.

The clove, scientific name: *Syzygium aromaticum*, in the Myrtaceae Family, common name Carnation is gases repellant, eliminates the spasm and it is one of the stimulants, antioxidants, antipyretic, natural anti-microbial, anesthetic, anti-throat inflammations and dental problems (Lemma et al., 2002).

Myrrh, scientific name *Commiphora myrrha* and *Commiphora molmol*, is in Bruseraceae family, common name: Myrrh; the resin of this plant in used in treating the cuts, bowel disorders, diarrhea, coughing and chest (Ghazanfar, 1994) and it is also used for treating (Serfaty and Itid, 1988). Rahman et al. (2008) stated that resin has been obtained from *C.molmol* which is active against many strains of *Staphylococcus aureus*. Hussien et al. (2011) performed a study in order to test the effect of aqueous plant extracts of cinnamon, cardamom, cloves, thyme, mustard and basil in inhibiting *Candida albicans* and *S. aureus*, they noticed that their ability in the inhibition is different, as they have positively affected the growth of *S. aureus* with rate of 10-20%, while they have no effect on *C. albicans*. Moreover, Takahashi et al. (2011) confirmed that ginger oil is more effective in inhibiting *C. albicans* yeast than the other oils and the minimum inhibiting concentration (MIC) is 200 mg/mL; while, the minimum inhibiting concentration (MIC) for the alcoholic extract of Myrrh which affects strains of *S. aureus* tested is 31.25 and 250 mg/mL (Abdallah et al., 2009). Al Ahmadi (2006) stated that the oils of Commiphora resins are rich in furanosesquiterpenoids compounds in a total number of 20 different compounds from such type. The separated compounds of furanosesquiterpenoids or extracts of Commiphora resin extracts have showed activity that resists the bacteria and fungi and they have anesthetic properties. Therefore, this study aimed at finding chemical materials from natural plant source that have effectiveness in inhibiting the growth of *C. albicans* and *S. aureus* whether they are sensitive or resistant to the known antibiotics and antifungal drugs to avoid human health hazards.

MATERIALS AND METHODS

Plant samples

The plant-samples, *C. myrrha* and *C. molmol*, were collected from retail-shops in Dammam city. The bought samples were kept at 4°C until tested.

Microbial isolates source

There are two isolates of *S. aureus* (MRSA) and *S. aureus* (MSSA) and fungal strain *C. albicans* (yeast, clinical isolate) employed for activity testing, were obtained from Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, University of Dammam. The microbial samples were subculture and kept at 4°C until used.

Preparing the extracts

Aqueous extract

Five grams powder of plant material was weighed in an Erlenmeyer of 200 ml to which 100 ml of distilled water were added which gave the final concentration of 5%. They were soaked at room temperature for 24 h, then filtered using layers of sterilized gauze (Boyraz and Ozcan, 2005).

Essential oils

Four essential oils obtained from perfumery shops in Dammam city,

(commercial producers of plant essential oils (80%) and aromatic substances) were used in this study. From each essential oil (cardamom – cinnamon- Ginger and clove), 2 ml were transferred to 9 ml ethyl alcohol (80%), after that concentrations 55.56, 83.3, 111, 250 and 1000 µ g/ml were prepared according to Prabuseenivasan et al. (2006), but 80% ethanol alcohol without oil, was used as a positive growth control.

Media preparation

Two media were used to evaluate the antimicrobial activity as follows: Nutrient Agar Oxiod was prepared for bacteria and Sabouraud Dextrose Agar Oxoid was used for yeast, which was incubated at 28°C, then transferred 250 ml into flasks and autoclaved at 121°C for 15 min.

Testing the effectiveness of aqueous extracts

The agar well diffusion method was used (Perez et al., 1990). From microbial inoculum, 1 ml was added in a sterile plastic Petri dish and then 10 ml of the medium was poured and left to harden. Then the plates incubated at temperature of 23-30°C for 48 h. The results were recorded by calculating the inhibited area. The inhibition zone was measured in mm (Amade et al., 1994).

Determination of minimum inhibitory concentration (MIC)

The antimicrobial activity of the extracts was determined by 2-fold dilution method using above media and MICs were read in µg/ml after overnight incubation at 37°C (Omura et al., 1993).

Detection of the composition of the chemical materials of Myrrha resin aqueous extract

Chemical composition analysis of myrrha was performed using mass spectrum, the GC–MS analyses of the volatile oils were carried out using a Hewlett-Packard 5890 gas chromatograph coupled to a VG Analytical 70-250S mass spectrometer, to determine the active principle compounds responsible for antimicrobial activities.

Statistical analysis

The statistical analyses were performed according to the fully randomized design and with three replicates for each treatment. The results were analyzed and compared at the 0.05 level of probability using the L.S.D. and the 16 version of SPSS program according to the method of Norusis (1999).

RESULTS AND DISCUSSION

Effect of the aqueous extracts of the tested plants

A study on effect of aqueous extracts of the tested spices on the three microbes using agar well diffusion technique was done for its quality, the ease of performing the same and the clarity of results thereof. The results were determined after 24-48 h by measuring the dimeters of inhibition zones. The results recorded in the Tables 1, 2 and 3) showed that there is no inhibiting effect of the growth of the tested microbes for the aqueous extract of cardamom, cinnamon, ginger and cloves; while the aqueous extract of both types of tested Myrrha was showed that there is inhibition zone for the microbes, the subject matter of the study, and that the resistant *S. aureus* (Table 1) was not affected by the first type of Myrrha, while its growth was inhibited by the three first concentrations of the Myrrha of the second type, *C. molmol*. The diameter of inhibition zone reached 0.874, 0.852 and 0.600 mm on the concentrations 1000, 250 and 111 µ g/ml successively. While that the sensitive *S. aureus* (Table 2) was the most affected. The present result showed that the sensitive *S. aureus* was the most affected by two types of aqueous extract of myrrha with a mean inhibition zone of 0.874 to 0.651mm with *C. myrrha* at 1000 and 83.3 MIC (µ g/ml) and 0.874 to 0.826 mm with *C. molmol* at 1000 and 250 MIC (µg/ml), respectively. Also, *C. albicans* has been affected by both types of Myrrha. The inhibited zone reached 0.776 and 0.749 mm for both concentrations of 250 and 1000 MIC (µg/ml) for the first type of Myrrha, while such zone reached 0.883 and 0.725 mm, successively for the second type of Myrrha. This result is in agreement with Rash and Al-Habib (2011) and Akintobi et al. (2013) and it disagrees with Hussien et al. (2011).

Impact of the essential oils of the tested plants

A study on essential oils impact of the tested spices on the microbes studied by the previous method and the obtained results as recorded in the Tables 1, 2 and 3 showed that the effectiveness of the plants essential oils toward the microbes was different according to the combination of the microbe. And *C. albicans* and *S. aureus* sensitively to the antibiotics have shown sensitivity more than the resisting bacteria which has not been affected by the essential oils of all the tested plants. The bacterial sensitivity to the antibiotics has shown a remarkable susceptibility to the essential oils of all the tested plants with all concentrations. The effectiveness of the cinnamon oil was greater followed by the clove then the ginger and finally the cardamom. The yeast was affected generally and its sensation to the cinnamon was greater followed by the cardamom, then the clove and finally the ginger.

The results obtained in relation to the sensation of bacteria and yeast of such extracts are in agreement with that obtained by many researchers (Arora and Kaur, 1999; Digraki et al., 1999; Okemo et al., 2001; Madamombe and Afolayan, 2003 and Akintobiet al., 2013). And disagreement with other such studies were made by Brandi et al. (2006), and Al-Rashedi and Al-Habib (2011) have confirmed that the ethanol extracts of the tested plants have a higher biological effects than the aqueous extracts on the growth of *Candida* sp. however,

Table 1. Effect of some of the aqueous and alcoholic extracts of certain plants on the growth of *S. aureus* (MRSA).

Treatment	Concentration (µ g/ml)	Aqueous extracts		Alcoholic extracts	
		Inhibition Zones*	MIC (µg/ml)**	Inhibition Zones*	MIC (µg/ml)**
Control	0	0.00	-	0.00	-
	1000	0.00		0.00	
	250	0.00		0.00	
Cardamom	111	0.00	-	0.00	
	83.3	0.00		0.00	
	55.56	0.00		0.00	
	1000	0.00		0.00	
	250	0.00		0.00	
Cinnamon	111	0.00	-	0.00	
	83.3	0.00		0.00	
	55.56	0.00		0.00	
	1000	0.00		0.00	
	250	0.00		0.00	
Ginger	111	0.00	-	0.00	
	83.3	0.00		0.00	
	55.56	0.00		0.00	
	1000	0.00			
	250	0.00			
Clove	111	0.00	-		
	83.3	0.00			
	55.56	0.00			
	1000	0.00			
Myrrh (*Commiphora myrrha*)	250	0.00			
	111	0.00	-		
	83.3	0.00			
	55.56	0.00			
	1000	0.874			
Myrrh	250	0.852			
(*Commiphora molmol*)	111	0.500	111		
	83.3	0.00			
	55.56	0.00			
L.S.D.#		0.050	-	0	-

*Mean diameter of inhibition zone (mm); **minimal inhibitory concentration.

it was discovered that anti-microbial compounds derived from plants hamper the growth of bacteria by processes separate from those currently utilized and anti-bacterial and they may have a great therapeutic value for the clinical diseases in resisting bacteria`s strains (Harborne, 1998).

The results of *Candida* and sensitive bacteria are positive, because these extracts contain some effective compounds antibacterial and antifungal compounds that act against these microbes like the volatile oils, turbines, phenols, flavonoids and saponins (Ellof, 1998; Ekwenye and Elegalam, 2005). The resistance of the *S. aureus* (MRSA) against the tested extracts may be referred to what is known as the combination of the same. Especially, the thickness of the mucous layer that surrounds the cell wall that has resulted from its adaptation with the excess and wrong use of the antibiotics resulting in increase in numbers of strains that resist the antibiotics whether those chemical or natural, regarding the sensitivity of 60 isolates of *S. aureus* are shown by having a mucus layer produced by the resisting isolates that was thicker than the layer of the sensitive strains, and the mucus layer covers the bacterial cell acting as insulator prevents the penetration of the

Table 2. Effect of some of the aqueous and alcoholic extracts of certain plants on the growth of *S. aureus* (MSSA).

Treatment	Concentration (µ g/ml)	Aqueous extracts		Alcoholic extracts	
		Inhibition zones*	MIC (µ g/ml)**	Inhibition zones*	MIC (µ g/ml)**
Control	0	0.00	-	0.00	-
	1000	0.00		1.374	
	250	0.00		0.716	
Cardamom	111	0.00	-	0.631	83.30
	83.3	0.00		0.599	
	55.56	0.00		0.00	
	1000	0.00		2.25	
	250	0.00		2.199	
Cinnamon	111	0.00	-	1.900	55.56
	83.3	0.00		1.533	
	55.56	0.00		0.631	
	1000	0.00		1.748	
	250	0.00		0.874	
Ginger	111	0.00	-	0.725	55.56
	83.3	0.00		0.623	
	55.56	0.00		0.573	
	1000	0.00		1.799	
	250	0.00		1.599	
Clove	111	0.00	-	1.574	55.56
	83.3	0.00		1.150	
	55.56	0.00		0.523	
	1000	0.826			
Myrrh	250	0.874			
(*Commiphora*	111	0.675	83.3		
myrrha)	83.3	0.651			
	55.56	0.000			
	1000	0.826			
Myrrh	250	0.874			
(*Commiphora*	111	0.000	250		
molmol)	83.3	0.000			
	55.56	0.000			
	L.S.D.#	0.001	-	0.900	-

*Mean diameter of inhibition zone (mm); **minimal inhibitory concentration.

antibiotic to inside the bacterial cell; it is resistant (Kirisits et al., 2007; Stapper et al., 2004).

Chemical effectiveness of the Myrrha resin in the aqueous extract

The obtained results showed that the aqueous extract of both types of Myrrha contains materials inhibiting the growth of the three microbes. In order to know the identity of such compounds, a chemical analysis was performed for Myrrha combination using the analysis method of mass spectrometry (Figures 1 and 2), as it has been proven that it contains three compounds known for their inhibiting effect as follows: 2-fluorodiphenylmethane, tribenzo-1,2,3,4,5,6-anthracene, 2-bromo-1-(4-bromophenyl)-ethanone. The inhibiting effect of the extract is attributed to the presence of the volatile oils

Table 3. Effect of some of the aqueous and alcoholic extracts of certain plants on the growth of *Candida albicans*.

Treatment	Concentration (µ g/ml)	Aqueous extracts		Alcoholic extracts	
		Inhibition zones*	MIC (µg/ml)**	Inhibition zones*	MIC (µg/ml)**
Control	0	0.000	-	0.000	-
Cardamom	1000	0.000	-	2.235	55.56
	250	0.000		2.199	
	111	0.000		2.077	
	83.3	0.000		1.250	
	55.56	0.000		0.000	
Cinnamon	1000	0.000	-	2.390	83.30
	250	0.000		1.250	
	111	0.000		0.820	
	83.3	0.000		0.709	
	55.56	0.000		0.000	
Ginger	1000	0.000	-	1.599	83.30
	250	0.000		1.324	
	111	0.000		1.184	
	83.3	0.000		0.736	
	55.56	0.000		1.599	
Clove	1000	0.000	-	1.749	55.56
	250	0.000		1.450	
	111	0.000		1.249	
	83.3	0.000		1.033	
	55.56	0.000		0.000	
Myrrh (*Commiphora myrrha*)	1000	0.776	250		
	250	0.749			
	111	0.000			
	83.3	0.000			
	55.56	0.000			
Myrrh (*Commiphora molmol*)	1000	0.883	250		
	250	0.725			
	111	0.000			
	83.3	0.000			
	55.56	0.000			
L.S.D.#		0.003	-	0.222	-

*Mean diameter of inhibition zone (mm); **minimal inhibitory concentration.

which are large single turbine compounds (Cowan, 1999).

These oils have the ability to inhibit the growth of yeast types and this is referred to the ability of the oil to analyze the cell wall and this also leads to weakening of the vital activities inside the cell by interfering with the function of the cytoplasmic membrane represented in the process of building the protein and thus inhibiting and stopping the process and also hindering the process of the effective transferring of the ions and salts through such membrane (Al-Qaysi, 2008).

Test for examining the sensitivity to the antibiotics

Eight pharmaceutically used bacterial antibiotics have

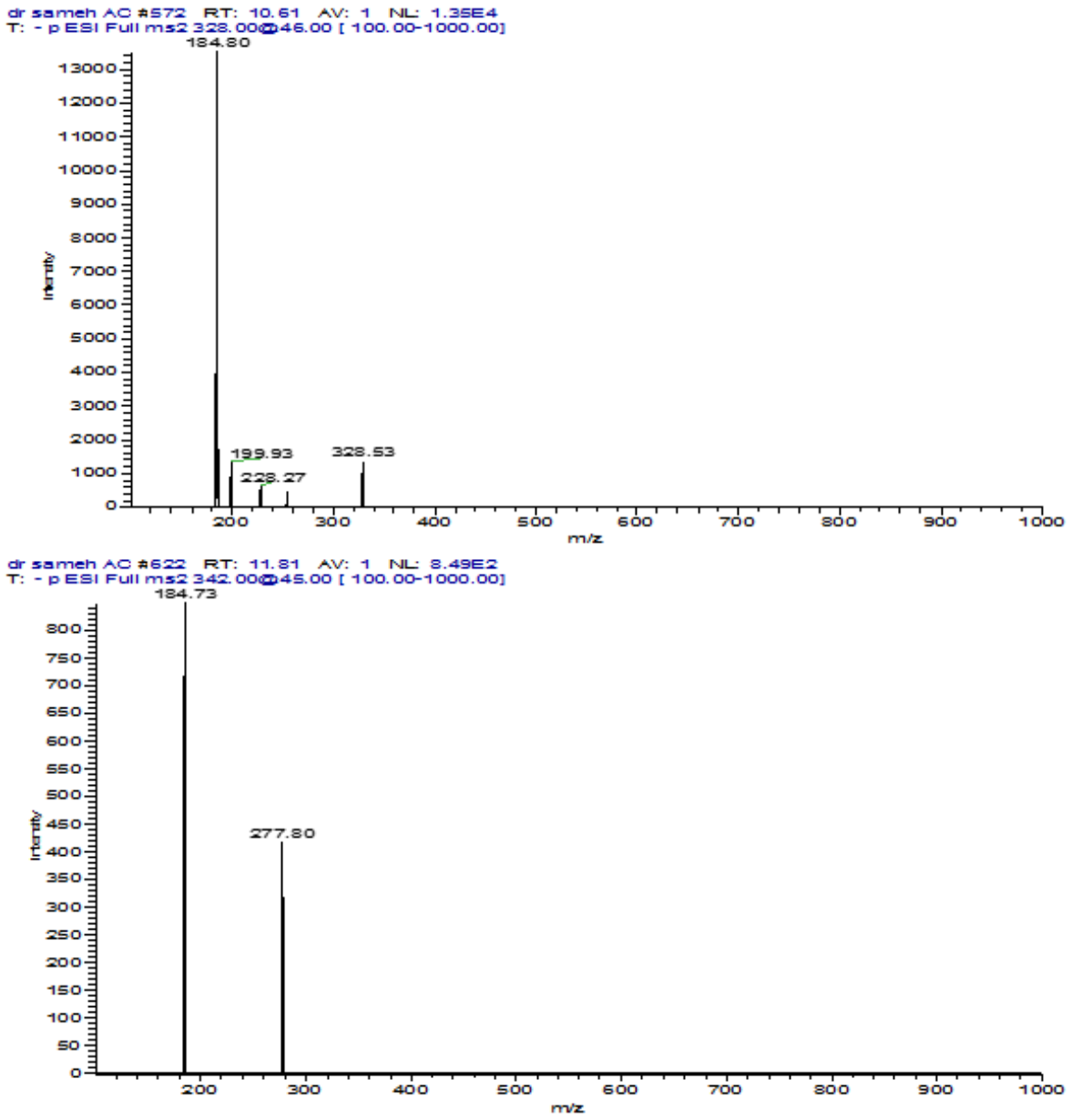

Figure 1. Chromatograms showing the main compound of *C. myrrha and C.molmol* by mass spectrum.

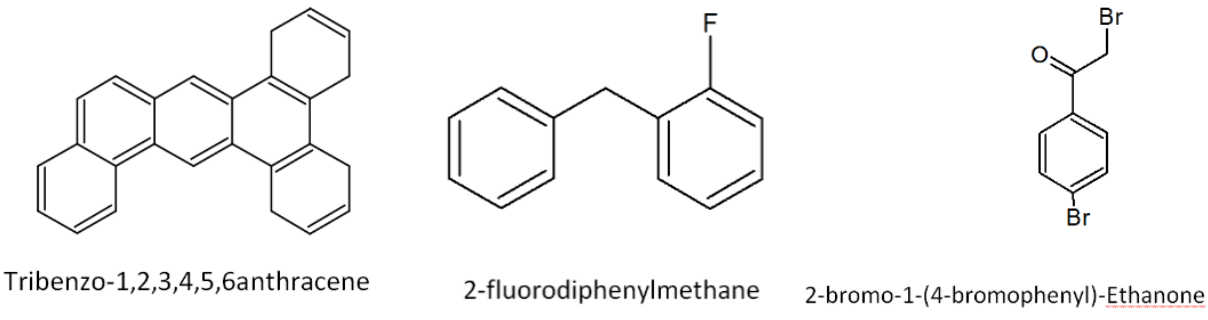

Figure 2: The chemical structures of three bioactive compounds derived from aqueous extract of Myrrh

Table 4. Impact of effectiveness of some antibiotics on the tested microbes.

Antibiotics	Mean diameter of inhibition zone (mm)		
	Candida albicans	***Staphylococcus aureus* (MRSA)**	***Staphylococcus aureus* (MSSA)**
Oxytetracycline	0.000	0.000	0.533
Gentamicin	0.000	0.000	1.350
Carbenicillin	0.000	0.000	0.000
Cotrimoxazole	0.000	0.000	0.000
Cephalothin	0.000	0.000	0.000
Tobramycin	0.000	0.000	0.175
Chloramphenicol	0.000	0.000	0.340
Polymyxin B	0.000	0.000	0.433
L.S.D.#	0.000	0.000	0.011

been used in order to know the sensitivity of the same microbes towards these antibiotics. The results shown in Table 4 show the resistance of *S. aureus* (MRSA) to all tested antibiotics. The obtained results are in agreement with what been obtained by Akintobi et al. (2013). Moreover, all the tested antibiotics have no effect on *C.albicans*. This is not odd, because the combination of the bacterial cells is different from the yeast, while five of the antibiotics affected the growth of the sensitive bacteria *S. aureus*, as the diameter of the inhibited zones reached 1.350, 0.550, 0.533, 0.433 and 0.400 mm for each of Gentamicin, Tobramycin, Oxytetracycline, Polymaxin B and finally Chloramphenicol, while the three antibiotics carbincilin, co-trimoxazole and sifalothin had no effect on the growth of the bacteria. Vasil (1986) stated that the resistance of *P. aeruginosa* to the antibiotics may result from different mechanisms including the production of enzymes able to destroy the antibiotics like the Betalactymize or to change in the penetration of cell membrane in order to prevent the access of the antibiotic to the target region as well as its ability to change the metabolic routes. Brown (1975) thinks that the reason for this is that some of the hospitals are restricted to the use of one antibiotic for curing their patients and this has resulted in the appearance of mutant strains resistant to these antibiotics.

In general, the mechanisms used by the micro-organisms to survive against the activity of the microbial antibiotics are still obscure and subject to discussion (Okemo et al., 2001). On the other hand, the chemical components of such plants may play a role in protecting the plants from the microbial attack inside the plant; however, some of them may have value as natural chemical materials used for defending the human body against the attack of the germs (Kubo et al., 1995). Therefore, this work recommends the performance of more studies, through which the natural effective components can be extracted and insulated from the plant which may act in saving the life of many peoples. In addition, this will lead to assistance in finding the alternatives in facing the appearance and spread of the strains that resist the known microbial antibiotics.

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Conflict of interests

The author has not declared any conflict of interests.

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Cytotoxicity, antifungal and antioxidant activities of the essential oil from *Eupatorium ballotifolium* Kunth (Asteraceae)

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This study aimed to characterize chemically and evaluate the cytotoxicity and antioxidant, antifungal and modulatory activities of the essential oil of *Eupatorium ballotifolium*, collected in the mountainous region of Meruoca in the state of Ceará. The antioxidant activity was investigated by the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and β-carotene/linoleic-acid oxidation model system. The lethality bioassay was performed using *Artemia salina* (brine shrimp). The evaluation of the antifungal activity *in vitro* was performed by broth microdilution using strains of dermatophyte fungi and yeasts. The modulatory activity assays were performed by the checkerboard technique using ketoconazole as standard. Chromatographic analysis associated with mass spectrometry showed the main constituents of *E. ballotifolium* essential oil were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). The brine shrimp lethality assay demonstrated potential biological activity. The essential oil showed better antioxidant action by the β-carotene/linoleic acid assay, with IC50 value of 19.47 µg/mL for essential oil, 11.32 µg/mL for thymol and 22.83 µg/mL for carvacrol, used as test standards. The broth microdilution test demonstrated that the essential oil inhibited fungal growth of all *Trichophyton rubrum* strains. In the modulation activity assay against strains of *Trichophyton rubrum,* there was synergism of essential oil on the strains of dermatophyte fungi when combined with ketoconazole.

Key words: Compositae, *Lourteigia ballotaefolia*, *Trichophyton rubrum*, antioxidant activity, antimicrobial activity.

INTRODUCTION

The Asteraceae family contains about 10% of the world's flora, and includes around 24,000 described species, grouped in 1,600 to 1,700 genera distributed in 17 tribes and 3 subfamilies (Funk et al., 2009; Petacci et al., 2012). It is the largest family of the eudicotyledons, and together with Calyceraceae, Campanulaceae, Menyanthaceae and Goodeniaceae, it forms a clade, the Asterales order (Pozner et al., 2012).

The genus *Eupatorium* L. (tribe Eupatorieae, subtribe Eupatoriinae) is significant in the Asteraceae family, comprising around 1,200 species. It is widely distributed, especially in Europe, Asia, North America and South America (Albuquerque et al., 2010). Brazil has around 250 native species with distribution in all geographical regions (Souza, 2007). *Eupatorium* species have been used to treat many diseases in folk medicine in various places in the world (Albuquerque et al., 2010).

Eupatorium ballotifolium Kunth (syn. *Lourteigia ballotaefolia* (Kunth) R.M. King & H.Rob.) is a perennial herbaceous species whose vertical growth ranges to 40 to 80 cm. It is an aromatic plant with few branches and many flowers, with pubescence and pink color with purple tones. Its leaves typically have purplish edges that are rich in oleíferas glands. The plant spreads through seeds (Silveira and Pessoa, 2005). It is popularly known in Brazil by many names, such as "maria-preta", "maria-preta-verdadeira" and "picão-roxo" (Cardoso et al., 2013).

Previous studies have demonstrated that the essential oil of the aerial parts of *E. ballotifolium* have anticholinesterase activity (Albuquerque et al., 2004), and two flavonoids were isolated from the aerial parts of the plant, nepetin and quercetin-3-O-glucoside, both showed antimitotic activity (Militão et al., 2004).

The therapeutic potential of many plants used in folk medicine has not been scientifically proved (Desoti et al., 2011). Hence, there is a need for chemical studies of natural products used in folk medicine to complement the studies to develop synthetic organic chemicals (Suffredini et al., 2006). Research of plants with antioxidant activity contributes to the development of new therapeutic strategies for inflammation, aging and chronic degenerative diseases (Fabri et al., 2011). Likewise, plants with potential antimicrobial activity can be a therapeutic alternative against multiresistant microorganisms to antimicrobial drugs (Bekele et al., 2015).

This study describes the chemical composition of the essential oil of *Eupatorium ballotifolium* (EOEB), and reports the antioxidant and antifungal activities against dermatophytes and yeasts, as well as the cytotoxicity, based on modulatory activity assays with ketoconazole.

MATERIALS AND METHODS

Plant material

The aerial parts of Eupatorium ballotifolium were collected in the flowering period in Brazil, Ceará, municipality of Alcântaras, in the Meruoca mountain region, in June, 2014 in a semideciduous forest environment located around 800 m above sea level. This region is located in the middle reaches of the Acaraú River, about 250 km from Fortaleza, the state capital. A voucher specimen (No. 3105) was deposited in Francisco José de Abreu Matos Herbarium (HUVA) and authenticated by Dr. Elnatan Bezerra de Souza of the Center for Agricultural Sciences and Biological Sciences, Vale do Acaraú State University.

Isolation of essential oil

Fresh aerial parts of *E. ballotifolium* (680 g) were subjected to hydrodistillation for 2 h in a modified Clevenger-type apparatus. The oil was dried over anhydrous Na_2SO_4 (~1 g), filtered and preserved in a sealed vial at 4°C prior to further analysis, with a yield of 0.1% (w/w).

Analysis of essential oil

The essential oil was analyzed using a Hewlett-Packard 5971 GC/MS instrument under the following conditions: dimethylpolysiloxane DB-5 fused silica capillary column (30 m × 0.25 mm i.d., 0.1 µm film thickness); carrier gas: helium (1 mL/min); injector temperature: 250°C; detector temperature 200°C; column temperature: 35 to 180°C at 4°C/min, then 180 to 250°C at 10°C/min; and mass spectra: electronic impact 70 eV. The identity of the components was achieved from their GC retention times relative to known compounds, calculated by linear interpolation relative to retention times of a series of n-alkanes and by comparison of their mass spectra with those present in the computer data bank (NIST) and published spectra (Adams, 2012).

In vitro antifungal assay

Fungal strains

The strains were obtained from the fungal collection of the Specialized Medical Mycology Center (CEMM), Federal University of Ceará, the URM Culture Collection of the Department of Mycology, Federal University of Pernambuco, and Hospital Santa Casa de Misericórdia de Sobral. In all these collections, the strains were maintained in saline (0.9% NaCl) at 28°C. At the time of the analysis, an aliquot of each suspension was taken and inoculated onto potato dextrose agar (Difco, Detroit, MI, USA), and then incubated at 28°C for 2 to 10 days.

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A total of four strains of *Trichophyton rubrum*, two strains of *Candida albicans*, one strain of *Candida parapsilosis* and one strain of *Candida tropicalis* were included in this study. *Candida* spp. strains were clinical isolates obtained from Hospital Santa Casa de Misericórdia de Sobral.

Preparation of inocula

For the broth microdilution method, standardized inocula (2.5 to 5 x 10^3 CFU mL^{-1} for *Candida* spp. and 5.0 x 10^4 CFU mL^{-1} for *T. rubrum*) were prepared by turbidimetry. Stock inocula were prepared on day 2 and day 10 for *Candida* spp. and *T. rubrum*, respectively, grown on potato dextrose agar at 28°C. Sterile saline solution (0.9%) was added to the agar slant and the cultures were gently swabbed to dislodge the conidia from the hyphal mat and from the blastoconidia for *T. rubrum* and *Candida* spp., respectively. The suspensions were diluted to 1:2000 for *Candida* spp. and 1:500 for *T. rubrum*, both with RPMI 1640 medium (Roswell Park Memorial Institute – 1640) with l-glutamine without sodium bicarbonate (Sigma Chemical Co., St Louis, MO, USA), and then buffered to pH 7.0 with 0.165 M MOPS (Sigma Chemical Co.), to obtain inocula of 2.5 to 5 x 10^3 CFU mL^{-1} and 5.0 x 10^4 CFU mL^{-1}, respectively.

Broth microdilution method

The minimum inhibitory concentration (MIC) for *Candida* spp. was determined by the broth microdilution method, in accordance with the Clinical and Laboratory Standards Institute (CLSI M27-A3, 2008). The broth microdilution assay for *T. rubrum* was performed as previously described (Sousa et al., 2009) based on the M38-A document (CLSI M38-A2, 2008).

The minimum fungicidal concentrations (MFC) for both *Candida* spp. and *T. rubrum* were determined according to the study of Fontenelle et al (2008). The EOEB was prepared in 100% mineral oil. Amphotericin B (AMB) and ketoconazole (Sigma, Chemical Co., USA) were prepared in distilled water. For the susceptibility analysis, the essential oil samples were tested in concentrations ranging from 0.002 to 2.5 mg/mL.

The microdilution assay was performed in 96-well microdilution plates. Growth and sterile control wells were included for the EOEB. The microplates were incubated at 37°C and read visually after 2 days for *Candida* spp. and 5 days for *T. rubrum*. The assay for the essential oil was run in duplicate and repeated at least twice. The MIC was defined as the lowest oil concentration that caused 100% inhibition of visible fungal growth. The results were read visually as recommended by CLSI. The MFC was determined by subculturing 100 µL of solution from wells without turbidity on potato dextrose, at 28°C. The MFCs were determined as the lowest concentration resulting in no growth on the subculture after 2 days for *Candida* spp. and 5 days for *T. rubrum* (Fontenelle et al., 2008).

Microdilution checkerboard assay

Assays were performed on all strains of *T. rubrum* according to the checkerboard technique (Johnson, 2004; Pyun and Shin, 2006), to determine the combined effect of the essential oil with the standard antifungal drugs. The interaction of the drugs was ascertained by calculating the fractional inhibitory concentration index (FICI). The FICI is calculated by adding the fractional inhibitory concentration (FIC) for each of the tested compounds, being defined as the addition of the MIC values of each drug in the combination divided by the MIC of the drug alone.

FIC^A = MIC of agent A in combination/MIC of agent A alone

FIC^B = MIC of agent B in combination /MIC of agent B alone

$FICI = FIC^A + FIC^B$

In the equations, A represents the EOEB and B the antifungal, ketoconazole. The turbidity of the fungal suspensions was adjusted to 0.5 McFarland standard (10^5 UFC/mL). In the solutions, the tested products were used at concentrations of their respective MICs. Initially, 50 µL of RPMI 1640 medium was added to all 96 wells of the microdilution plate. Then 50 µL of essential oil was added in the first column, in which serial dilutions were made in the plate until the 8th column, with the essential oil concentrations ranging from 5 to 0.03 mg/mL. In the vertical lines, 50 µL of standard antifungal ketoconazole was placed in concentrations ranging from 16 to 0.125 µg/mL.

Finally, 100 µL of inoculum was added to all wells. RPMI 1640 medium with inoculum was used as a negative control, while the antifungals and essential oil separately were used as positive controls at the respective MIC values. The microplates were incubated at 37°C and read visually after five days for dermatophytes. Assays were performed in triplicate. The FICI was interpreted as indicating a synergistic effect at values ≤ 0.5, an indifferent effect at values > 0.5 or ≤ 4.0, and an antagonistic effect at values > 4.0 (Odds, 2003; Johnson, 2004).

Determination of DPPH radical scavenging activity

To evaluate the antioxidant activity of the essential oil, the study used the free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging assay, according to Fenglin et al. (2004), with modifications. The activity was investigated by the ability to scavenge the DPPH radical through variation of absorbance obtained for a stoichiometric color loss of the radical solution in the presence of antioxidant substances present in the essential oil sample.

In a test tube, 3.9 mL of a methanol solution of free radical DPPH 6.5 x 10^{-5}M was combined with 0.1 mL of the methanol solution of essential oil in the concentrations to be tested. After 60 min, the absorbance was determined with an UV-VIS spectrophotometer at a wavelength of 515 nm. Assays were performed in triplicate. The inhibition of free radical DPPH was calculated in percent using the following equation:

IP% = Abs(DPPH) - Abs(sample) / Abs(DPPH) x 100

Where IP% is the inhibition percentage; Abs (DPPH) is the absorbance of the DPPH solution and Abs (sample) is the absorbance of the solution containing the essential oil at a particular concentration. IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated by the regression equation of the concentration of the essential oil, and percentage inhibition of free radical formation/percentage inhibition DPPH was calculated (Bajpai et al., 2009).

Determination of antioxidant activity using β-carotene/linoleic acid assay

The evaluation of oxidation inhibition by β-carotene/linoleic-acid oxidation system was performed by means of a spectrophotometric assay based on the discoloration of β-carotene due to oxidation induced by oxidative degradation products of linoleic acid (Silva et al., 1999; Alves et al., 2010). The antioxidant activity was determined by measuring the ability of the volatile organic compounds to inhibit the conjugated diene hydroperoxide formation from linoleic acid and β-carotene coupled oxidation in an emulsified aqueous system, which loses its orange color when reacting with the radicals (Alves et al., 2010; Lopes-Lutz et al., 2008).

The β-carotene (Sigma, St. Louis, MO) was dissolved in 5 ml of chloroform (0.3 mg/mL), followed by adding 20 µL of linoleic acid (Sigma, St. Louis, MO) and 200 µL of Tween 40 (Sigma, St. Louis, MO). Chloroform was completely evaporated using a vacuum evaporator. After removal of $CHCl_3$, 100 ml of distilled water saturated with oxygen under constant agitation was added to form an emulsion. The solution was adjusted in the spectrophotometer to a wavelength of 470 nm. The final emulsion had absorbance between 0.6 and 0.7 nm. Then 5 ml aliquots of the emulsion were placed in test tubes followed by 100 µl of dilutions of previously prepared methanol solutions of the essential oil, at concentrations of 500 to 25 mg/ml. Sample readings were taken 2 min after contact of the methanol solutions having varied concentrations with the emulsion. Then the samples were put in a water bath at 50°C for 120 min, and a second reading was performed. The negative control consisted of 5 ml of emulsion alone (Andrade et al., 2012). The percentage inhibition was calculated from the data with the formula:

$$IP\% = [(Abs_{sample(0)} - Abs_{sample(120)}) / (Abs_{system(0)} - Abs_{system(120)})] \times 100$$

$$\% \text{ Protection} = 100 - IP\%$$

Where IP% is the inhibition percentage; $Abs_{sample(0)}$ is the absorbance of the essential oil at t=0 min; $Abs_{sample(120)}$ is the absorbance of the essential oil at t=120 min; $Abs_{system(0)}$ is the absorbance of the system at t=0 min and $Abs_{system(120)}$ is the absorbance of the system at t=120 min. Thymol and carvacrol, oxygenated monoterpenes present in many essential oils, were used as positive controls. Samples were read against a blank containing the emulsion minus beta-carotene. Each assay was repeated three times and the IC_{50} values (concentration sufficient to obtain 50% of a maximum effect estimate in 100%) were calculated by the regression equation of the concentration of the essential oil, and percentage of protection (Lopes-Lutz et al., 2008; Andrade et al., 2012).

Brine shrimp lethality bioassay

The lethality assay against *Artemia salina* Leach (Crustacea, Artemiidae) was performed according to the method proposed by Meyer et al. (1982) with adaptations. The eggs of *A. salina* were incubated at room temperature (between 22 to 29°C) in artificial brine consisting of 23 g/L of sea salt and 0.7 g/L of sodium bicarbonate in distilled water for a period of 48 h in a tank fitted with a dark compartment and another clear one.

Using a light source, the nauplii were attracted to the light, collected with a Pasteur pipette and transferred to a beaker with saline water. Extraction solutions were prepared with the solvents methanol, DMSO and saline water in concentrations of 10,000 to 1 µg/mL. The positive control was prepared with potassium dichromate ($K_2Cr_2O_7$) and saline solution, and the negative control with saline solution and DMSO. Then 10 larvae were added to test tubes containing 5 ml of each tested solution and negative and positive control solutions (Costa et al., 2009).

Assays were performed in triplicate and the number of dead larvae was counted after contact for 24 h with the solutions. For counting the number of nauplii, the study considered those that remained immobile for more than 10 s after gentle agitation of the tubes (Lhullier et al., 2006).

Hemolysis assay

Fresh blood (10 ml) was collected in EDTA tubes and centrifuged at 1000 g for 10 min at 4°C. After plasma removal, the pellet containing the red blood cells (RBCs) was washed five times with PBS and then re-suspended in PBS to obtain an 8% (v/v) suspension. Then 100 µL of this suspension was added to different microcentrifuge tubes with 100 µL of 2-fold serial dilutions of essential oil, ranging from 0.005 to 2.5 mg/ml. Final concentrations were 4% (v/v) of erythrocyte suspension, and the essential oil concentration range was 0.1 to 100 µM. The resulting suspensions were incubated with agitation for 60 min at 37°C. After incubation, the samples were centrifuged for 2 min at 1000 g. The supernatants were transferred to 96-well plates and the hemoglobin release was measured by absorbance at 540 nm, using the Biotek Synergy HT multiplate reader. Triton X-100 at 1% and 4% (v/v) RBCs in PBS with no essential oil (untreated) were used as positive and negative controls, respectively. Percentage hemolysis was determined as [(Abs_{540nm} sample-treated – Abs_{540nm} untreated)/ (Abs_{540nm}1% Triton X-100 – Abs_{540nm} untreated)] x 100, and experiments were carried out in triplicate (Ahmad et al., 2010).

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). One-way ANOVA with the Tukey test was performed followed by multiple comparisons testing where appropriate. LC_{50} values were obtained using statistical package for social sciences (SPSS) 14.0 for Windows (SPSS Inc. Chicago, USA). Significance of difference was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The essential oil yield was 0.1% (w/w) dry weight. The chemical compositions of the volatile constituents of the EOEB and the respective percentages are reported in Table 1. The essential oil contained 25 components, accounting for 93.84%, among them mono- and sesquiterpenes, with a prevalence of sesquiterpenes. The main constituents were β-caryophyllene (23.59%), thymol methyl ether (12.28%), germacrene D (6.56%) and bicyclogermacrene (6.47%). Among these main constituents, only one monoterpene was found, thymol methyl ether. Sesquiterpene hydrocarbons predominate in the chemical composition.

A previous study of the chemical composition of the aerial parts of EOEB identified 27 components, representing 91.2% of the total content (Albuquerque et al. 2001). In this study, the EOEB showed a high content of monoterpenes, limonene (15.3%), (E)-β-ocimene (10.5%), followed by β-caryophyllene sesquiterpene (7.5%) and myrcene monoterpene (7.3%) (Albuquerque et al., 2001). Variations in the chemical composition of essential oils, secondary metabolism products of the plant, occur due to changes in abiotic factors, as well in the geographic location of the plant, season of the year and even time of day when the plants are sampled (Dudareva et al., 2004; Gobbo-Neto; Lopes, 2007). In other Eupatorium species, the chemical analysis showed a corresponding composition. In *E. capillifolium* (Lam.) Small. ex Porter & Britton, the chemical composition of the essential oil of the aerial parts contained thymol methyl ether (36.3%) as the main constituent (Tabanca et al., 2010).

Table 1. Chemical composition of the aerial parts of the essential oil from *E. ballotifolium* (EOEB).

Compound[a]	RI Lit[b]	RI[c]	EOEB
Sabinene	975	975	1.88
β-Myrcene	990	992	0.51
α-Phellandrene	1002	1004	1.32
p-Cymene	1024	1025	3.41
Limonene	1029	1023	0.88
(Z)-β-Ocimene	1037	1039	1.40
(E)-β-Ocimene	1050	1050	5.05
Terpinen-4-ol	1177	1186	2.13
Thymol methyl ether	1245	1235	12.28
δ-Elemene	1338	1350	1.87
α-Copaene	1376	1373	2.29
β-Elemene	1390	1389	5.47
β-Caryophyllene	1419	1427	23.59
α-Humulene	1454	1450	1.58
epi-Caryophyllene	1466	1471	0.76
Germacrene D	1481	1490	6.56
Bicyclogermacrene	1500	1499	6.47
Germacrene A	1509	1513	1.08
δ-Cadinene	1523	1529	5.54
Germacrene B	1561	1560	0.97
Caryophyllene oxide	1583	1583	1.38
Globulol	1590	1591	2.85
1,10-de-*epi*-Cubenol	1619	1621	0.73
epi-α-Muurolol	1642	1634	2.40
α-Cadinol	1654	1644	1.44
Total identified	-	-	93.84

[a] Order of elution on DB-5 capillary column. [b] RI_{lit} refers to the retention index taken from Adams, 2012. [c] RI_{ca} refers to the retention index experimentally calculated using C_7–C_{26} n-alkanes.

Regarding the *in vitro* antifungal activity of the EOEB, the results are summarized in Table 2. The essential oil showed no significant activity when tested against strains of *Candida* spp (*C. albicans* LABMIC 0201, *C. albicans* LABMIC 0202, *C. parapsilosis* LABMIC 0301 and *C. tropicalis* LABMIC 0401). However, the results showed MIC values ranging from 2.5 to 1.25 mg/ml for the strains of dermatophytes (*T. rubrum* LABMIC 0101, *T. rubrum* LABMIC 0102, *T. rubrum* CEMM 05-1-08 and *T. rubrum* CEMM 05-1-034). Ketoconazole was used as positive control.

There are no reports in the literature of antimicrobial activities of the *E. ballotifolium* species, so this study is groundbreaking in investigating the antifungal activity against dermatophytes, which are medically important fungi responsible for superficial skin infections, with *T. rubrum* being the species most often found to cause dermatophytosis (Cafarchia et al., 2013).

Previous studies with some of the major constituents in the composition of EOEB report antifungal activity for β-caryophyllene (Tampieri et al., 2005; Skaltsa et al., 2003; Bougatsos et al., 2004) and bicyclogermacrene (Silva et al., 2007), both sesquiterpene hydrocarbons. These results indicate that the antifungal activity found in our study may represent a combined effect of these main EOEB constituents. For the *Eupatorium* genus, numerous studies have been conducted of the antimicrobial activity, mainly involving extracts and essential oils. The data are still incipient, although *Eupatorium* has been found to be one of the largest genera of the family Asteraceae (Garcia-Sanchez et al., 2011; Roque and Bautista, 2008).

In testing the modulatory activity, the study used the strains of *T. rubrum* LABMIC 0101 and *T. rubrum* LABMIC 0102. The results (Table 3) demonstrate that the combination of ketoconazole with EOEB reduced the MICs for both strains of *T. rubrum* (Table 3). Mutual synergistic potentiation of antifungal activity of EOEB and ketoconazole occurred, with a significant reduction in the MIC of ketoconazole of 1.0 to 0.125 µg/mL on the strain *T. rubrum* LABMIC 0102. The most significant reduction occurred in the tests with strain LABMIC 0102, whose fractional inhibitory concentration index (FICI) was low

Table 2. Minimum inhibitory concentration of essential oils from *Eupatorium ballotifolium* against *Candida* spp and *Trichophyton rubrum.*

Strains	Essential oil of *E. ballotifolium*		Drug (µg/mL)	
	MIC (mg/mL)	MFC (mg/mL)	Amphotericin B	ketoconazole
C. albicans LABMIC 0201	NI	NI	2.0	–
C. albicans LABMIC 0202	NI	NI	1.0	–
C. parapsilosis LABMIC 0301	NI	NI	4.0	–
C. tropicalis LABMIC 0401	NI	NI	2.0	–
T. rubrum LABMIC 0101	1.25	–	–	1.0
T. rubrum LABMIC 0102	2.5	–	–	1.0
T. rubrum CEMM 05-1-08	2.5	–	–	1.0
T. rubrum CEMM 05-1-034	2.5	–	–	1.0
Geometric mean of *T. rubrum*	2.69	–	–	–

LABMIC, Microbiology Laboratory; CEMM, Specialized Centre of Medical Mycology.

Table 3. MIC of the ketoconazole in the presence and absence of essential oil from *Eupatorium ballotifolium* against *Trichophyton rubrum.*

Plant essential oil/Drug	*T. rubrum* LABMIC 0102			*T. rubrum* LABMIC 0101		
	MIC (µg/ml) alone	MIC (µg/ml) combined	FIC indexa	MIC (µg/ml) alone	MIC (µg/mL) Combined	FIC index
E. ballotifolium	5000	39	0.1	2500	39	1.0
ketoconazole	1.0	0.125		1.0	1.0	

[a]FIC index, index fractional inhibitory concentration (FICI).

Table 4. Antioxidant activity of the essential oil from *E. ballotifolium* and the thymol and carvacrol, tested standard.

Methods	β-carotene / linoleic acid	DPPH
Compound	IC_{50} (µg/mL)	IC_{50} (µg/mL)
E. ballotifolium	19.47	222.17
Thymol	11.32	21.71
Carvacrol	22.83	25.5

IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%.

(0.1) compared with strain LABMIC 0101, whose FICI value was 1.0. So, for LABMIC 0102 the modulatory activity was synergistic and for LABMIC 0101 the activity was indifferent.

The mechanisms of inhibiting microbial growth may be related to the hydrophobic nature of the constituents of the essential oil. Such compounds can act on the plasma membrane, causing it to become more permeable to antifungal agents, affecting the mitochondrial respiratory chain and cellular energy production. Thus, this mechanism can occur due to combined action of the antifungals with natural products at subinhibitory concentrations (Nogueira et al., 2014; Tintino et al., 2014). This is the first report of modulatory activity of a standard antifungal drug combined with EOEB.

The results presented here corroborate with other studies involving essential oils of *Thymus vulgaris* L. and *Cinnamomum cassia* L. (Pekmezovic et al., 2015), *Ocimum sanctum* L. (Amber et al., 2010), *Myrtus communis* L. (Mahboubi and Bidgoli, 2010), *Melaleuca alternifolia* (Maiden & Betche) Cheel and *Lavandula angustifolia* Mill. (Cassella et al., 2002). These studies demonstrate the ability of essential oils used to modify the antibiotic activity of drugs through the checkerboard technique. IC_{50} values of the EOEB obtained from the antioxidant assays investigated by the free radical DPPH (1.1-diphenyl-2-picrylhydrazyl) scavenging assay and β-carotene/linoleic-acid oxidation model system are shown in Table 4.

In the DPPH scavenging assay, the IC_{50} value was 222.17 µg/mL. This result indicates that the essential oil exhibited weak antioxidant capacity when compared to the positive controls: thymol, whose IC_{50} was 21.71 µg/mL and carvacrol, with IC_{50} of 25.5 µg/mL. In the test

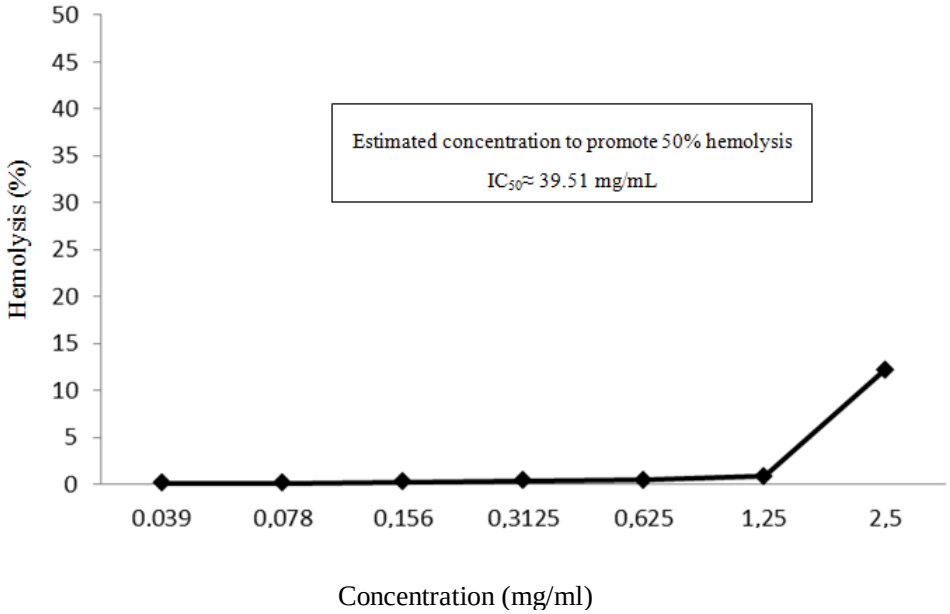

Figure 1. Hemolytic activity of the essential oil from *Eupatorium ballotifolium* (EOEB).

with β-carotene/linoleic-acid, the IC_{50} of the essential oil was 19.47 µg/mL, an excellent antioxidant action, while the IC_{50} of thymol was 11.32 µg/mL and carvacrol was 22.83 µg/mL.

The discrepant results between the two tests can be explained by the purpose of each method. The DPPH scavenging assay is based on the ability of the tested substance to sequester DPPH free radicals, which are reduced to the hydrazine compound and are routinely used to evaluate the antioxidant activity in plant extracts and pure substances such as terpenoids and flavonoids (David et al., 2007; Alves et al., 2010). The β-carotene/linoleic-acid assay is employed specifically to investigate the ability of a sample to minimize the complete oxidation of linoleic acid and β-carotene in an aqueous-lipid system which loses its orange color when it reacts with radicals produced by oxidative degradation of fatty acids (Alves et al., 2010). This method is used to investigate the antioxidant capacity of lipophilic substances, such as essential oils (Kulisic et al., 2004). Another property that makes it a useful method to test essential oils is that it does not require high temperatures, which allows determination of the antioxidant activity of thermo-sensitive substances such as essential oils (Silva et al., 1999).

Sesquiterpenes were the constituents identified in greatest quantities in the study essential oil, and the literature indicates antioxidant potential of plant-derived sesquiterpenes (Xu et al., 2008; Sghaier et al., 2011). β-caryophyllene, the main constituent found in the study (23.59%), was previously shown to have antioxidant activity and protective effect on liver fibrosis and the ability to inhibit activation of hepatic stellate cells (Calleja et al., 2013). The isolated action of β-caryophyllene or the synergism between the main constituents of the essential oil can be also be related to the antioxidant action (Candan et al., 2003). These results of antioxidant activity of the EOEB, detected by two different methods, corroborate previous studies that have indicated antioxidant potential of the genus *Eupatorium*, with activity for many species, such as *E. adenophorum* (Ahluwalia et al., 2014), *E. odoratum* (Raman et al., 2012; Chakraborty et al., 2010), *E. polystachyum* DC. (Souza et al., 2007) and *E. triplinerve* Vahl (Melo et al., 2013).

In the brine shrimp lethality bioassay, mortality rates to the EOEB varied between 0 and 100%, and the concentration required to kill 50% of the larvae (LC_{50}) was 28.89 µg/mL. LC_{50} values less than 1000 µg/mL indicate a possible spectrum of biological activities of some of the constituents, combined and/or isolated (Meyer et al., 1982). The anticholinesterase activity has been investigated of the EOEB from leaves and bark (Albuquerque et al., 2010). Lethality bioassays with A. salina have also been used in prospecting studies to screen plants with possible pharmacological activity, in Brazil (Quignard et al., 2004), India (Krishnaraju et al., 2006) and Nicaragua (Coe et al., 2010).

The tests to determine the in vitro hemolytic activity of EOEB showed that at concentrations of 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 mg/mL respectively the percentage of hemolysis ranged from 0.1 to 12.16% (Figure 1). Based on these results, the study can extrapolate hemolysis values and estimate the IC50 of 39.51 mg/mL for the essential oil. Regarding hemolytic

activity, the statistical analysis showed a significant difference at the level p = 0.05 when compared to the positive control Triton X-100 with the EOEB concentrations used.

Methods to measure the hemolytic activity *in vitro* enable determining the cytotoxic profile of the studied substance, and consist of checking for potential damage caused by the substances present in essential oils to the membranes of erythrocytes, which when undergoing lysis release hemoglobin (Miyazaki et al., 2013). At the essential oils' MIC values, the study observed hemolysis percentages between 0.8 and 12.2%. Comparison of the IC_{50} value of hemolytic activity with the MIC indicated that the oil concentration responsible for the fungistatic activity is lower than the concentration required to damage red blood cells by rupture of their membrane. However, according to the test of hemolytic activity, the EOEB showed low cytotoxic effect at the concentrations that inhibited microbial growth.

Conclusion

Chemical analysis of the EOEB identified 25 components, accounting for 93.84% of the substances, with predominance of sesquiterpene hydrocarbons. The brine shrimp lethality bioassay (*A. salina*) used as for screening allowed establishing a correlation with other potential biological activities, and the hemolytic activity assay showed that the essential oil has low cytotoxicity.

The essential oil showed *in vitro* antifungal activity against the dermatophyte *T. rubrum*, and when tested in combination with ketoconazole, the EOEB interacted synergistically, increasing its antifungal action. Antioxidant activity was evidenced by the DPPH scavenging assay, with IC_{50} of 222.17 µg/mL, while the IC_{50} value was 19.47 µg/mL in the test with β-carotene/linoleic-acid. The antioxidant potential can be related to the high content of sesquiterpenes and especially β-caryophyllene, germacrene D and bicyclogermacrene, the main constituents found in this study.

It is necessary to investigate the mechanism of action in the fungal cells and perform tests to identify the components responsible for the biological activities. It is also important to carry out tests with isolated constituents of EOEB against strains of dermatophyte fungi, modulatory activity assays with other antifungal drugs used in antifungal therapy and to investigate new toxicological aspects of the essential oil.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Therapeutic and preventive effects of *Commiphora gileadensis* against diethylnitrosamine-induced hepatic injury in albino rats

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Commiphora gileadensis **is a tree in the burseraceae family, cultivated widely in Hadhramaut governorate (Yemen) and known locally as "Besham" or Balsam" used traditionally for many ailments. The aim of this study was to evaluate the therapeutic and preventive effects of *C. gileadensis* against diethylnitrosamine (DEN) -induced hepatic injury in albino rats. 40 albino rats were divided randomly into five groups (each contains 8 animals). Group I (Negative control) was given normal saline i.p and distilled water, group II (Positive control) toxicity-initiated with single dose of DEN 200 mg/kg i.p and promoted after 2 weeks with 0.05% of phenobarbitone in drinking water to complete 10 weeks. Group III was given 500 mg/kg extract of *C. gileadensis* bark for 10 weeks. Groups IV (preventive group) pretreated with 500 mg/kg extract of *C. gileadensis* bark and injected with DEN 200 mg/kg i.p for 10 weeks. Groups V (Treatment group) was given single dose of DEN same like group II but for 6 weeks, then treated with 500 mg/kg *C. gileadensis* bark extracts orally for an additional 4 weeks. All doses were used according to the effective dose fixation. Liver function enzymes, complete blood count (CBC) and lipid profile were measured. In addition, fasting blood sugar (FBS) and total body weight were taken weekly. At the end of experiment relative weight of liver was calculated. *C. gileadensis* showed significant hepatoprotective effect as it reduced the liver function enzyme's level, this effect was supported by hepatic histopathological improvement against DEN-induced hepatic injury. In addition, it demonstrated potent anti-platelets activity. The outcomes of this study suggested that *C. gileadensis* has novel hepatoprotective and remarkable anti- platelets effect.**

Key words: *Commiphora gileadensis*, diethylnitrosamine, phenobarbitone, hepatic injury.

INTRODUCTION

Commiphora gileadensis (opobalsamum L.) or the Arabian balsam tree is one of a famous plant spread in the Arabian Peninsula especially Yemen, Saudi Arabia and Oman. The ancient product that is secreted from this

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tree like resins or even the uses of woods cutting from plants were used for making perfumes, incense and different medical products (Jones, 1924). It is a member of the highly studied and commercially used resinous plant family burseraceae, comprising, among others, the biblical frankincense and myrrh (Zohary, 1973). It contains flavonoids, saponins, volatile oils, sterols and triterpenes (Zohary, 1982).

In Yemen, especially in Hadhramaut governorate this plant is used for thousands of years for many different ailments like skin disorders (wounds and burns), respiratory disease, gynecological purposes as contraceptive, labor pain, laxative and diuretic effect (Pliny, 1989). Nitrosamines are chemical compounds with the chemical structure R1N (- R2)-N=O. They are used in the manufacturing of some cosmetics, pesticides, and most rubber products. Nitrosamine occurs in latex products such as balloons (Altkofer et al., 2005) and in many foods, and other consumables. Diethylnitrosamine chemically belonging to the N-Nitrosamine family is proved to be one of the potent carcinogens that is primarily metabolized by the cytochrome P-450 enzymes to reactive electrophiles (O6 alkyl-guanine and N7 alkyl-guanine) which are proved to be cytotoxic (Archer, 1989), carcinogenic (Swann and Magee, 1971) and mutagenic (Magee and Barnes, 1967). Moreover, phenobarbitone may promote the toxic effects induced by diethylnitrosamine (DEN) (Mohammed et al., 2014).

The aim of the present study is to evaluate the therapeutic and preventive effects of *C. gileadensis* against DEN-induced hepatic injury in albino rats.

MATERIALS AND METHODS

Drugs and natural product

N-Nitrosodiethylamine (DEN) and phenobarbitone were purchased from Sigma-adrich Co. USA. *C. gileadenesis* bark was freshly collected in December, 2014 from Hadhramaut governorate. The plant was identified and authenticated at Botany Department, College of Science-, Sana'a University.

Animals

Experimental animals being Wister albino rats (Rattus norvegicus albinus), with average weight of 260±20 g and age of 3 to 4 months were obtained from the animal house of Biology Department, Sana'a University. They were allowed for one week to acclimatize and maintained in 12 h dark/light cycle. They were kept under free water and normal rat chow.

Preparation of extract

1500 g of air-dried bark of *C. gileadensis* was powdered and macerated in 4 L of 99.9% of methanol for one week. The macerated barks then were put in Orbital shaker (0S10B-IKA®-Werke- Germany) for further 48 h to mix all the contents of macerated preparation. Then filtered and the filtrate evaporated under reduced pressure using rotary evaporator (RE3022C-Stuart) at 40°C. Repeated steps were done till the extraction of bark was completed. This process yielded about 24.39 g dark brownish color semi solid extract that was dissolved freshly in distilled water and given to animals (Al Howiriny et al., 2004).

Animal study design

Forty albino rats were divided randomly into five groups (each contains 8 animals). Group I (**Negative control**) was given normal saline i.p and distilled water. In group II (**Positive control**) toxicity was initiated with single dose of DEN 200 mg/kg i.p and promoted after 2 weeks with 0.05% of phenobarbitone in drinking water to complete 10 weeks. Group III was given 500 mg/kg extracts of *C. gileadensis* bark for 10 weeks. Groups IV (**Preventive group**) was pretreated with 500 mg/kg extract of *C. gileadensis* bark and injected with DEN 200 mg/kg i.p for 10 weeks. Groups V (**Treatment group**) was given single dose of DEN same like group II but for 6 weeks, then treated with 500 mg/kg *C. gileadensis* bark extracts orally for further 4 weeks. All doses were used according to the effective dose fixation according to method of Kalaiselvan et al. (2013). Aspartate aminotransferase (AST) (Schumann et al., 2002) and alanine aminotransferase (ALT) (Sonntag and Scholer, 2001), Alkaline phosphatase (ALP) (Abicht et al., 2001), lipid profile (Stein and Myers, 1995; Pisani et al., 1995; Bachoric, 2000) and complete blood count (CBC) were measured. In addition, fasting blood sugar (FBS) (Knudson and Weinstock, 2001) and total body weight were taken regularly. At the end of experiment relative weight of liver was calculated.

Dose fixation study

Different doses of *C. gileadenesis* extract (50, 100, 250, 500 and 750 mg/kg body weight) were used for 4 weeks in albino rats. The effective dose was based on the biochemical studies including liver and kidney function tests. The doses of 500 and 750 were found to be effective, but the minimum effective dose (500 mg/kg) was chosen and fixed throughout this study.

Acute toxicity study

Another twelve albino rats (330±10 g) were randomly divided into 3 groups, each contained 4 animals (the least no. was used for toxicological study). First group served as control group were only drunk distilled water, second and third groups were given extract of *C. giladenesis* bark 2 g/kg and 3 g/kg dissolved in distilled water. All animals were given the tested extract through oral gavage for 72 h according to the method of Jaykaran (2008). The following parameters were observed and measured:

1. Observational parameters: Mortality of animals, motor activity, tremors, convulsion, posture, spasticity, ataxia, writhing, skin color, diarrhea, salivation, lacrimation and respiration. Additionally, daily body weight and food intake were also noted. These observational parameters were monitored immediately at 0, 2, 4, 8, 24, 48 and 72 h of given tested extract.

2. Biochemical parameters: After 72 h of closed observation, animals were sacrificed by decapitation under anesthesia. Blood samples were collected for biochemical studies AST, ALT, CBC and FBS. Liver and heart were dissected out for histopathological examination. All the study procedures were in accordance with the guidelines for the care and use of laboratory animals, and approval was received prior to the experiments from the Institutional Research and Ethics Committee, UST.

Table 1. Effect of *C. giladenesis* on the (mean± SE) liver function enzymes (AST, ALT and ALP) for 10 weeks in albino rats (n=.8).

Treatment	Mean± SEM		
	AST (U/l)	ALT(U/l)	ALP(U/l)
Control	153.2±3.94	37.6±3.86	101.4±10.33
DEN	281.0±23.87*	45.6±8.22	162.0±4.93*
Plant	158.2±6.47**	35.2±1.06	95.8±7.72**
Preventive	208.6±3.59**	42.7±4.85	106.2±19.88**
Treatment	197.4±20.38**	44.2±5.75	103.4±18.12**

*Significant as compared with control at (P< 0.05), ** significant as compared with DEN- induced liver damage at (P< 0.05), AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase.

Table 2. Effect of *C. giladenesis* on the (mean± SE) body weight (g) and relative weight of liver (g) for 10 weeks in albino rats (n=8).

Parameter	Mean± SEM				
	Control	DEN	Plant	Preventive	Treatment
Initial body weight (g)	272.7±12.1	274.2±14.0	273.0±15.5	277.8±14.2	275.5±21.4
Final body weight (g)	320±12.7	252±11.3	290±17.4	280±6.68	285±19.6
Weight of liver (g)	8.1±1.68	13.0±1.00*	8.8±0.75**	9.50±1.5**	10.3±1.12**
Relative weight of liver	2.5	5.1*	3.03**	3.39**	3.61**

*Significant as compared with control at (P< 0.05), ** significant as compared with DEN-induced liver damage at (P< 0.05).

Table 3. Effect of *C. giladenesis* on the (mean± SE) lipid profile for 10 weeks in albino rats (n=8).

Treatment	Mean± SEM			
	Cholest (mg/dl)	LDLc(mg/dl)	TG(mg/dl)	HDL(mg/dl)
Control	74.2±2.75	18.2±0.80	53.4±3.05	62.0±3.57
DEN	114.6±14.1*	26.0±3.0	64.6±12.1	36.8±10.7*
Plant	63.8±9.29**	19.2±1.25**	42.0±5.05	64.4±7.32**
Preventive	70.4±4.05**	20.3±1.33**	49.0±6.82	63.8±4.38**
Treatment	80.7±6.98**	20.8±0.91**	46.6±9.45	60.8±4.96**

*Significant as compared with control at (P< 0.05), ** significant as compared with DEN- induced liver damage at (P< 0.05), Cholest.: cholesterol, LDL-c: low density lipoprotein lipids, TG: Triglyceride, HDL: High density lipoprotein lipids

Statistical analysis

Data were summarized as means ± SEM. One way analysis of variance (ANOVA) followed by Dunnet's multiple comparison test was used to conduct the significance of association using statistical package for the social sciences (SPSS) program version 21. Differences were considered significant at P values of less than 0.05.

RESULTS

Hepatoprotective effect

Methanolic extract of *C. gileadenesis* showed significant amelioration of DEN-induced liver injury as it reduced the level of liver function enzymes AST, ALT and ALP (Table 1). This effect was supported by reduction in the relative weight of liver and lipid profile (Tables 2 and 3) as well as tissue and hepatocytes improvements. In addition, *C. gileadenesis* showed significant reduction in platelets aggregation and lymphocyte levels as seen in Table 4 and 5. Moreover, continuous administration of *C. gileadenesis* in albino rats for 10 weeks counteracted the weight loss caused by DEN, and improved the health state of animals without harmful effect on blood glucose level as shown in Figures 1 and 2.

Toxicological study

There were no acute mortality and biochemical toxicity observed after oral administration of *C. gileadenesis* even

Table 4. Effect of *C. giladenesis* on the (mean± SE) complete blood count (CBC) for 10 weeks in albino rats (n=8).

Treatment	Mean± SEM					
	Hb (g/dl)	RBC (X10^12/L)	MCV (Femtoliters)	MCH (pg)	MCHC (g/dl)	Platelets (X10^9/L)
Control	16.0±0.45	8.77±0.488	52.0±0.23	17.1±0.07	31.6±0.65	494.0±64.4
DEN	17.0±0.17	9.87±0.298	54.5±1.28	17.3±0.29	32.2±0.58	837.0±126*
Plant	16.1±0.39	8.63±0.750	53.7±2.07	16.7±0.21	31.9±0.56	553.0±11.3**
Preventive	15.9±0.98	8.35±9.428	54.4±0.81	17.8±0.57	31.7±0.41	593.064.2**
Treatment	16.8±0.47	9.19±0.257	53.6±1.26	17.2±0.14	32.6±0.41	616.0±30.6**

*Significant as compared with control at (P< 0.05), ** significant as compared with DEN-induced liver damage at (P< 0.05), Hb: hemoglobin, RBC: Red blood cell, MCV: mean cell volume, MCH: mean corpuscular hemoglobin. MCHC: mean corpuscular hemoglobin concentration.

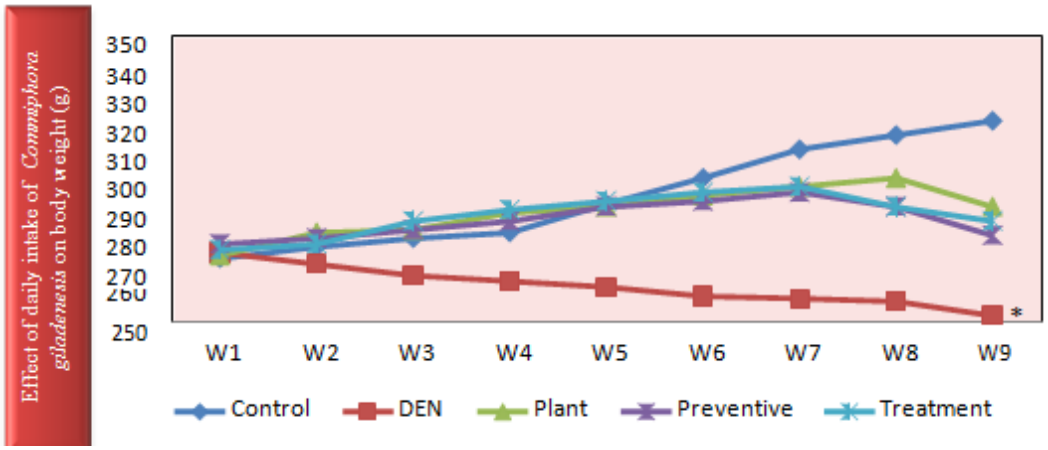

Figure 1. *C. giladenesis* on the (mean± SE) body weight (g) for 10 weeks in albino rats (n=8),*Significant as compared with control at (P< 0.05), W: week's number.

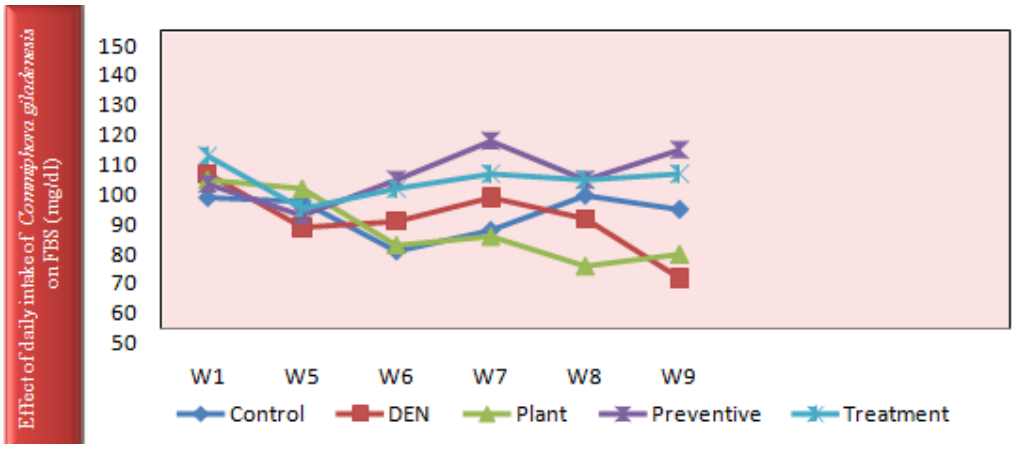

Figure 2. Effect of daily intake of *C. giladenesis* on the (mean± SE) fasting blood sugar (mg/dl) for 10 weeks in albino rats.

at a dose of 3 g/kg. All animals were found to be normal at the end of 72 h with no significant differences between control and treated groups in the measured parameters (Tables 5 and 6).

Table 5. Effect of *C. giladenesis* on DEN-induced alteration in CBC in male albino rats.

Treatment	Mean ±SE				
	T.WBC (×10^9/L)	Lymphocyte (%)	Monocyte (%)	Neutrophil (%)	Eosinophil (%)
Control	9.1±0.63	64.3±5.16	0.7±0.16	20.5±2.91	1.7±0.29
DEN	11.4±0.84*	80.6±5.08*	0.9±0.23	22.2±1.19	1.9±0.23
Plant	8.78±0.55**	60.8±2.39**	0.7±0.16	20.7±1.01	1.5±0.33
Preventive	9.52±0.92	68.3±2.40**	0.8±0.18	19.5±1.42	1.1±0.30
Treatment	10.3±0.399	67.6±2.91**	0.6±0.20	20.8±1.89	1.3±0.14

*Significant as compared with control at (P< 0.05), ** significant as compared with DEN-induced liver damage at (P< 0.05), TWBC: total white blood cell.

Table 6. Acute toxicity study of extract of *C. gileadenesis.*

Test	Mean ±SE		
Animal groups	**Control**	**Extract 2 g/kg**	**Extract 3 g/kg**
Clinical observation	No effect	No effect	No effect
Body weight (g) Pretreatment	323.0±26.5	314.0±15.0	330.0±14.9
After 24 hours	323.7±27.3	314.8±15.1	330.5±15.3
After 48hours	325.1±28.1	316.2±14.9	332.0±14.8
After 72 hours	325.9±28.3	316.7±14.6	334.5±15.3
Food intake (g)			
Pretreatment	79.0±3.23	76.1±3.48	77.5±3.84
After 24 hours	78.9±3.20	77.8±2.19	81.1±2.57
After 48hours	80.2±3.32	78.6±2.70	82.7±2.40
After 72 hours	81.0±3.46	80.0±2.65	89.6±3.17
Biochemical studies			
Hematology	12.4±0.63	11.9±1.07	14.1±0.54
Hb (g/dl)	10.2±0.41	10.5±0.29	9.33±1.01
Total W.B.C liver function enzymes			
AST	62.3±19.4	55.0±20.4	37.3±11.3
ALT	56.0±19.5	51.3±21.6	39.7±8.66
Fasting blood sugar			
Pretreatment	119.3±4.66	126.0±12.0	113.7±9.56
Post -treatment	117.5±13.9	120.7±3.17	91.0±6.51
Histophathology study	Normal	No change	No change
Liver Heart	Normal	No change	No change

DISCUSSION

In the present study, diethylnitrosamine was used as a model of hepatic injury. It is an N-nitroso alkyl compound, categorized as a potent hepatotoxin and hepato-carcinogen in experimental animals (Figure 3a-j) (Jose et al., 1998). This compound is metabolized in liver by P450 enzymes to form reactive electrophiles which cause oxidative stress leading to cytotoxicity, mutagenicity and carcinogenicity (Archer, 1989). It is considered as a pollutant found in environment, foods, alcoholic beverages and pharmaceutical agents (Sivaramkrishnan et al., 2008; Gupta et al., 2010).

The result of this study showed that DEN elevated significantly the liver function enzymes as well as it caused severe histopathological changes in the liver tissues. DEN led marked elevation of serum enzyme levels of AST, ALT and ALP is good indication of hepatocellular damage. However, elevation of these enzymes may lead to liver necrosis due to leakage of these enzymes to blood stream (Ala-Kokko et al., 1987; Al-Rejaie et al., 2009). Furthermore, an accumulation of connective tissue

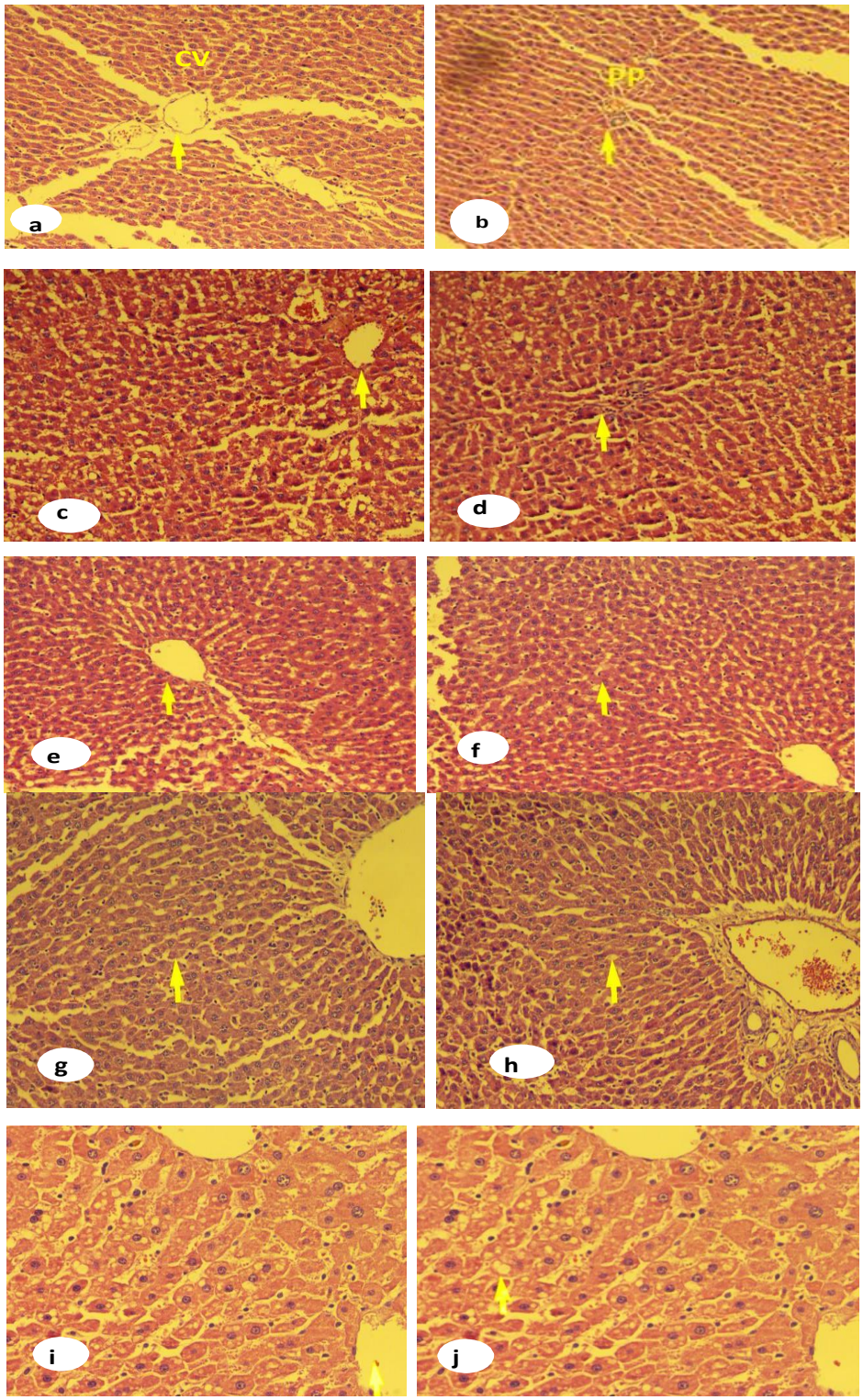

Figure 3. a and b: Control group section showing liver with preserved architecture with normal preportal, central vein and trabeculae of hepatocytes separated by blood sinusoids (H&E; original magnification: X 200); c and d: DEN treated group, showing inflammatory cells, the hepatocytes show macro and micro steatosis involving zone 3 in few acinus (H&E; original magnification: X 200); e and f: Plant treated group, showing normal liver architecture of central vein and mid zone (H&E; original magnification: X 200); g and h: Preventive group, showing marked improvement of liver with no evidence of steatosis in mid zone and preportal area (H&E; original magnification: X 200); i and j: Treatment group showing marked improvement with mild steatosis in zone3 (H&E; original magnification: X 200).

protein especially collagen, have been reported in DEN induced liver injury (George and Chandrakasan, 1996). Moreover, decreased synthesis of collagenolytic in the impaired hepatocytes may cause an accumulation of collagen (George et al., 2001). All these effects were ameliorated by the using of methanolic extract of *C. gileadenesis* bark. This plant showed significant preventive and therapeutic effects against DEN-induced hepatic injury through reduction of the liver function enzymes like AST, ALT and ALP as well as lipid profile especially cholesterol, LDL-c supported by the histopathological tissue improving in commiphora-treated groups.

However, the mechanism underlying the improvement of hepatic functions after continuous using of this plant may be referred to prevent the formation of reactive metabolite of DEN through direct inhibition of the hepatic cytochrome P450, additionally, it inhibits lipid peroxidation processes, stabilizes the hepatocyte membrane and enhances protein synthesis (Al-Howiriny et al., 2003). The presence of phytochemical antioxidant constituents like flavonoids, volatile oils, saponins, triterpenes and sterols are considered as key role for the protective effects and free radical scavengers in this plant (Vogel, 1977; Kikuzaki et al., 2000).

Regarding the effect of *C. gileadenesis* on complete blood count, it was found that this plant has significant reduction of platelets levels caused by DEN-induced toxicity. The precise reason for diminished platelets aggregation is still unknown. This worthwhile and remarkable anti-platelets activity may be due to either anti-inflammatory effect (Al-Howiriny et al., 2003) through the action on inflammatory mediators like thromboxane A2, 5HT and ADP or direct action on GPIIb/IIa receptors of platelets.

Conversely, the outcome of the present study was that, this plant counteracted the weight loss in DEN- treated group when it was administered for preventive or therapeutic purposes, this effect was not accompanied with any change in fasting blood sugar level. It suggested that this plant is characterized by appetite modulating effect without harmful metabolic changes as it reduced lipid profile especially cholesterol and low density lipoprotein (bad lipids). In contrary, it raised high density lipoprotein level which is known as a good lipid (Al-Amoudi, 2009). All these magic effects make this plant as a novel complementary medicine for many ailments. Additionally, beside its worthwhile health benefits, it is also considered as a safe plant assessed by preliminary toxicological study. It saves up to 3 g/kg/d (2.5 fold of the dose used) without any observational, biochemical side effects and/or histopathological changes.

Conclusion

From the outcomes of the present study, it is suggested that *C. gileadenesis* possesses protective effect against DEN-induced liver injury and anti -platelets activity. The presence of phytochemical antioxidant constituents in this plant like flavonoids, volatile oils, saponins, triterpenes and sterols may be responsible for its protective effects as they working as free radical scavengers. Additionally, the toxicological study showed that this plant can be used safely without any harmful effects. Further studies using more technical methods to elucidate the exact constituent (s) responsible for these benefits without side effects are required in order to approve and expand these findings.

Conflict of interests

The authors have not declared any conflict of interests.

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