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Antibacterial, Antifungal and Toxicity of Rare Iranian Plants

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Abstract: As a part of our drug discovery program, an effort to introduce new effective medicinal plants, with antibacterial, antifungal and cytotoxic properties was made. The extracts of aerial part of 8 plants, collected in southeastern Iran, were investigated against standard strains of *Bacillus subtilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Salmonella entrica* subsp. *entrica* ser. Typhi, *Escherichia coli*, meticillin resistant *Staphylococcus aureus* (isolated from patients), *Fusarium oxyosporum*, *Aspergilus niger,* and *Aspergilus fumigatus*. We used brine shrimp (*Artemia salina*) cytotoxicity bioassay in order to provide a better base for introducing the extracts as the new therapeutic candidates. *Capparis deciduas* (Forsk.) Edjw. and *Cleome oxypetala* Boiss. (both from Capparidaceae family) were recognized as having the potential for development of new antibiotics. On the other hand, *Cistanche tubulosa* (Schrenk) R.Wight, could be worthy of attention for finding anticancer phytochemicals.

Key words: Antibacterial, antifungal, *Capparis deciduas*, *Cleome oxypetala*, plant, toxicity

INTRODUCTION

The emergence of pathogens resistant to antibiotics as a result of excessive use of them in clinical and veterinary applications, represent a serious problem for public health. Despite the existence of efficient antibiotics, drug resistant or multi-drug resistant strains are steadily appearing and require longer and more expensive treatments (Alanis, 2005). These facts play a driving force for scientists to explore new anti-infective agents for developing new drugs. Among several options, plants are the most known source of healing compounds. Plants have limitless ability to synthesize substances, many of them with anti-infective activity. Nowadays, scientists all over the world are engaged to explore bioactive properties of plants with the hope of adding new therapeutic agents (Gurib-Fakim, 2006). Iran, in addition to having a very honorable tradition of plant-healing remedies (Gorji and Khaleghi Ghaderi, 2002), is one of the richest countries in terms of its plant biodiversity. In an attempt to introduce plants with bioactive compounds, we selected and gathered several plants for their desired bioactivity and collected them. After identification, the plant extracts were evaluated through various bioassays to assess the anti-microbial potency as well as their cytotoxic property. Such study is part of our expanded bioactive lead discovery program from natural products and their introduction for potential anti-infective or anticancer agents.

The potential and rational for collections of plants stemmed from their potential of previous usage and novelty in the report of investigated bioactivity. Capparidaceae is a vast family with several genera. Many of plants which have been used in traditional medicine belong to this family particularly to *Cappris* and *Cleome* genera (Jeruto *et al*., 2008; González-Tejero *et al*., 2008). *Capparis deciduas* is a well known plant in Saudi Arabia with a broad range of medical usage (Rahman *et al*., 2004). In addition to *C. deciduas* we have investigated *C. oxypetala* from Capparidaceae. *Cleome* genus includes many species with vast applications in herbal remedies. *Cleome gynandra* has been used against malaria as well as stomach congestion in Kenya (Jeruto *et al*., 2008). *Cleome arabica* is mentioned as a plant with versatile medical uses in Saudi Arabia (Rahman *et al*., 2004). There are many other plants of *Cleome* genus with documented traditional medical application. *Tamarix dioica* from Tamaricaceae family was among the collected plants. Actually, *T. dioica* is known as an important medicinal

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plant in India and has been investigated due to richness of bioactive phytochemicals (Pamar *et al*., 1994). Other species from *Tamarix* genus have reputable therapeutic applications too. For example *Tamarix aphylla* and *Tamarix gallica* are used to alleviate several physiological distresses and infectious diseases in the Central Sahara region in Africa (Hammiche and Maiza, 2006). Plants from genus *Taverniera* have been studied for search of valuable phytochemicals particularly saponins. Many pharmacological activities have been reported about saponins such as antibiotic, antifungal and antiviral activities. *Taverniera aegyptica* is indicated to have various kinds of this bioactive phytochemical (Ibraheim *et al*., 2003). *Taverniera cuneifolia* (Roth) Arn. exhibited promising anti-inflammatory, anti-tumor, anti germ tube formation (Zore *et al*., 2008). In this context, *T. glabra* was investigated for assessment of antimicrobial activity in this study. *Rumex* genus belongs to Polygonaceae family and several species of this genus has indicated noteworthy therapeutic potentials. *Rumex cyprius*, the plant from this genus which we assessed its antimicrobial activity has shown antiviral activity against HIV through inhibition of reverse transcriptase (Vermani and Garg, 2002). *Blepharis edulis* is an Indian medicinal plant which is source of several bioactive phytochemicals such as blepharin (Pratt *et al*., 1995). Extracts of *Blepharis ciliaris* has indicated significant antimicrobial activity and is used in folk medicine of Saudi Arabia (Harraz *et al*., 1996). *Blepharis panduriformis* Linda, another member of blepharis genus is applied in form of decoction for dysentery treatment in Tanzanian traditional medicine (Maregesi *et al*., 2007). *Convolvulus sericeus* is collected and assessed for anti infective activity. Some members of *Convolvulus* genus are among traditional medicinal plants of some cultures. For example, *Convolvulus arvensis* is known as carminative, antiseptic and stimulant in Egypt (Atta and Mouneir, 2004). The Orobanchaceae parasitic plant, *Cistanche tubulosa* (Schrenk) R. Wight, is widely distributed in North Africa, Arabia and Asian countries and has been traditionally used as a promoting agent of blood circulation and treatment of impotence, sterility, lumbago, body weakness and tonic (Yoshikawa *et al*., 2006). Another member of *Cistanche* genus is *Cistanche deserticola* which is a Chinese traditional herb. The dried whole plant is used for the treatment of kidney pain, gynecological diseases, intestinal infection and constipation (Dong *et al*., 2007).

MATERIALS AND METHODS

Plant material: Plant materials were collected in March 2005, from Chabahar, in Sistan and Baluchestan, the southeastern province of Iran (Table 1). The identification was carried out by our team members and confirmed by Dr. S. Sardari. The voucher specimens were deposited in our herbarium. After identification, aerial parts of each specimen were dried in ambient temperature for 4 days and mill ground to a fine powder using an electric grinder.

Preparation of the plant extracts: About 10 g of each plant sample was extracted three times with 100 mL of 80% ethanol in water each time. The plant extracts were filtered through Whatman No. 1 filter paper. The filtrates were concentrated to total dryness in a rotary evaporator at 40°C until a constant dry weight for each extract was obtained, all in dark brown color. The residues were stored at 4°C. The extraction yield was between 25-32%. For *Capparis deciduas* the alcohol extract was further fractionated by dichloromethane. After concentration, the extract was dissolved in ethanol and the remaining undissolved extract was further dispersed in dichloromethane.

Antibacterial and antifungal screening: *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* NCTC 8213, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella entrica* subsp. *entrica* ser. Typhi NCTC 5761, *Escherichia coli* ATCC 8739, methicillin resistant *Staphylococcus aureus* (isolated from patients), *Aspergillus niger* N 402, *Aspergillus fumigatus* PTCC 5009 and *Fusarium oxyosporum* CBS 620.87 were used as test strains. The plant extracts were dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 10 mg mL^{-1}. The broth microdilution method was performed for antibacterial and antifungal activity tests.

Table 1: Scientific names of collected plants, their family and their abbreviations used in this study

Plant species	Family	Abbreviation	Voucher No.
Capparis deciduas (Forsk.) Edjw.	Capparidaceae	CD	CH-6
Taverniera glabra Boiss.	Papilionaceae	TG	CH-17
Convolvulus sericeus Burm.	Convolvolaceae	CS	CH-19
Rumex cyprius Murb.	Polygonaceae	RC	CH-20
Tamarix dioica Roth.	Tamaricaceae	TD	CH-26
Cistanche tubulosa (Schrenk) R.Wight.	Orobanchaceae	CT	CH-28
Blepharis edulis (Forssk.) Pers.	Acanthaceae	BE	CH-29
Cleome oxypetala Boiss.	Capparidaceae	CO	CH-35

The absorbance was read at 530 nm for fungi and 600 nm for bacteria inoculums to reach the suitable density of pathogen or test microorganisms. From the prepared stock fungal culture, a 1:1000 dilution with broth (e.g., 10 μL stock fungal culture: 10 mL broth) was prepared, which is called working fungal culture. Modified antimicrobial susceptibility testing based on NCCLS M27-A method was performed. From the prepared stock culture, a 1:1000 dilution with broth medium (10 μL stock culture: 10 mL broth) was prepared, which is called working microorganism culture. Mueller-Hinton broth was used for the screening of antimicrobial activity against the bacterial test strains. Tween 20 (0.1% w/v) was added to facilitate the preparation of fungal spores suspensions of the fungi (Cuenca-Estrella *et al*., 2002; Gomez-Lopez *et al*., 2005). Mueller-Hinton broth and Sabourod Dextrose broth were used for the screening of antimicrobial and antifungal activities, respectively. Broth medium (100 μL) was added to each well of a 96-well microplate and then 40 μL of plant extract and 60 μL broth were added to well (A), then a solution (100 μL) serially diluted from well (A) by taking 100 μL into (B) was obtained. This two-fold dilution was continued down the plate and 100 μL from the last well (H) was discarded. Then all the wells were filled with 100 μL of working microorganism suspension. Fluconazole was used as a reference in fungal test whereas Penicillin, Kanamycin and Ciprofloxacin were references for bacterial tests.

For this experiment the following controls were prepared: no inoculum was added to a number of wells were used for control of sterility; control of inoculum viability, no plant extract was added to wells containing inoculum and nutrient medium and control of the DMSO inhibitory effect, DMSO was added to some wells containing inoculum and nutrient medium. The plates were covered and incubated at 37°C for 24 h. The MIC values were obtained by reading the concentration of the well with no growth.

Toxicity screening: Extracts of all plants were screened for toxicity with larvae (nauplii) of *Artemia salina* (brine shrimp). In order to improve the exposure of nauplii with the extract; we made some changes to conventional method of McLaughin *et al.* (1998). The test was performed in triplicate in vials with extract concentrations of 10, 100 and 1000 μg mL^{-1}. The eggs were placed in an aerated bottle, using electric pump with a flow of 3-4 L min^{-1}, containing 33 g L^{-1} NaCl saline and natural lighting. After two days of hatching period at room temperature, the nauplii were ready for the experiment. To obtain a concentration of 5 mg mL^{-1}, relative mass of each extract was accurately measured and dissolved in appropriate volume of solvent (acetone or ethanol). From the stock solutions 10, 100 and 1000 μL were placed in vials (three vials for each concentration). A mixture of 33 g L^{-1} saline and polyethylene glycol (PEG) 6000 (3% w/v) was prepared to have a better dispersion of the plant extract. After evaporation of solvent, the above mixture was added to vials making the volume up to 5 mL to make the final concentrations of 10, 100 and 1000 μg mL^{-1}. The mixture of saline and PEG 6000 was used as control solution. Ten shrimps were added to each vial. For better aeration, the vials were put horizontally on a shaker to be moved slowly. After 24 h, the survivors were counted under microscope and recorded. The data were analyzed by SPSS version 10 for probit analysis to determine LC_{50} values.

RESULTS AND DISCUSSION

The hydro-alcoholic extract of ten Iranian plants were screened for their antimicrobial and toxicity properties. The effect of extracts was compared to control agents. At the same time, the solvent of the extracts was tested to identify the level of inhibition that is attributable to the solvent and the results are shown in Table 2 and 3. There was no inhibition by the DMSO solvent for microorganisms tested. Between the plants extracts tested, *C. tubulosa*, *R. cyprius* and *C. deciduas* (extracted by CH_2Cl_2) had maximum toxicity against *A. salina* (Table 4). The extracts of these plants may not be used as antimicrobial agents for further therapeutic research in spite of their good antimicrobial activities, unless we indicate that the toxicity is originated from different materials present in the extract than the antimicrobials. However, the extracts of such plants are potential cases for finding anticancer phytochemicals. *T. glabra*, *T. dioica*, *B. edulis* and *C. oxypetala* and ethanolic phase of *C. deciduas* extracts had milder toxicity. It is useful to remember that caffeine showed LC_{50} 300 μg mL^{-1} against *A. salina* (Meyer *et al*., 1982). However, *B. edulis*, *T. dioica* and *C. sericeus* do not show acceptable antimicrobial activity. *Cleome oxypetala* and ethanolic phase of *C. deciduas* extract had potent antibacterial activity against methicillin resistant *S. aureus* (11 times more than penicillin and kanamycin and 47 times more than ciprofloxacin). It should be remembered that methicillin resistant *Staphylococcus aureus* (MRSA) has shown relatively high resistance when exposed to reference antibiotics. These plant extracts can be useful for further researches.

Compared to fluconazole, *C. oxypetala* is more effective against *F. oxyosporum* and *A. niger*. Between the two *Aspergillus* species tested, *A. niger* N 402 showed more susceptibility to plant extracts than,

Table 2: Antimicrobial activity of extracts expressed as Minimum Inhibitory Concentration (MIC)

	Extract/MIC (μg mL^{-1})											
Microorganism	CD[1]	CD[2]	TG	CS	RC	TD	CT	BE	CO	P	K	C
SA	666.7	1333.3	666.7	1333.3	333.3	1333.3	1333.3	1333.3	666.7	55.6	55.6	13.9
BS	166.7	666.7	166.7	666.7	333.3	333.3	1333.3	333.3	166.7	<1.7	<1.7	<0.9
EF	>1333.3	1333.3	1333.3	666.7	1333.3	1333.3	>1333.3	666.7	>1333.3	13.9	27.8	3.5
PA	1333.3	1333.3	1333.3	1333.3	>1333.3	1333.3	>1333.3	1333.3	1333.3	55.5	6.9	1.7
SE	>1333.3	>1333.3	1333.3	1333.3	>1333.3	>1333.3	1333.3	1333.3	1333.3	55.5	6.9	3.5
EC	1333.3	1333.3	>1333.3	>1333.3	>1333.3	>1333.3	>1333.3	1333.3	>1333.3	13.9	27.8	<0.9

CD[1]: *Capparis deciduas* ethanolic phase, CD[2]: *Capparis deciduas* dichloromethane phase, SA: *Staphylococcus aureus*, BS: *Bacillus subtilis*, EF: *Enterococcus faecalis*, PA: *Pseudomonas aeruginosa*, SE: *Salmonella entrica* subsp. *entrica* ser. Typhi, EC: *Escherichia coli*, P: Penicillin, K: Kanamycin, C: Ciprofloxacin

Table 3: Antifungal activity of extracts expressed as Minimum Inhibitory Concentration (MIC)

	Extract/MIC (μg mL^{-1})									
Microorganism	CD[1]	CD[2]	TG	CS	RC	TD	CT	BE	CO	F
AN	1000	500	416.7	833.3	333.3	500	666.7	583.3	416.7	>1000
AF	833.3	1000	1000	833.3	1000	250	666.7	1000	1000	>1000
FO	125.0	125	500	125	500	500	250	125	250	1000

AN: *Aspergillus niger*, AF: *Aspergillus fumigatus*, FO: *Fusarium oxyosporum*, F: Fluconazole

Table 4: The results of cytotoxic effect of plant extracts on *A. salina*

		Confidence limit (ppm)	
Plant	LC_{50}	Min.	Max.
CD[1]	955.72	587.150	2674.60
CD[2]	223.13	56.406	691.10
TG	471.39	283.100	781.17
CS	768.64	485.570	1477.43
RC	203.58	101.030	343.90
TD	500.13	300.950	841.29
CT	62.95	49.260	79.05
BE	942.99	759.080	1240.10
CO	974.65	642.890	2078.48

A. fumigatus PTCC 5009. *R. cyprius* and *T. dioica* represent the least MIC among the extracts against *A. niger* N 402 and *A. fumigatus* PTCC 5009, respectively. Due to *A. fumigatus* PTCC 5009 strikethrough susceptibility to *T. dioica*, it could be an option for more studies. Though all the extracts showed considerable inhibitory effect against *F. oxyosporum* CBS 620.87, *C. deciduas*, *C. sericeus* and *B. edulis* act in 8 times less concentration in comparison with the reference antibiotic fluconazole. The extracts show weak effects against bacteria as compared to conventional antibiotics, but we should not neglect the least MICs that guide us to know extracts with potential ingredients against bacterial pathogens. In a general view, *B. subtilis* is the most susceptible bacterial organism among the others. *C. deciduas* (the ethanolic phase), *T. glabra* and *C. oxypetala* show the strongest effect against *B. subtilis*. Another considerable result is the effect of *R. cyprius* extract against meticillin resistant *Staphylococcus aureus*, which has shown relatively high resistance when exposed to reference antibiotics. Among the extracts, the ethanolic phase of *C. deciduas*, with the least MICs against two pathogens, *F. oxyosporum* and *B. subtilis* and relatively, low toxicity in comparison with other extracts is considered as a promising material that merit more detailed studies. In the same manner, *C. oxypetala* extract with low MIC against the two above mentioned microorganisms and the least cytotoxicity among the extracts, could be another option for antibiotic development. *C. tubulosa* extract, has the highest cytoxicity among the others, a trait which introduce *C. tubulosa* as a potential case for finding anticancer phytochemicals. We intend to continue the research by further study on the promising plants towards the active ingredients and the bioactivity profile.

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