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Abstract

Ten methanolic, petroleum ether and water plant extracts from botanical species used in traditional medicine in Egypt to cure different diseases have been subject to a screening study to detect the potential antimicrobial activity of their extracts against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger.* The antimicrobial activity of the extracts were evaluated using colonies growing on solid medium, establishing the minimal concentration required to inhibit their *In Vitro* growth (MIC). The results revealed that the extracts from the plants under study using methanol exhibited strong antimicrobial activity than that of petroleum ether or water The methanolic plant extracts revealed that extracts of *Ambrosia maritime*, *Zizyphus Christi* and *Salvia officinalis* possess strong *In Vitro* antimicrobial activity against the tested organisms than the other plant extracts used in this study. The separation, purification and characterization of the active agent(s) from *Salvia officinalis* leaves was carried out using thin layer chromatography. The physicochemical studies of the purified active agent(s) including, spectroscopic characteristics and chemical reactions was conducted. The biological activities of the purified active agent(s) i.e. MICs values were also estimated.

Keywords: Antimicrobial assay, Medicinal plants, Anti-infective plant extracts.

Introduction

The use of natural plant secondary metabolites having therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main source of drugs **(Pasquale 1984)**. Plants can produce antimicrobial metabolites to protect themselves from biotic attack that could be essential for microbial infection resistance **(Peng *et al.*, 2001).** Alternative mechanisms of infection prevention and treatment should be included in the initial activity screenings **(Cowan 1999)** and **Natarajan,** and **John (2005).** One approach that has been used for the discovery of antimicrobial agents from higher plants is based on the evaluation of traditional medicine The need for new antimcrobial drug has apparent in the past few years, especially for the treatment of infections where microbial resistance to antibiotics has developed **(Penna *et al*. 1996)**. In Egypt

traditional medicine, the use of plants in the form of crude extracts, infusions or plasters is a widespread practice to treat common disease. Different plant extracts used for treating gastrointestinal, respiratory, urinary and skin infections **(Martinez 1944; Diaz 1977; Baytelman 1980; Lozoya 1987; Argueta *et al* 1994; Batanouny 1999** and **Buwa, Staden 2006** and **Rocío *et a*l 2009).** However, there is still a lack of experimental scientific studies confirming the possible antibiotic properties of a great number of these remedies **Dimayuga** and **Garcia 1991**.This study deal with *In Vitro* antimicrobial screening methods provide the required preliminary observation to select, among the crude plant product, those with potentially useful properties for further chemical and pharmacological studies

Materials and method

Plant material

All plant samples were collected from the local market of medicinal plants and herbs Cairo-Egypt. Botanical identification was carried out identify by staff member of Botany and Microbiology Dept. Fac. Sci., Al Azhar Univ.

Preparation of extracts.

The dried powdered plant organs were subjected for extraction by maceration in methanol, petroleum ether and water (10g/100ml) for seven hours and the process was repeated twice. After wards, the solvent was distilled under reduced pressure in a rotary evaporator until the extracts become completely dry. The percentage yield for each extract was determined, and the products were dissolved in 10% tween 80 and added to melted agar culture medium in petri-dishes at the following final concentration: 1.0, 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 mg/ml.

Extract and fraction preparation

The *Salvia officinalis* (Lamiaceae) leaves (150 g) was dried in a current air, finely ground and the powder was subjected for soaking for 12 h. in 1000 ml methanol. The extract was filtered and distilled under reduced pressure in a rotary evaporator until the extracts become dry. The percentage yield for each extract was determined as 15.2 g of crude extract. TLC chromatography and mobile phase (n-butanol: acetic acid: water 1:1:1)) was used to evaluate the separation of the agent(s) of study. Spots were visualized by spraying by vanillin-sulfuric acid reagent.

Compound identification

Physiochemical properties:

Ultra violet (UV) spectra of the purified metabolite(s) was conducted using Unicom SP 1570 ultra violet spectrophotometer. Infrared (IR) spectra was recorded using potassium bromide (kBr) disk using Infrared spectrophotometer model PYE Unicom SP 1100. The GC–MS was conducted at the micro analytical center of Cairo University Egypt. Different color reactions was carried out to detect the presence of certain chemical groups in the metabolite of study using the method of (Hawk *et al*., 1954), (Kohn, 1961) and (Smith 1969).

Microorganisms

The tested microbial species are: *Bacillus subtilis* NCTC 10416, *Staphylococcus aureus* NCTC 7447, *Escherichia coli* NCTC10416, *Pseudomonas aeruginosa* ATCC 10145, *Candida albicans* IMRU 3669 and *Aspergillus niger* ATCC6275.The bacterial species were maintained and tested on nutrient agar (NA), yeast sabouraud-dextrose agar (SDA) and the filamentous fungi on Dox agar (DA).

Antimicrobial activity

The inoculum for each organism (bacteria and yeast) was prepared fresh from broth cultures containing 10^5 colony-forming units (CFU) and the plates were incubated at 37 ºC for 24h. The plates used for fungus were inoculated with spore suspension technique and incubated at 30 ºC for 72h. Ciprofloxacin and nystatin (10-100µg/ml) were used as reference standards. Observations were performed in duplicate and result expressed as the lowest concentration of plant extract that produced a complete suppression of colony growth, the minimal inhibitory concentration (IMC) (Rios *et al*., 1988).

Results

The results obtained from table (2) revealed that the plant organs employed, percentage yield from extraction with methanol and MICs values. Are in concentrations of 2-12 mg/ml of crude extract *Bacillus subtilis* NCTC 10416, *Staphylococcus aureus* NCTC-7447, of 5-26 mg/ml of crude extract against Gram-negative bacteria: *Escherichia coli* NCTC-10416, *Pseudomonas aeruginosa* ATCC-10145, of 6-24 mg/ml of crude extract against, unicellular fungi: *Candida albicans*

IMRU -3669 of 9-30 mg/ml of crude extract against multicellular fungi: *Aspergillus niger* ATCC-6275. The plant organs extract with methanol from *Solenostemma arghel* failed to exhibit activity against Gram-negative bacteria or unicellular and multicellular fungi.

The ethnobotanical data (name, local name, family name and popular use) of the selected plant was displayed in table (1).

The results obtained from table (3) indicate the plant organs employed, percentage yield from extraction with petroleum ether and MICs values.

Petroleum ether extract possess a moderate antimicrobial activity otherwise extraction with water.The MICs values were found in concentration of 3-21 mg/ml of crude extract against Gram-positive bacteria: *Bacillus subtilis* NCTC 10416, *Staphylococcus aureus* NCTC 7447, of 5-29 mg/ml of crude extract against Gram-negative bacteria: *Escherichia coli* NCTC10416, *Pseudomonas aeruginosa* ATCC 10145, of 6-25 mg/ml of crude extract against, unicellular fungi: *Candida albicans* IMRU 3669 of 9-33 mg/ml of crude extract against, multicellular fungi: *Aspergillus niger* ATCC6275. The plant extract with petroleum ether from *Solenostemma arghel* failed to exhibit effect on Gram-negative bacteria, unicellular and multicellular fungi. The results obtained from table (4) shows the yield from extraction with water and MICs values. The extraction all plants used on this study with water exhibit low antimicrobial activity on (bacteria and yeast) except *Salvia officinalis* and no effect of all plant extract on multicellular fungi. The MICs values were found in concentration of 5-35 mg/ml of crude extract against Gram-positive bacteria: *Bacillus subtilis* NCTC 10416, *Staphylococcus aureus* NCTC 7447, of 9-52 mg/ml of crude extract against Gram-negative bacteria: *Escherichia coli* NCTC10416, *Pseudomonas aeruginosa* ATCC 10145, of 12-51 mg/ml of crude extract against, unicellular fungi: *Candida albicans* IMRU 3669. The plant extract with water from *Solenostemma* failed to exhibit any effect on Gram-negative bacteria and yeast.

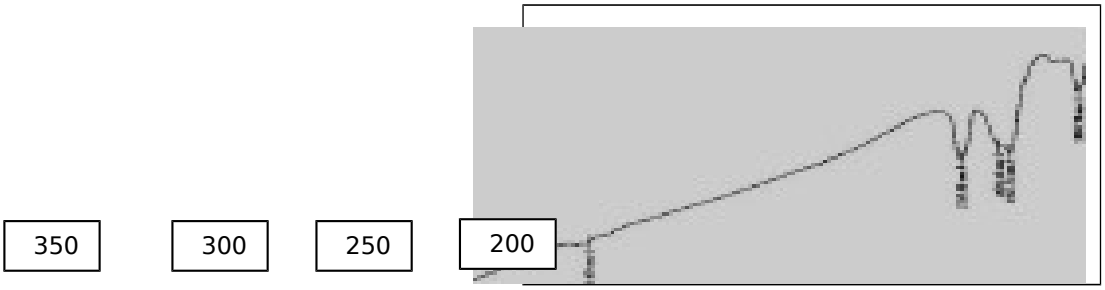

Fig (1) : Infrared spectra of active substance

Isolation and purification

The Sage *Salvia officinalis* (Lamiaceae) leaves was extracted with methanol in the ratio 2.0 w/v. From the 150 g of leaves 15.2 g of dry crude extract principal was obtained. The crude compound was purified through preparative The active compound was subjected to TLC and showed two bands (n-butanol: acetic acid: water 1:1:1 as mobile phase) plates was used. Spots were visualized by spraying vanillin-sulfuric acid reagent). The major band showing R_f 0:85 and the adjacent minor showing R_f 0:82. Elution of major band was done with ethanol and assayed for antimicrobial activity and checked for homogeneity by TLC. After evaporation 500 mg the of sample active principal compound was obtained. Results indicated that the active compound under investigation was found to be soluble in methyl and ethyl alcohols, dimethylformanide acetone and water. It showed positive responses to tannins test, and Fehling test, UV absorption spectra of the purified active compound exhibit maximum absorption peak at wave length 270.6 nm in ethanol. The Infrared absorption spectrum (IR) showed (Fig. 1) peaks at 3650, 2300, 2154, 2045, 1675 and 1600 cm_1. GC–MS: GC analysis showed at m=z 357 corresponds to a molecular formula of $C_{18}H_{14}O_8$

Table (1): List of medicinal plant organs used in the antimicrobial assay

Species Family	Local name	Popular use
Ambrosia maritima Compositae	Demsisa	For rheumatic pain, asthma, bilhrziasis, diabetes, expelrenal, stones, as stimulant, stomach bronchitis cold **(Batanouny 1999)**
Artemisia judaica Compositae	Shih	For stomach, cough, healing wound, burns, bronchitis and antiseptic of intestinal (Soliman 1995) and **(Batanouny 1999)**
Solenostemma arghel Asclepiadaceae	Harggal	For cough, gastrointestinal cramps stomachic, anticolic, colds and urinary tract antisyphilic **(Soliman 1995)** and **(Batanouny 1999)**
Thyme capitatus Labiatae	Zaater	For acute and chronic bronchitis, whooping cough, catarrh of the upper respiratory tract, gastrointestinal infection and mouth wash **(Soliman 1995)** and **(Batanouny 1999)**.
Balaniles aegyptiaca *Zygophyllaceae*	Heglig	For anthelmentic, purgative vermifuge herpes, malaria wounds syphilis **(Batanouny 1999)**.
Zizyphus Christi Rhamnaceae	Nabq	For cold, hypertension, antidiarrhea, stomachic, sores, heal wound skin disease, fever, sex disease and cough **(Soliman 1995)** and **(Batanouny 1999)**
Eletteria cardamomum Zingiberaceae	Habhin	For gastro intestinal cramps, stomach, stimulant sex, and oral antiseptic **(Pintz** and **Tatar1996)**
Calotropis procera Aslepiadaceae	Osher	For cold, cough, asthma, scabies teeth to loosen and skin disease and antidysentery **(Mahran, 1984)** and **(Batanouny 1999)**
Salvia officinalis Lamiaceae	Sage	antiviral, antibacterial, anti-inflammatory and antioxidant astringent, eupeptic and anti-hydrotic effects. hypoglycaemic effects
Achillea fragrantissma Compositae	Qaysum	For relaxation of contraction rats proximal aorta, trachea, urinary bladder, uterus and antimicrobial **(Barel *et al*.1991)** and **(Mustafa *et al*.1992)**

Table (2) : antimicrobial activity of crude methanolic extract.

Botanical species	Plant part tested	Extracted yield%	antimicrobial activity MIC (mg/ml)					
			B.s.	S.a.	E.c.	P.a.	C.a.	A.n.
Ambrosia maritima	whole plant	4.6	4.0	2.0	5.0	7.0	6.0	12
Artemisia judaica	whole plant	5.2	6.0	4.0	8.0	8.0	7.0	16
Solenostemma arghel	Leaves	4.1	6.0	5.0	----	----	----	----
Thyme capitatus	Leaves	5.7	7.0	5.0	9.0	11.0	9.0	23
Balaniles aegyptiaca	Fruits	8.3	15.0	12.0	18.0	22.0	20.0	25
Zizyphus christis	Leaves	4.5	3.0	3.0	5.0	7.0	8.0	14
Eletteria cardamomum	Fruits	3.7	8.0	5.0	12.0	16.0	16.0	27
Salvia officinalis	Leaves	10.5	3.0	2..0	5.0	6.0	6.0	9.0
Calotropis procera	Fruits	4.3	7.0	5.0	11.0	13.0	12.0	22
Achillea fragrantissma	whole plant	6.2	12.0	10.0	22.0	26.0	24.0	30
ciprofloxacin	------	-----	0.02	0.01	0.04	0.04	---	----
nystatin	------	-----	----	----	----	----	0.01	----

B.s.= *Bacillus subtilis* NCTC 10416, S.a.= *Staphylococcus aureus* NCTC 7447 E.c.= *Escherichia coli* NCTC10416, P.a.= *Pseudomonas aeruginosa* ATCC 10145, C.a.= *Candida albicans* IMRU 3669 and A.n.= *Aspergillus niger*ATCC6275.

Table (3): antimicrobial activity of crude petroleum ether extract

Botanical species	Plant part tested	Extracted yield%	antimicrobial activity MIC (mg/ml)					
			B.s.	S.a.	E.c.	P.a.	C.a.	A.n.
Ambrosia maritima	whole plant	2.4	5.0	3.0	7.0	9.0	7.0	12
Artemisia judaica	whole plant	3.3	8.0	6.0	9.0	11.0	8.0	19
Solenostemma arghel	Leaves	2.1	7.0	7.0	----	----	----	----
Thyme capitatus	Leaves	2.9	9.0	5.0	10.0	11.0	11.0	23
Balaniles aegyptiaca	Fruits	6.4	21.0	13.0	18.0	22.0	19.0	21
Zizyphus christis	Leaves	4.4	6.0	4.0	8.0	9.0	9.0	17
Eletteria cardamomum	Fruits	2.0	9.0	7.0	14.0	17.0	19.0	19
Salvia officinalis	Leaves	9.1	3.0	3..0	5.0	6.0	6.0	9.0
Calotropis procera	Fruits	3.7	8.0	5.0	13.0	19.0	17.0	28
Achillea fragrantissma	whole plant	4.9	14.0	12.0	27.0	29.0	25.0	33
Ciprofloxacin	------	-----	0.02	0.01	0.04	0.04	---	----
Nystatin	------	-----	----	----	----	----	0.01	----

Table (4): antimicrobial activity of crude water extract

Botanical species	Plant part tested	Extracted yield%	antimicrobial activity MIC (mg/ml)					
			B.s.	S.a.	E.c.	P.a.	C.a.	A.n.
Ambrosia maritima	whole plant	2.0	14.0	6.0	22.0	25.0	23.0	----
Artemisia judaica	whole plant	5.7	17.0	9.0	36.0	38.0	34.0	----
Solenostemma arghel	Leaves	6.1	12.0	12.0	----	----	----	----
Thyme capitatus	Leaves	4.9	20.0	16.0	33.0	37.0	31.0	----
Balaniles aegyptiaca	Fruits	8.4	35.0	30.0	41.0	44.0	51.0	----
Zizyphus christis	Leaves	8.5	13.0	11.0	26.0	28.0	36.0	----
Eletteria cardamomum	Fruits	4.0	18.0	15.0	47.0	52.0	46.0	----
Salvia officinalis	Leaves	8.7	6.0	5..0	9.0	9.0	12.0	----
Calotropis procera	Fruits	2.5	24.0	21.0	27.0	30.0	32.0	----
Achillea fragrantissma	whole plant	3.5	29.0	29.0	45.0	48.0	43.0	----
Ciprofloxacin 30µg	------	-----	0.02	0.01	0.04	0.04	---	----
Nystatin	------	-----	----	----	----	----	0.01	----

B.s.= *Bacillus subtilis* NCTC 10416, S.a.= *Staphylococcus aureus* NCTC 7447 E.c.= *Escherichia coli* NCTC10416, P.a.= *Pseudomonas aeruginosa* ATCC 10145, C.a.= *Candida albicans* IMRU 3669 and A.n.= *Aspergillus niger*ATCC6275.

Table (5): Summery of the response of the purified plant extracts to certain chemical reactions.

No	Chemical test	Result	Remark
1	Tannins test	+ve	presence of tannins
2	Flavonoid test	-ve	no flavonoid
3	Terpenes test	-ve	no terpenes
4	Saponins test	-ve	no fat
5	Glycoside test	-ve	no glycoside
6	Molish test	-ve	no carbohydrates
7	Fehling test	+ve	present keton or aldhyde free
8	Test Biuret test	-ve	no protein
9	Ehrlish test	-ve	no indolic
10	Mayer reaction	-ve	no nitro group

Table (6) MIC of rosmarinic acid extracted from *Salvia officinalis* leaves

Microorganisms	MIC (mg/ml)
Bacillus subtilis NCTC 10416	2.0
Bacillus cereus	2..0
Staphylococcus aureus NCTC 7447	2.0
Escherichia coli NCTC10416	4.0
Pseudomonas aeruginosa ATCC 10145	5.0
Candida albicans IMRU 3669	6.0
Aspergillus niger ATCC6275	8.0

Discussion

The obtained results can be considered very promising for the extraction and isolation some compounds with antimicrobial activities of some medicinal plants used in traditional medicine of Egypt people to show that the therapeutic properties of some plants used in traditional medicine. This study is a preliminary evaluation of antimicrobial activity of the plants. It indicates that several plants have the potential to generate novel metabolites. Previous reports an antimicrobial activity have been carried out for *Thymus vulgaris* **L. (Janssen, *et al,*1986; Caceres, *et al,* 1987; Panizzi, *et al,* 1993; Batanouny, 1999, Alanís e*t al* 2005),** *Piper nigrum* **(Lentz *et***

***al* 1998, Dorman and Deans 2000, Lemos *et al.*, 2000, Supayang, 2004 and González *et al* 2009), *Zizyphus* (Shahidi 2004),** *Artemisia* **(Alanís *at el* 2005), *Ambrosia maritime* (Amin,1990, Abadome *at el*,1994),** *Achillea fragrantissma* **(Aboutabl *et al*,1986 and Barel *et al* 1991)** *Solenostemma arghel*, **(Osbron 1968)** *Salvia officinalis* **(Maike and Monique 2003).** In classifying the antibacterial activity as Gram-positive or Gram-negative, it would generally be expected that a much greater number would be active against Gram-positive than Gram-negative bacteria **(McCutcheon *et al*., 1992).** The activity against both the types of bacteria may indicative of the presence due to broad-spectrum antibiotic compounds or simply general metabolic toxins. However, in this study, a large number of the extracts (9) were active against both (Gram-positive and Gram-negative bacteria), unicellular and multicellular fungi, while a relatively less number (1) was active against Gram-positive bacteria alone. From the fragmentation pattern of the GC MS, IR, UV, and chemical reaction it was found that the active purified compound is consider as rosmarinic acid analogue closely related to rosmarinic acid reported by **Hippolyte, *et al*., (1992), Maike** and **Monique (2003).** Victor *et al* 1996 considered a strong response to exist when the extract produced an effect at concentration of 10 mg/ml or below for *Staphylococcus aureus*, between 10-20 mg/ml for *Escherichia coli* and *Pseudomonas aeruginosa*, and below 20 mg/ml for *Candida albicans* therefore consider most active substances in plant extract under study have strong antimicrobial activity when extracted with methanol and petroleum ether. Three susceptible bacteria species were *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* most prevalent burn-patients pathogen capable of causing life-threatening illnesses. Some strains causing septicemia and pneumonia in cystic fibrosis and immunocompromised patients are becoming difficult to treat with currently available antimicrobial agents **(Lory,1990).** Intensive use of antibiotics often resulted in the development of resistant strains **(Sydney *et al*., 1980)**. The multi-resistance of *Pseudomons aeruginosa*, there is a lack of active antibiotics effective against this bacterium, resulting in an increase in nosocomial infections and high mortality. It is also an opportunist associated with respiratory and urinary tract, wounds, and bacteremia **(Giamarellos-Bourboulis *et al*., 1999)**. Because of this drug resistance, the search for new antibiotics continues unabated. In this connection plants continue to be a rich source of therapeutic drugs. The active principles of many drugs are found in plants or are produced as secondary metabolites. The remarkable contribution of plants to the drug industries was

possible, because of the large number of the phytochemical and biological studies all over the world. Egypt has a great variety of plants used in folk medicine and only a few of these have been studied for there.

Conclusion

This investigation indicated that some of the plants used in Egypt herbal medicine do possess antimicrobial activity against the organisms tested. These plants after evaluate them against bacterial and fungal strains can be used for microbial control of food preservation and treatment burn and infectious diseases with antibiotic resistant on ointment and cream form or isolation active compound goal to find new therapeutic principles.

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