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Full Length Research Paper

Antimicrobial potential of licorice: Leaves versus roots

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Medicinal plants play a vital role in covering the basic health needs. They may offer a new source of antibacterial agents. The aim of this study was to screen *in-vitro* the antimicrobial activity of some Egyptian medicinal plants against clinical methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from different hospitals in Egypt followed by studying the MIC and cytotoxicty of the most active one. Screening of antimicrobial activities of 70% ethanolic extracts obtained from 19 plants against 59 MRSA clinical isolates were tested by agar well diffusion method. Licorice showed the highest antimicrobial effect against all 59 MRSA isolates (leaves were more active than roots). Minimum inhibitory concentrations (MICs) of licorice leaves were 8 µg/ml, whereas that of Flucloxacillin range between 32 to >128 ug/ml when tested against five MRSA strains. Colorimetric cytotoxicity assay of licorice leaves extract was done on HEPG2 and HCTI16 cell lines and revealed that the IC_{50} were 19.5 and 15 µg/ml respectively. Separation of the components in licorice leaves using thin layer chromatography (TLC) results in two active fractions identified with the help of spectroscopic analysis as inflacoumarin A and Licochalcone A. Our results reveal that the Egyptian licorice leaves extract represent a new candidate for antimicrobial agent against MRSA more than that achieved by root. This is the first report which highlights the antimicrobial activity of licorice leaves.

Key words: Plant extracts, licorice, MRSA, antimicrobial.

INTRODUCTION

Methicillin-resistant *S. aureus* (MRSA) is considered one of the major causes of nosocomial infections. The problem has been magnified by the rapid spread and high incidence of MRSA in intensive-care units (ICUs) (Cepeda et al., 2005) which has led to increase attention to develop novel anti-MRSA agents and vaccines (Cutler and Wilson, 2004; Hancock, 2007).

Plant has traditionally provided a source of novel drug compounds (Betoni et al., 2006; Shinwari, 2010). Medicinal plants have two special advantages which distinguish them from chemical drugs; the use of crude herbs and the ability of prolonged usage. A single herb may contain many natural constituents which have been found to have antimicrobial properties. Experience has showed many benefits in long-term use of whole medicinal plants and their extracts, because the constituents in them work in conjunction with each other (Xiaorui, 1998). So many researchers turn their attention to medicinal plants, hopping to develop better drugs against different microbial infections (Benkeblia, 2004; Shinwari et al., 2009; Upadhyay et al., 2010; Alpsoy, 2010, Sedighinia et al., 2012).

The aim of this study was to screen the *in vitro* antimicrobial activity of some Egyptian medicinal plants against some clinical MRSA strains isolated from different hospitals in Egypt followed by further studying the minimal inhibitory concentration (MIC) and antitumor activity of the most active extract.

MATERIALS AND METHODS

Bacterial isolates

Two hundred and nineteen *Staphylococcus* strains were collected from different hospital samples over a period of one year, through

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2009. The colonies on different culture media (Blood agar, Mannitol salt agar, Nutrient agar, Nutrient broth, Baried Barker agar (Oxoid Ltd.), *Staphylococcus* medium 110 (Difco)) were isolated and identified according to the conventional methods described by Bergey's Manual of Systematic Bacteriology (1989). One hundred and eleven isolates were identified as *Staphylococcus aureus.* Screening of MRSA was done by detection of resistance to oxacillin by disc diffusion method on Muller Hinton agar (Difco) and culturing the resistant isolates on Oxacillin Resistance Screening Agar Base (ORSAB) medium (Oxoid, Ltd.), the ORSAB medium supplement SR195 (Oxoid Ltd.) was supplied and contained Oxacillin (1mg) and polymyxin B (25000IU) in each vial sufficient to supplement 500 ml of the medium. Fifty nine isolates were found to be MRSA and were maintained for completion of this work by sub-culturing on nutrient agar overnight at 37°C, every 15 days and stored at 4°C. Before use**,** sub-culture for 24 h was done**.** *Staphylococcus aureus* ATCC 29737 was used as sensitive control.

Antibiotic susceptibility testing

For further classification of MRSA strains to multidrug resistant (MDR) isolates (resistant to three or more non β- lactam antibiotics) and non multidrug resistant (NMDR) isolates (resistant to less than three non β- lactam antibiotics) (Merlino et al., 2002), the MRSA isolates were tested for their resistance to different antibiotics by disc diffusion method using the following antibiotic discs (Oxoid, Ltd.): amikacin (30 ug), amoxicillin (25 ug), ampicillin (10 ug), cefepime (30 ug), cefoperazone(75 ug), cefotaxime (10 ug), cefprozil (30 ug), oxacillin (1 ug), clarithromycin (15 ug), doxacyclin (30 ug)e, erythromycin (15 ug), levofloxacin (5 ug), ofloxacin (5 ug), tobramycin (10 ug) and vancomycin (30 ug) on Muller Hinton agar (Difco). Isolates were classified as susceptible, intermediate, or resistant, in accordance with the National Committee for Clinical Laboratory Standards (2000).

Antimicrobial activity of plants extracts

Screening of antimicrobial activities of 70% ethanolic extracts obtained from 19 plants (Fennel, Anise, Coriander (fruit and leaves), Parsley, Dill, Peppermint (Piperita and Spicata), Rosmarinus, Marjoram, Lavenders, Thyme, Chamomile (Flower and Herb), Lantana, Guava, Carob gum, Licorice (root and leaves), Acacia, Lemon grass, Basil, Oregano) against 59 MRSA isolates were tested by agar well diffusion method according to NCCLS (2000).

Polymerase chain reaction (PCR) for detection of *mecA* gene of Multi-drug resistant MRSA isolates

Five randomly selected MDR MRSA isolates undergone detection of *mecA* gene by PCR. Extraction of DNA was performed by QIAamp DNA minikit (QIAGEN) according to the manufacturer's instructions. The PCR method used was modified from the protocol published before (Predari et al., 1991), giving amplification of a 528 bp *mecA* fragment. The oligonucleotide primers were based on published sequence of PBP 2a gene: Met 1 (5′-dGGGATCATAGCGTCATTATTC-3′) nucleotides 516 to 536, Met 2 (5′dACGATTGTGACACGATAGCC-3′) nucleotides 1024 to 1044. Primers were synthesized using ABI 392 DNA/RNA synthesizer (Applied Biosystem, USA). Target DNA (1 ug) was added to 45 µl of master mix (Perkin Elmer, Foster City, CA) containing: PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% (W/V) gelatin), 0.1 mM of each deoxynucleotide (Perkin Elmer) and 0.3 µM of each of the *mecA* primer. The tube were placed in a Gene Amp PCR systems 2400 (Perkin Elmer) and the DNA denaturated at 94˚C for 1 min, then 1.0 U AmpliTaq DNA polymerse (Perkin Elmer), in Taq buffer (50 mM KCl, 10mM Tris HCl [pH 8.3] and 0.01% (w/v) gelatin [GenAmp 1 × PCR – buffer II, Perkin Elmer) was added to each PCR reaction. This was followed by denaturation at 95°C for 45 s, annealing at 55°C for 45 s and elongation at 72°C for 60 s. Samples were amplified by a final extension at 72°C for 3 min. The PCR product was visualized by electrophoresis, using 2% agarose gel (Amersham-Biosciences AB), stained with ethidium bromide and viewed with UV light.

Determination of minimum inhibitory concentration (MIC) of multi-drug resistant MRSA isolates using Flucloxacillin

The MIC of the five selected MDR MRSA isolate were determined by the agar dilution method on Mueller Hinton agar medium (Difco) according to NCCLS (2000). Briefly; the Flucloxacillin powder (Oxoid) was dissolved in distilled H_2O, and diluted to give serial two-fold dilutions ranging from 512 to 4 µg/ml. Before gelling, 20 ml of agar medium were added to each of the Petri dishes containing the antibiotic. Each bacterial suspension adjusted to 0.5 McFarland standard was diluted 1:10 in sterile broth to obtain a concentration of 10^7 CFU/ml. Subsequently, 2 µl of each bacterial suspension were inoculated on the Mueller Hinton agar surface. The final concentration on the agar will be approximately 10^4 CFU/ml.

Antimicrobial activity of different extracts of licorice leaves and roots against MRSA isolates

Ethanol Licorice roots and leaves extracts had the higher antimicrobial activity against 59 MRSA isolates at 70%; we however wondered if the extraction with other organic solvents will have the same antimicrobial effect on tested MRSA strains. Extraction by different organic solvents; *n*-hexane, dichloromethane, ethyl acetate and 70% ethanol; from roots and leaves of Egyptian Licorice was tested against five MDR MRSA isolates giving positive *mecA* gene with PCR and *Staphylococcus aureus* ATCC 29737 was used as sensitive control (MSSA) by well diffusion assay. This was followed by MIC testing of ethyl acetate extract of licorice leaves (being the most potent extract) against the same MRSA isolates by agar dilution method (NCCLS, 2000).

Cytotoxicity assay of licorice leaves extract

Cytotoxicity assay of licorice leaf extract was done using a colorimetric cytotoxicity assay (Skehan et al., 1990). Briefly; cell lines HEPG2 (liver carcinoma) and HCTI 16 (colon carcinoma) were harvested from exponential phase cultures by trypsinization, counted and plated as cell monolayer in 96 multi well plate (10^4 cell/well) for 24 h . Cells were cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% heat inactivated fetal calf serum and 1 mM L-glutamine. Different concentrations of the licorice leaves extract (0, 10, 25, 50 and 100 µg/ml in DMSO) were added to HEPG2 and HCTI 16 cell lines in 6 replicate in 96 multi well tissue culture plate. Doxorubicin was used as positive control. The plate was incubated for 48 h at 37°C with 5% CO_2. After 48 h, cells were fixed with TCA, washed and stained with Sulfo-Rhodamine B (SRB) stain (Sigma Chemical CO. Louis, MO, U.S.A.). Excess stain was washed with acetic acid and the attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader at 564 nm. The relation between surviving fraction and plant extract concentration was plotted to get the survival curve of each tumor cell line after specified dilution of the extract using Graph pad prismp software version 5 (Graph- Pad, UK). IC_{50} which is the concentration of the compound that reduces cell survival to 50% were calculated.

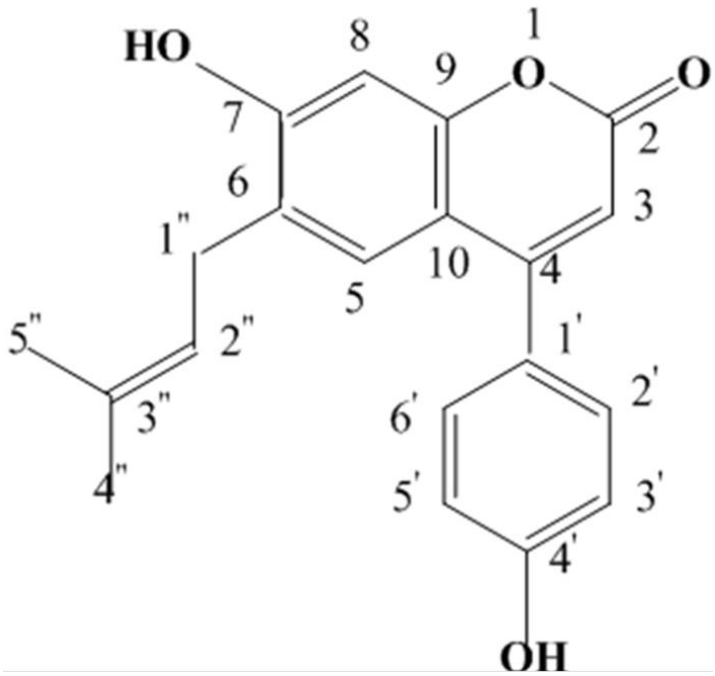

Figure 1. The structure of inflacoumarin A.

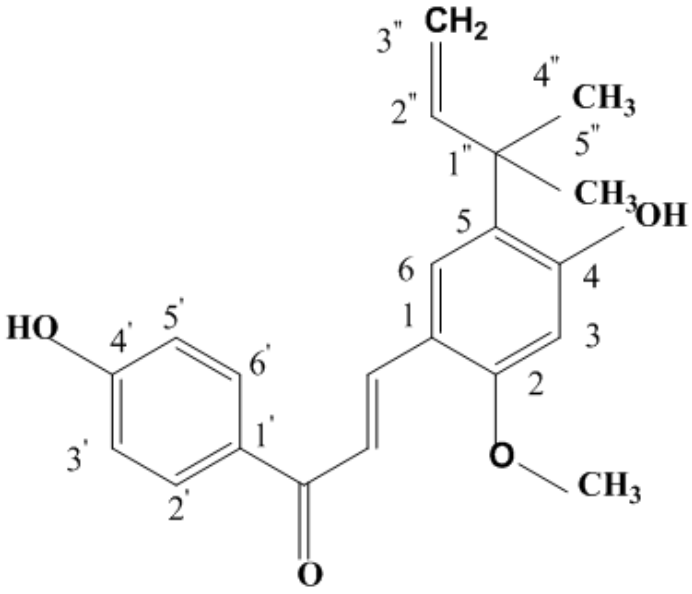

Figure 2. The structure of licochalcone A.

Separation, fractionation and purification of the active antimicrobial components from Licorice Leaves

Ethyl acetate extract from Licorice leaves (the most active extract against MRSA) undergone separation using thin layer chromatography (TLC) and column chromatography. Separation results in two active fractions. These fractions were identified by measuring physicochemical properties with the help of spectroscopic analysis as inflacoumarin A and Licochalcone A (Figures 1 and 2).

RESULTS

Antibiotic susceptibility of MRSA isolates

The highest resistance of 59 MRSA isolates appeared

Table 1. Antibacterial activity of alcoholic extracts of 19 medicinal plants against 59 MRSA isolates.

Plant		Number of MRSA giving diameter of inhibition zone: in mm			
		0-15	16-20	21-25	26-30
Fennel		59	0	0	0
Anise		39	20	0	0
Coriander	Fruit	31	27	1	0
	leaves	52	6	1	0
Parsley		59	0	0	0
Dill		51	8	0	0
Peppermint	Piperita	59	0	0	0
	Spicata	59	0	0	0
Rosmarinus		1	56	2	0
Marjoram		28	26	5	0
Lavenders		36	21	2	0
Thyme		42	17	0	0
Chamomile	Flower	31	28	0	0
	Herb	58	1	0	0
Lantana		50	9	0	0
Guava		0	54	5	0
Carob gum		2	55	2	0
Licorice	root	0	8	40	11
	leaves	0	3	44	12
Acacia		59	0	0	0
Lemon grass		54	5	0	0
Basil		57	2	0	0
Oregano		42	17	0	0

with Macroloids (erythromycin 74.5% and clarithromycin 67.7%) followed by levofloxacin (44%), vancomycin and amikacin (42.3%), tobramycin (38.9%), ofloxacin and doxacyclin (33.8%). As expected no susceptibility appeared with ampicillin, amoxicillin, oxacillin and all generation of cephalosporins (cefepim, cefoperazone, cefotaxime and cefprozilal). 23 isolates were MDR MRSA.

Antimicrobial activity of alcoholic extracts of different medicinal plants against 59 MRSA isolates

The antimicrobial activities of 70% ethanolic extracts obtained from 19 plants against 59 MRSA isolates are shown in Table 1. Licorice ethanolic extract showed the highest antimicrobial effect against all MRSA isolates (both leaves and roots); 40 isolates for roots and 44 for leaves extract showed inhibition zone ranging from 21-25 mm and 11 isolates for root and 12 for leaves showed higher inhibition zone against MRSA (26-30 mm). The lowest inhibitory activity was recorded for extracts of Fennel, Peppermint (Piperita and Spicata), and Parsley and Acacia plants. All ethanol extracts of these plants exhibited relatively lower activity against 59 MRSA isolates with zone of inhibition ranging from 10-15 mm.

Antimicrobial activity of extracts of licorice leaves and roots against MRSA isolates using different organic solvents

Each organic extract from roots and leaves of Egyptian licorice was tested against five randomly selected MDR MRSA isolates (after detection of *mecA* gene by PCR; Figure 3) and one sensitive *S. aureus* ATCC 29737strain (MSSA) by well diffusion assay. Results are presented in Table 2. Ethyl acetate and dichloromethane extracts showed the highest activity with inhibition zone of 23-35 mm. Ethyl acetate leaves extracts had higher antimicrobial activity in comparison with that of root extracts; it showed 31-33 mm inhibition zone against the five MRSA isolates whereas *n*-hexane leaves extract was found without activity against tested MRSA isolates (Table 3).

Cytotoxicity assay of licorice leaves extract on human cell lines

The survival fraction of each cell line was plotted against the different concentration (0, 10, 25, 50 and 100 µg/ml) of leaves extract. The concentration of leaves extract that reduced survival of carcinoma cell line of liver

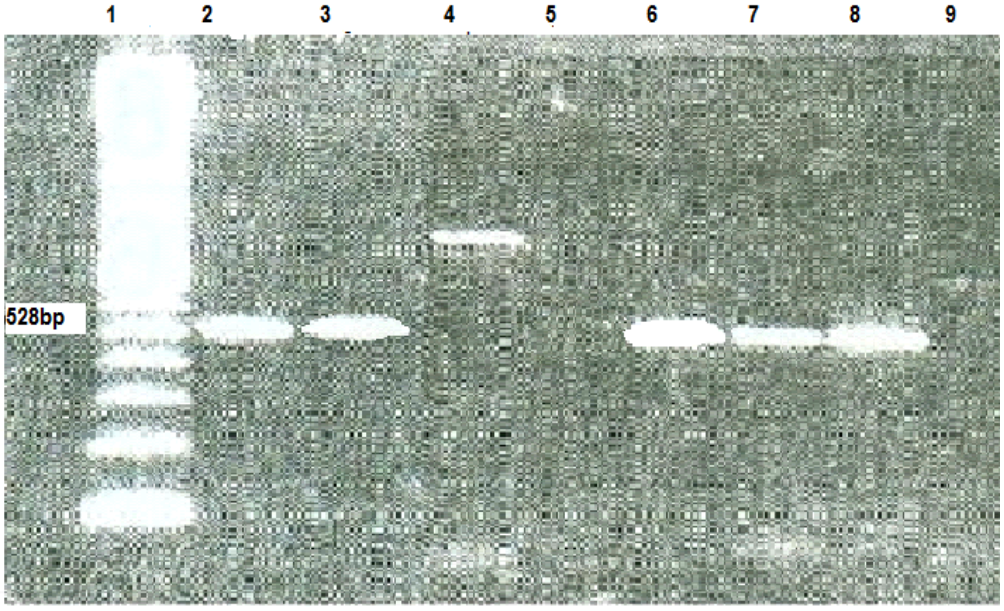

Figure 3. Agarose gel electrophoresis containing the amplification products of 5 MRSA isolates. Lane 1 marker. Lane 2, 3, 6, 7 and 8 represent *mecA* positive isolates, lane 5 negative control, lanes 4 and 9 represent *mecA* negative isolates.

(HEPG2) and colon carcinoma (HCT 116) to 50% were 19.5 and 15 µg/ml respectively. The highest inhibitory effect was that on liver carcinoma cell line (Figures 4 and 5).

DISCUSSION

Much attention has been directed recently towards plant extracts and biologically active compounds isolated from popular plant species. The use of medicinal plants play a vital role in covering the basic health needs and these plants may offer a new source of antibacterial, antifungal, antiviral and anticancer agents with significant activity against pathogenic microorganism (Upadhyay et al., 2010; Alpsoy, 2010; Shinwari and Qaisar, 2011).

S. aureus is the most problematic Gram positive bacteria in public health and health care associated infections not only because it is highly prevalent but also because it has become resistant to almost all available antibiotics except vancomycin and techplanin (Chambers, 2001). Recently susceptibility even to vancomycin has been decreased in several countries (Mukn et al., 2011). The emergence of these multidrug resistant bacteria increased the need for new antimicrobial agents especially from natural sources or modification of older ones. To achieve this goal, screening of plants used in popular medicine for its antimicrobial effect must be taken in considerations.

A total of 23 alcoholic extracts representing 19 Egyptian medicinal plants were submitted in the preliminary screening of this study. The antimicrobial efficacy against 59 clinical MRSA strains was assessed. 70% ethanolic extracts of all plants screened showed inhibitory effect against all tested MRSA and the diameter of inhibition zones varied between 10-30 mm. Among all plants tested licorice (*Glycyrrhiza. Glabra*) extracts (both roots and leaves) were found to be the most effective as they have the highest inhibition zone against all tested MRSA isolates ranging between 21 and 30 mm. Because the roots of this plant became very important for their beneficial effects, they have been reviewed previously (Shinwari et al., 2012); Sedighinia et al. (2012) tested the antimicrobial activity of licorice root extract and found that in agar dilution method minimum inhibitory concentration (MIC) for *Streptococcus mutans, Actinomyces viscosus* and *Enterococcus faecalis* were 12.5 mg/ml and for *Escherishia coli* and *Staphylococcus aureus* were 35 mg/ml. Also our results are consistent with previous study which indicated that concentration of licorice 16-126 µg/ml can inhibit growth and reduce staphylococcal enterotoxin as well as the expression of hemolysin (Qiu et al., 2010). Extracted compounds from licorice showed potent antibacterial activity specially to *S. aureus and Micrococcus luteus* in another study (Nowakowska, 2007).

A large number of MRSA were sensitive to Coriander fruits, Marjoram, Guava, Anise, Rosmarinus, Chamomile flowers, Carob gum and Lavenders plant extracts with high growth inhibition zone ranged from 16-25 mm. Similar to our findings, other researches reported that thyme and Romarinus extracts was active against MRSA (Nita et al., 2002; Abu-Shanab et al., 2004). Also Chao and his colleges reported that essential oils of myrtle,

Table 2. Antibacterial activity of Egyptian licorice roots extracts against 5 MRSA isolates and one sensitive strain.

Organic extract	Diameter of inhibition zone in mm											
	MRSA 7		MRSA 30		MRSA 48		MRSA 57		MRSA 59		Sensitive *S. aureus*	
	Root	Leave	root	leave	root	leave	root	leave	root	leave	root	leave
n-Hexane	16	0	14	0	14	0	16	0	16	16	17	0
Dichloromethane	23	28	23	27	23	26	23	28	24	23	25	30
Ethyl acetate	24	33	23	31	23	33	24	33	24	24	25	35
70% ethanol	21	21	20	19	21	20	21	21	21	21	23	22

Table 3. Comparison of MIC of licorice leaves ethyl acetate extract with Flucloxacillin.

MRSA isolates	MIC of Flucloxacillin (µg/ml)	MIC of licorice leaves ethyl acetate extract (µg/ml)
MRSA 7	64	8
MRSA 30	>128	8
MRSA 48	>128	8
MRSA 57	32	8
MRSA 59	64	8

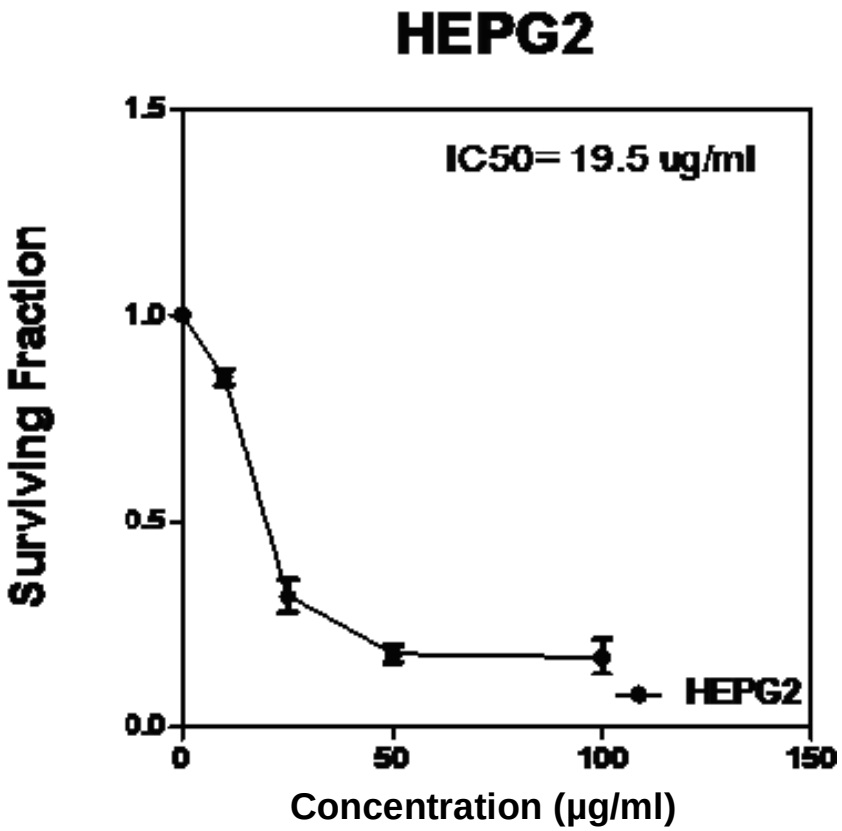

Figure 4. Cytotoxicity assay of licorice leaves extract on human cell line HEPG2 (liver carcinoma) with LC_{50} = 19.5 µg/ml.

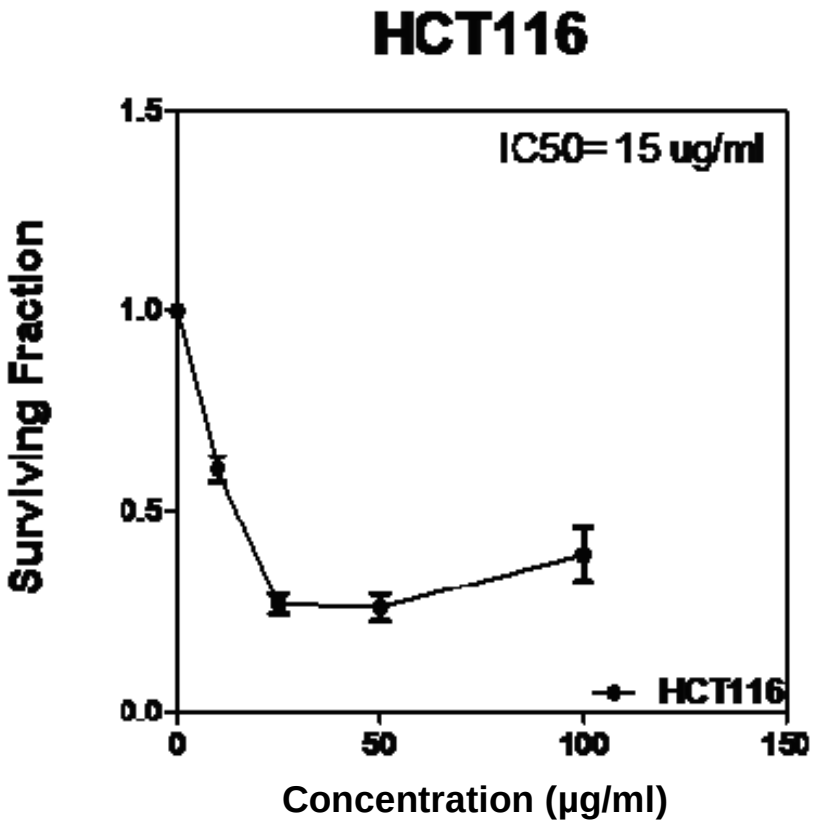

Figure 5. Cytotoxicity assay of licorice leaves extract on human cell line HCT116 (colon carcinoma) with 1 C_{50} 15 µg/ml.

Eucalyptus globulus, *Eucalyptus australiana*, *Eucalyptus radiata*, marjoram, pine, cypress, lavender, spruce, peppermint, *Eucalyptus*, *citriodora,* Roman chamomile, spruce, lavender, frankincense, clove, orange and thyme alone and in combination can inhibit MRSA (Chao et al., 2008).

The present results (Table 1) revealed that the licorice extracts exhibited the most potent antimicrobial activity against MRSA; therefore it was selected for performance of further steps. Powdered samples of leaves and roots of licorice were extracted with different solvents using successive method. The antimicrobial activity of the extracts was tested against five randomly selected MDR MRSA after detection of *mecA* gene by PCR as well as one sensitive strain. Results indicated that dichloromethame, ethyl acetate and ethanolic extracts of roots and leaves showed inhibitory activity against all tested MRSA isolates as well as the sensitive strain. This similarity of sensitivities between methicillin sensitive and resistant strains may indicate that resistance to licorice extracts has not been developed yet which make it a new candidate for antimicrobial therapy specially for MDR MRSA. The ethyl acetate was the most favorable solvent for extracting the active constituents. This is can be explained by the fact that the composition of extracts depends on the polarity of the extracting solvent (Velickovic et al., 2007). Earlier reports indicated that the effectiveness of the extraction by methanol yielded higher antimicrobial activity than *n*-hexane and ethyl acetate (Febles et al., 1995). In agreement with the present results, Gangadevi and his colleges reported that ethyl acetate extracts of leaves and roots of *Acalypha indica* recorded higher activity than the methanol and hexane extracts (Gangadevi et al., 2008).

The result which surprised us was that ethyl acetate and dichloromethane leaves extracts showed higher inhibition zones against tested MRSA in comparison with that of roots extracts, as to our knowledge all previous researches of antimicrobial effect of licorice focused on roots only and this may be the first report on antimicrobial activity of licorice leaves extract. Therefore, the MIC and the cytotoxicity assay against liver and colon tumor cell lines of leaves extracts of licorice were determined. Although the MIC of flucloxacillin of tested MRSA strains ranging from 32 µg/ml to more than 128 µg/ml, the ethyl acetate leaves extract was highly active. Separation of components of licorice leaves resulted in two active fractions identified as inflacoumarin A and Licochalcone A.

Our results showed that MIC of the leaves extract was found to be 8 µg/ml which is in agreement with other researches that found two phenolic compounds isolated

from licorice root showed remarkable antibacterial effects as the MICs were 8 ug/ml for MRSA strains as well as methicillin-sensitive *S. aureus* (Hatano et al., 2000). Also the antimicrobial effects of licoricidin isolated from Licorice which found to be directly effective against MRSA infections, when coupled with oxacillin, licoricidin reduced the MIC of oxacillin required to effectively combat MRSA infections. A licoricidin concentration of 4 ug/mL reduced the MIC of oxacillin from 128-256 ug/ml to 8-16 ug/ml and a licoricidin concentration of 8 ug/mL reduced the MIC of oxacillin to less than 0.5 ug/ml (Hatano et al., 2005).

Some previous studies reported that medicinal plants could be a source for anticancer agents, so we investigate the anticancer effect of licorice leaves. Our results reveal the ability of crude ethyl acetate extract to inhibit carcinoma cells of liver ($HEPG^2$) and colon (HCT 116) *in-vitro*. The concentrations of licorice extract that reduced survival of carcinoma cell line of liver and colon carcinoma to 50% were 19.5 and 15 µg/ml respectively. Although these results are for the effect of licorice leaves, but still in-agreement with other reports of licorice root extracts, chalcone components separated from licorice root, has antiproliferative and anti-inflammatory properties in human and murine cell lines, they increase efficacy and improve patient outcomes in colorectal and prostate cancers and also has potent antimetastatic activity (Yo et al., 2009; Kim et al., 2010), they can induce cell cycle arrest and up-regulate p21 expression in a lung cancer cell line (Ii et al., 2004), suppress pulmonary metastasis of mouse renal cell carcinoma through activation of the immune system (Yamazaki et al., 2002), and induce apoptosis in human gastric cancer cells (Ma et al., 2001).

It is important to notice that the cytotoxicity effect of licorice leaves extract was achieved at higher concentrations (19.5 ug/ml for liver cell line and 15 µg/ml for colon cell line) than that needed for inhibition of MRSA strains (8 ug/ml) which may reflect its safety when used in low dose as antimicrobial agent. Further researches are needed to confirm this point.

In conclusion, our results showed that the Egyptian licorice leaves extracts represent a new candidate for antimicrobial agent against MDR-MRSA and also has an *in vitro* antitumor activity against different human cell lines when used at high concentrations. We believe that these findings will be helpful to many researchers in the field of the evolution of antibacterial and anticancer activities in plant.

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