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In vitro Antimicrobial Activity of Five Egyptian Plant Species

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ABSTRACT

In this study, five Egyptian species were tested for their *In vitro* antimicrobial activities. The antimicrobial screening was carried out via disc diffusion method toward four strains of the clinical antibiotic resistant pathogens including *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus niger*. Among the methanolic extracts screened, *Azadirachta indica*, *Tectona grandis* and *Ficus sycomorus* showed a broad antimicrobial spectrum against three strains with inhibition zones between 13-27 mm followed by *Gmelina arborea* and *Ficus microcarpa* with inhibition zones between 11-17 mm, all plants showed no activity against *Aspergillus niger* except *Gmelina arborea* with inhibition zones 12 mm. Penicillin G was used as positive control at concentration of 100 µg/disc with inhibition zones (*Staphylococcus aureus* 28mm, *Escherichia coli* 22mm, *Candida albicans* 25mm and *Aspergillus niger* 0mm). Owing to the high activity of the methanolic extracts, these extracts were defatted via petroleum ether then were fractionated via; chloroform, ethyl acetate and n-butanol. The n-butanol of *Azadirachta indica* was the most active against *Candida albicans* (25 mm), ethyl acetate of *Ficus sycomorus* against *Staphylococcus aureus* (18 mm), n-butanol of *Gmelina arborea* against *Staphylococcus aureus* (17 mm) and n-butanol of *Ficus microcarpa* against *Staphylococcus aureus* (15 mm). These results suggest that the tested plants may be effective potential sources of natural antimicrobials, and are potent inhibitors of antibiotic resistant pathogens.

INTRODUCTION

Since the coming of antibiotics in the 1950s, the use of medicinal plants and herbs derivatives and isolates as a source of antimicrobial agents has been practically nonexistent (Marjorie, 1999). Plants have been used for thousands of years to flavor and conserve food, to treat health disorders and to prevent diseases including epidemics. The knowledge of their healing properties has been transmitted over the centuries within and among human communities. Active compounds produced during secondary vegetal metabolism are usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases. Data on the antimicrobial activity of numerous plants, so far considered empirical, have been scientifically confirmed, concomitantly with the increasing number of reports on pathogenic microorganisms resistant to antimicrobials. Products derived from plants may potentially control microbial growth in diverse situations and in the specific case of disease treatment, numerous studies have aimed to describe the chemical composition of these plant antimicrobials and the mechanisms involved in microbial growth inhibition, either separately or associated with conventional antimicrobials (Silva and Fernandes, 2010). The use of plants for treating diseases is as old as the human species. Popular observations on the use and efficacy of medicinal plants significantly contribute to the disclosure of their therapeutic properties, so that they are frequently prescribed, even if their chemical constituents are not always completely known (Maciel *et al.*, 2002; Silva and Fernandes, 2010). Herbal medicine is the use of plants for their therapeutic or medicinal value. Plants contain a variety of chemical substances that act upon the body to prevent, relieve and treat illnesses (Wijesekera, 1991). Medicinal plants are important for pharmacological research and drug development. Not only plant constituents are used directly as therapeutic agent but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Mukherjee, 2003). Nowadays, the resistance of pathogens against antibiotics develops much faster than ever.

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The search for new antimicrobial and antioxidant substances from nature is on great demand (Ying-Jang *et al.*, 2008). The present study was to investigate the antimicrobial and antioxidant activities of five Egyptian plants in the evaluation of its potential to be a preservative from natural source. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient (Gislene *et al.*, 2000).

Infectious disease caused by bacteria, viruses, fungi and parasites are still a major threat to public health, despite the tremendous progress in human medicine. The past three decades have seen a dramatic increase in microbial resistance to antimicrobial agents. Such situation stimulates the development of new anti-microbial agents in order to treat the infectious disease in an effective manner. So this matter continued to an era to identify the potential antimicrobial agent from the natural resources. The edible plants that used for traditional medicine contain a wide range of substance that can be used to treat abundant of infectious disease with reduced side effects (Subramanion *et al.*, 2010). The previous reported studies indicated that, the tested plants showed wide range of biological activities and also, numerous bioactive secondary metabolites were isolated from various parts of the selected species which may be responsible for such activities (Ephraim *et al.*, 2008; Abdel-Hameed *et al.*, 2009; Mortada *et al.*, 2009; Mortada *et al.*, 2010; Mortada *et al.*, 2011; El-Sayed *et al.*, 2011; Ghareeb *et al.*, 2013; Ghareeb *et al.*, 2014; Mosad *et al.*, 2014; Shoeb *et al.*, 2014).

MATERIALS AND METHODS

Plant Material and Chemicals

The leaves of the plants under investigation were collected from Zoo Garden, and El-Orman Botanical Garden, Giza, Egypt in August 2014. The identity of the plant was established by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimens (given number GA, TG, AI, FS and FM) were kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI). The plants materials were air-dried in shade place at room temperature and then powdered by electric mill, finally kept in tightly closed container in a dark place till the extraction process. All solvents and reagents used were of analytical grade. all solvents and acids (methanol, petroleum ether, chloroform, ethyl acetate, n-butanol), were purchased from (Sigma-Aldrich Co.).

Equipment and Chemicals for Antimicrobial Assays

Low temperature incubator SHEL-LAB model 2005 sheld on manufacturing. Inc, NUAJARE Biological safety cobient, LABSCO oven Laboratory Supply Company, Olmon and Cokg Germany, Autoclave la Astell Heorson Germany, Refregerator Toshiba (no frost model FR-GF40P). Nutrient agar medium (LAB M, UK), sucrose (Oxford), Na NO_3 (S.D. Fine Chem. Ltd), $MgSO_4$ (S.D. Fine Chem. Ltd), KCl (S.D. Fine Chem. Ltd), $FeSO_4$ (S.D. Fine Chem. Ltd), K_2HPO_4 (MERCK), agar-agar bacto (S.D. Fine Chem. Ltd), *Staphylococcus aureus* (ATCC 6538-P), *Cadida albicans* (ATCC 27853), *Peudomonas aeroginosa* (ATCC 10231), and *Aspergillus niger* (NRRL A-326). All the test microbes were obtained from the culture collection at Microbial Chemistry Department, National Research Center.

Extraction and Fractionation

Extraction process was carried out via taking five samples from dry powder of fresh leaves of each plant (200 g) soaking it in (2000 ml), then extracted separately with 85% MeOH in room temperature with shaking day by day followed by filtration and again extraction for four times (two weeks). Then each extract was filtered using Whatmann filter paper No.1 and concentrated by using a rotary evaporator (Buchi, Switzerland) at (40 ± 2ºC) affording known weight of each crude methanol extract.

The crude extracts were collected and stored at room temperature in the dark for the further process. The 85% methanolic crude extracts (20-30 g) were defatted by washing several times with petroleum ether (60-80°C). Twenty gram of the defatted methanol extracts were undergoes fractionation process via different organic solvents; $CHCl_3$; EtOAc and n-BuOH (4 x 150 ml solvent).

Antimicrobial Assay

Disc agar plate method was done to evaluate the antimicrobial activity of different methanol extracts and their derived sub-fractions from the selected plants. The antimicrobial activities of 0.5-cm-diameter filter paper disc saturated with about 1mg sample were tested against four different microbial strains, i.e., *Staphylococcus aureus* (G +ve bacteria), *Escherichia coli* (G -ve bacteria), *Candida albicans* (yeast) and *Aspergillus niger* (fungi). Penicillin G was used as positive control at concentration of 100 µg/disc. Both bacterial and yeast test microbes were grown on nutrient agar (DSNZ 1) medium (g/l): beef extract (3), peptone (10), and agar (20). Whereas fungal test microbe was grown on Szapek-Dox (DSMZ130) medium (g/l): sucrose (30), $NaNO_3$ (3), $MgSO_4.7H_2O$ (0.5), KCl (0.5), $FeSO_4.7H_2O$ (0.001), K_2HPO_4 (1) and agar (20). The culture of each microorganism was diluted by sterile distilled water to 10^7 to 10^8 CFU/ml to be used as inoculum.1ml of the previous inoculum was used to inoculate 1l of agar medium (just before solidification) then poured in Petri-dishes (10cm diameter containing 25ml). Discs (5 mm diameter) were placed on the surface of the agar plates previously inoculated with the test microbe and incubated for 24 h for bacteria and yeast but for 48 h for fungus at 37 and 30°C, respectively (Bauer *et al.*, 1996).

RESULTS AND DISCUSSION

Yield (%) of the extracts and fractions

In our current study there is a remarkable variation in the yield percentages of crud 85% methanolic extracts and their derived sub-fractions (petroleum ether, methylene chloride- ethyl acetate and n-butanol) of the five species under investigations, and such phenomena may be return to the variation in nature of the chemical constituents to be extracted in each plant (Table 1).

Table 1: Yield (%) of the different extracts and their derived fractions from the five species under investigation.

Extract/Fraction	Yield (%)[1]
A. indica Me.	18.0
A. indica Pt.	3.20
A. indica Met.	3.0
A. indica Et.	3.5
A. indica n-Bu	5.0
T. grandis Me.	19.5
T. grandis Pt.	2.0
T. grandis Met.	1.5
T. grandis Et.	2.5
T. grandis n-Bu	4.3
F. sycomorus Me.	14.0
F. sycomorus Pt.	2.8
F. sycomorus Met.	3.25
F. sycomorus Et.	1.60
F. sycomorus n-Bu	5.65
G. arborea Me.	21.30
G. arborea Pt.	1.75
G. arborea Met.	2.0
G. arborea Et.	2.25
G. arborea n-Bu	4.75
F. microcarpa Me.	22.45
F. microcarpa Pt.	2.90
F. microcarpa Met.	1,65
F. microcarpa Et.	3.40
F. microcarpa n-Bu	4.85

[1]Yield (%): (total extractable content TEC).
Me.= Methanol; Pt.= Petroleum ether; Met.= Methylene chloride; Et.= Ethyl acetate and n-Bu.= n-BuOH.

Antimicrobial activity

Penicillin G was used as positive control at concentration of 100 µg/disc with inhibition zones (*Staphylococcus aureus* 28mm, *Escherichia coli* 22mm, *Candida albicans* 25mm and *Aspergillus niger* 0mm). The inhibition zones against three strains of different tested fractions of *T. grandis* were ranged from 7-24 mm and there is no any effect against *A. niger*. From our results the methanolic extract showed the strongest action against *E. coli* (18 mm), *S. aureus* (24 mm) and *C. albicans* (20 mm) (Table 2). Shalini, 2009; reported that the antifungal activity of the methanolic extract of *T. grandis* was investigated against *Alternaria cajani*, *Curvularia lunata*, *Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp., and such activity may be attributed to the various phytochemical constituents present in the crude extract (Shalini, 2009). Furthermore, the antibacterial activity of methanolic extract of *T. grandis* was investigated based on the synergistic activity with tetracycline against different bacteria both Gram-positive and Gram-negative species (Purushotham *et al.*, 2010). Sumthong *et al.*, 2006, 2007; showed that the antimicrobial activity may be due to the presence of certain bioactive secondary metabolites like quinones (Sumthong *et al.*, 2006, 2007). The inhibition zones against three strains of different tested fractions of *G. arborea* were ranged from 5-17 mm against *E. coli*, *S. aureus* and *C. albicans* as well as a characteristic effect against *A. niger* with inhibition zone (12 mm) (Table 2). El-Mahmood *et al.*, 2010; showed that the crude extracts of the leaves and stem bark of the *G. arborea* showed *In vitro* antimicrobial activity against *Escherichia coli* and *Salmonella typhi* (El-Mahmood *et al.*, 2010). Also, Amrutha *et al.*, 2010 reported on the antimicrobial activity of the methanol and chloroform extracts of *G. arborea* (Amrutha *et al.*, 2010). On the light of such results *G. arborea* provided the scientific bases for the folkloric application as a medicinal plant and can be used as source for newer antibiotic substances for the possible control of infections associated with bacteria.

The results revealed that the methanolic extract of *F. sycomorus* showed very strong activity against *S. aureus* (27 mm) and moderate activity against *E. coli* and *C. albicans* of inhibition zones 14 and 16 mm respectively. On the other hand, the ethyl acetate fraction showed strong activity against *S. aureus* (18 mm) (Table 2). Our results were supported and reinforced via previous studies which showed that the *In vitro* antifungal activity of hexane, petroleum ether and chloroform extracts of stem bark of *F. sycomorus* was studied against different fungal species and the results exhibited that the hexane extract of the plant was active on *Microsoporum gypseum, Aspergillus niger, Aspergillus flavus* and *Candida albicans*. The chloroform extract of the plant showed very high inhibitory activity on only *Trichophyton mentagrophytes* and *Trichophyton rubrum*. The petroleum ether extract did not exhibited significant activity on the tested fungal species (Hussan *et al.*, 2007).

The methanolic extract of *A. indica* exhibited very strong activity against *S. aureus* and *C. albicans* with inhibition zones 21 and 20 respectively. For its derived sub-fractions, the n-butanol showed potent activity against *C. albicans* (25 mm) and *S. aureus* (19 mm) followed by the ethyl acetate sub-fraction with inhibition zone (17 mm) against *C. albicans* (Table 2). Evaluation of antimicrobial activity of different fractions of leaves of *A. indica* against eight strains of Gram positive bacteria; *Micrococcus glutamicus, Lanctobacillus, streptococcus faecorlris, staphylococcus aureus, Bacillus sterothemmophrlus, Staphylococcus pyrogenes, Micrococcus luteus, Bacillus cereus* and two strains of Gram negative bacteria; *E. coli* and *Pseudomonas aeruginosa* was carried and the results showed that all plant extracts exhibit significant antibacterial activity against all the tested microorganisms (Rajasekaram *et al.*, 2008). Also, Biswas *et al.*, 2002, reported that neem leaves and seeds exhibited antibacterial activity against a wide spectrum of Gram-Positive and Gram- Negative microorganisms (Biswas *et al.*, 2002).

Ficus microcarpa showed the lowest antimicrobial activity among all tested plants with inhibition zones ranged from (9-17 mm). Both of methanolic and n-butanol extracts showed moderate activity against *S. aureus* with inhibition zones 17 and 15 mm respectively (Table 2). Our results were in agreement with the previous studies which showed that methanol extracts of bark,

fruits and leaves of *F. microcarpa* exhibited excellent antibacterial activity against tested Gram-positive and Gram-negative bacteria. Ethyl acetate fraction of bark extract exerted strong antibacterial effects and the inhibition zones against *Bacillus brevis*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* and *Achromobacter polymorph* were 18.0, 15.5, 16.5, 16.0 and 8.0 mm, respectively. The strong antibacterial activities of *F. microcarpa* bark extract may be attributed to its high level of phenolic compounds which were identified via GC-MS and HPLC analyses (Changwei *et al.*, 2008).

Table 2: Antimicrobial activity of the defatted 85% methanolic extracts of five Egyptian plants as well as their derived sub-fractions.

Sample	Clear Inhibition zone (Θmm)			
	E. coli	***S. aureus***	***C. albicans***	***A. niger***
A. indica Me.	**13**	**21**	**20**	**-**
A. indica Pt.	**-**	**7**	**-**	**-**
A. indica Met.	**8**	**-**	**-**	**-**
A. indica Et.	**10**	**14**	**17**	**-**
A. indica n-Bu	**15**	**19**	**25**	**-**
T. grandis Me.	**18**	**24**	**20**	
T. grandis Pt.	**8**	**8**	**-**	**-**
T. grandis Met.	**-**	**7**	**-**	**-**
T. grandis Et.	**10**	**9**	**11**	**-**
T. grandis n-Bu	**12**	**17**	**10**	**-**
F. sycomorus Me.	**14**	**27**	**16**	**-**
F. sycomorus Pt.	**-**	**-**	**9**	**-**
F. sycomorus Met.	**9**	**-**	**7**	**-**
F. sycomorus Et.	**10**	**18**	**12**	**-**
F. sycomorus n-Bu	**14**	**13**	**15**	**-**
G. arborea Me.	**12**	**16**	**11**	**-**
G. arborea Pt.	**-**	**5**	**-**	**-**
G. arborea Met.	**7**	**7**	**-**	**12**
G. arborea Et.	**8**	**7**	**10**	**-**
G. arborea n-Bu	**14**	**17**	**12**	**-**
F. microcarpa Me.	**12**	**17**	**12**	**-**
F. microcarpa Pt.	**-**	**-**	**-**	**-**
F. microcarpa Met.	**9**	**-**	**11**	**-**
F. microcarpa Et.	**10**	**13**	**11**	**-**
F. microcarpa n-Bu	**14**	**15**	**12**	**-**
Penicillin G	**22**	**28**	**25**	**-**

The results of samples against ***E. coli***= *Escherichia coli* (G-ve bacteria)*;* ***S. aureus***= *Staphylococcus aureus* (G+ve bacteria); ***C. albicans***= *Candida albicans* (yeast); ***A. niger***= *Aspergillus niger* (fungus); **(-)**; inactive.. Penicillin G as positive control.

CONCLUSION

The present study demonstrates that the methanolic extracts of fives Egyptian plants as well as their derived sub-fractions showed promising *In vitro* antimicrobial activities against four stains with different strength which represented by the inhibition zones. This finding provides an insight into the usage of the tested species as antimicrobial agents.

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