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Full Length Research Paper

Anti-bacterial and anti-fungal investigation of *Astragalus atropilosulus* subsp. *abyssinicus*

Sulaiman A. Alrumman[1], Mahmoud F. M. Moustafa[1,2] and Saad A. Alamri[1]*

[1]Department of Biology, College of Science, King Khalid University, Abha, Saudi Arabia.
[2]Department of Botany, Faculty of Science, South Valley University, Qena, Egypt.

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Antimicrobial properties of *Astragalus atropilosulus* subsp. *abyssinicus* leaf extract were carried out against some pathogenic bacteria and fungi by detecting the zone of inhibition using agar well diffusion method. Fresh *A. atropilosulus* subsp. *abyssinicus* leaf extracts were prepared using various solvents (hot and cold water, acetone, ethanol, methanol, 1/1 ethanol/methanol, 1/1 ethanol/acetone and 1/1 acetone/ methanol). Except the cold water extract, all extracts demonstrated broad-spectrum activity against all bacteria tested with inhibition zones in the range of 9.33-35.0 mm. The minimal inhibitory concentration (MIC) values of different plant extracts against the tested bacteria were found to range from 12.50-17.5 mg/ml. Except the cold water extract, all extracts exhibited antifungal activity against *Candida* sp., *Drechslera halodes, Fusarium oxysporum* and *Pythium ultimum* with inhibition zone within the range of 6.56-20.3 mm. This study shows that leaf extracts of *A. atropilosulus* subsp. *abyssinicus* could be source of compounds, which can be used to combat pathogenic microorganisms.

Key words: *Astragalus atropilosulus* subsp. *abyssinicus*, antibacterial, antifungal, Infrared spectra.

INTRODUCTION

Astragalus atropilosulus (Hochst.) Bunge subsp. *abyssinicus* (Hochst.) J. B. Gillett (Fabaceae - Leguminosae) is an annual herb, stems firm, erect, 3–4 feet or little high, sparingly branched, the branches firm, glabrous, terete. Stipules cordate acuminate, unequal-sided and free to the base. Leaves nearly have no pedicel, leaflets in 10–15 pairs, linear-oblong, glabrous. Flowers in dense racemes on short erect glabrous peduncles (for further details, see http://plants.jstor.org/flora/flota001971; Gillett, 1964). It commonly occurs in waste places, borders of cultivated fields and roadsides in Abha governorate, Kingdom of Saudi Arabia (Collenette, 1985). In recent years, researchers have shown an increased interest in exploring extracts and natural compounds from plants to replace existing synthetic drugs (Alamri and Moustafa, 2010, 2012; Wink et al., 2005). The reason for this, is the belief that medicinal plants are considered a significant source of unknown chemical compounds with potential healing effects. Moreover, many countries use naturally-occurring substances from plants as pharmaceutical preparations in extracted or pure forms. Recently, many studies have described substances present in medicinal plants that are used to cure human illnesses, substances that have therapeutic value (Nostro et al., 2000).

According to previous studies, the antimicrobial activity of plants extracts could be attributed to a number of different chemical components including aldehyde and phenolic compounds and many of the plants' metabolites have activities against human pathogens (Ao et al., 2008; Rios and Recio, 2005). These natural compounds in many cases have a synergistic effect making the crude plant extracts activities higher than the pure individual compounds (Bandyopadhyay et al., 2005). For example, extracts obtained from *Quercus ilex*, displayed activity against several bacterial strains (Berahou et al., 2007). A similar example is *Cordia curassavica,* a traditional medicinal plant that showed antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Vibrio cholerae, Yersinia enterocolitica*

*Corresponding author. E-mail: amri555@yahoo.com.

and *Escherichia coli* (Hernández et al., 2007), therefore, it has been claimed that plants are one of the bedrocks for modern medicine to attain a new principle (Evans et al., 2002).

In view of these, *Astragalus* species represent very old and well known drugs in folk medicine for its usage as an antiperspirant, tonic and diuretic and has been adopted in the medication of diabetes mellitus, nephritis, leukemia and uterine cancer (Tang and Eisenbrand, 2009). However, to date, there are no attempts to study the chemical composition and antimicrobial potential of extract from *A. atropilosulus* subsp. *abyssinicus* against pathogenic bacteria and fungi. Therefore, the present study was undertaken to investigate the antimicrobial activity of a Saudi Arabian's weed, *A. atropilosulus* subsp. *abyssinicus* extract and the data are presented here.

MATERIALS AND METHODS

Plant material

Healthy, mature leaves of *A. atropilosulus* subsp. *abyssinicus* were collected locally from roadside vegetation of Abha governorate, Aseer region (KSA) and were used for preparation of different extracts. A voucher specimen of the plant has been deposited in the herbarium of Biology Department, Faculty of Science, King Khalid University, Saudi Arabia.

Preparation of extract

Around 290 g of thoroughly washed mature leaves of *A. atropilosulus* subsp. *abyssinicus* were ground in a grinding machine (Thomas Wiley laboratory mill, model # 4, screen size-1 mm) for 15 min and filtered through two-layered muslin cloth. The filtrates were divided into nine equal aliquots, each containing twenty four grams and extractions were done by the addition of seventy milliliters of solutions of either acetone or ethanol, methanol, 1/1 ethanol/methanol, 1/1 ethanol/acetone and 1/1 acetone/ methanol and water (hot and cold).

Each sample was placed in a rotary shaker at 37°C for 7 2 h, and then solvent was removed in each sample by air-drying at 30°C. Each extract was weighed and stored at 4°C for furth er use (Akueshi et al., 2002).

Maintenance and growth of microorganism

Bacterial cultures of *Pseudomonas aeruginosa, Staphylococcus aureus, E. coli, Acinetobacter* sp., *Proteus* sp., *Klebsiella pneumoniae, Micrococcus* sp., *S. epidermidis* and *B. subtilis* were maintained on nutrient agar medium. Fungal cultures of *Alternaria alternatra*, *Candida* sp., *D. halodes*, *Fusarium oxysporum*, *Macrophomina phaseolina, Pythium ultimum* and *Rhizoctonia solani* were maintained on Potato dextrose agar (PDA) medium. All cultures were obtained from the Biology Department, Faculty of Science, King Khalid University, Saudi Arabia.

Inoculum preparation

Various bacterial strains include Gram-positive (*B. subtilis, S. aureus, S. epidermidis* and *Micrococcus* sp.) and Gram-negative bacteria (*E. coli, P. aeruginosa, Acinetobacter* sp., *Proteus* sp. and *K. pneumoniae*) were pre-cultured in nutrient broth (NB) for 12 h in a rotary shaker at 37°C, centrifuged at 10,000 rpm for 5 min, the pellet was resuspended in distilled water, and the density of the cells were standardized to OD 600 nm spectrophotometrically. The fungal inoculums (*A. alternatra*, *Candida* sp., *D. halodes*, *F. oxysporum*, *M. phaseolina, P. ultimum* and *R. solani*) were prepared from seven-day old colonies grown on Potato dextrose agar (PDA) medium.

Antimicrobial activity

Different leaf extracts of *A. atropilosulus* subsp. *abyssinicus* were tested by agar-well diffusion method against four standard Gram-positive bacteria: *B. subtilis, S. aureus, S. epidermidis* and *Micrococcus* sp. and five standard Gram-negative bacteria: *E. coli, P. aeruginosa, Acinetobacter* sp., *Proteus* sp. and *K. pneumonia* and seven fungi: *A. alternatra*, *Candida* sp., *D. halodes*, *F. oxysporum*, *M. phaseolina, P. ultimum* and *R. solani* (Okeke et al., 2001). Bacterial strains cultured for 24 h were seeded into Mueller Hinton Agar Medium (MHA) by the spread plate method and potato dextrose agar plates were inoculated with 10 old days' fungal cultures by point inoculation (Mahesh and Satish, 2008), then, three cup-shape wells (5 mm diameter) were made in each plate using a sterile cork-borer. The inoculum densities were approximately 5×10^5 cfu/ml and $0.5\text{-}2.5\times10^3$ cfu/ml for bacteria and fungi, respectively, which they swabbed uniformly over the agar surface of each plate (Didem et al., 2007). The dried extracts were dissolved in sterile dimethyl sulfoxide (DMSO) to a final concentration of 100 mg/ml. 100 µl volume of each extract was poured in triplicate wells made in inoculated plates. The plates were held at room temperature (24°C) for one hour to allow diffusion of the extract into the agar. Gentamicin (10 µg/disc) and Fluconazole (30 µg/disc) were used as positive reference for bacteria and fungi, respectively, and the same solvent employed to dissolve the extracts (DMSO) (100 µl) used as negative control (Lalitha et al., 2011). All antimicrobial assay plates were kept at 37°C for 24 h for bacteria and 48 h for fungi under microaerofilic conditions. After incubation, the antimicrobial activity was assessed based on the size of the inhibition zone diameter (mm) and minimum inhibition concentration (MIC) of each extract was noted as the lowest concentration of the extract, which inhibits any visual microbial growth (Aneja et al., 2009a, b). All tests were performed in triplicates and the mean values of the diameter of clear zones with ± standard deviation were estimated.

RESULTS

Antibacterial activity

The results of antimicrobial activity of acetone, ethanol, methanol, 1/1 ethanol/methanol, 1/1 ethanol/acetone and 1/1 acetone/methanol and water (hot and cold) extracts of leaves of *A. atropilosulus* subsp. *abyssinicus* by agar well diffusion method revealed that all solvent extracts except cold water contained compounds with inhibitory effects against all bacterial strains tested. Tables 1 and 2, show that the largest mean zone of inhibition was produced by the mixture of (1/1 ethanol/acetone) extract with inhibition zone of (35.0) mm and a MIC of 13 mg/ml followed by ethanolic extract (27.7) mm and a MIC of 13 mg/ml, 1/1 acetone/ methanol (27.6 mm) and a MIC of 12.5 mg/ml, acetone extract(23.6 mm) and a MIC 13

Table 1. Antibacterial activity of different extracts of *Astragalus atropilosulus* subsp. *abyssinicus* against some human pathogenic bacteria at 10 mg of extract. (Zone of inhibition measured in mm).

Zone of inhibition (mm)										
Test bacteria	**Acetone**	**Ethanol**	**Methanol**	**1/1 ethanol/methanol**	**1/1 ethanol/ acetone**	**1/1 acetone/ methanol**	**Water**		**DMSO**	**Gentamicin (10 µg/disc)**
							Cold	**Hot**		
Pseudomonas aeruginosa	15.3±0.51	13.9±0.25	12.73±0.4	14.6±0.31	11.2±0.41	13.9±0.76	N I	10.9±0.10	NI	31.33 ±0.67
Staphylococcus aureus	10.13±0.2	14.8±0.30	14.9±0.68	16.6±0.47	14.8±0.87	14.7±0.54	N I	9.45±0.45	NI	29.33 ±0.32
Escherichia coli	10.9±0.51	12.7±0.49	14.7±1.18	16.7±0.41	14.7±0.95	12.8±0.97	N I	10.1±0.27	NI	32.33 ±0.53
Proteus sp.	11.1±0.45	10.8±0.30	12.8±0.45	10.9±0.18	11.1±0.49	14.7±0.58	N I	10.7±0.32	NI	23.77±0.93
Klebsiella pneumoniae	9.80±0.89	10.7±0.41	11.8±0.25	14.7±0.63	14.8±0.90	14.8±0.70	N I	9.33±0.22	NI	36.33±0.79
Micrococcus sp.	23.6±2.25	27.7±3.61	20.6±0.89	19.5±0.77	35.0±1.78	27.6±2.25	N I	19.3±0.54	NI	31.67±0.47
Staphylococcus epidermidis	17.0±0.89	19.6±1.37	14.8±0.45	15.8±0.83	14.7±0.65	14.7±0.73	NI	13.8±0.17	NI	31.77±0.86

NI (no zone of inhibition); DMSO, dimethyl sulfoxide. Data are means (n = 3) ± standard deviation of three replicates.

Table 2. Minimum inhibitory concentration (MIC) of *Astragalus atropilosulus* subsp. *abyssinicus.*

Source	**Extract**	**Lowest concentration of extract that is effective against a bacterial strains (mg/ml)**						
		P. aeruginosa	***S. aureus***	***E. coli***	***Proteus* sp.**	***K. pneumoniae***	***M. luteus***	***S. epidermidis***
Astragalus atropilosulus subsp. *abyssinicus*	Acetone	13.00	13.00	13.00	13.00	13.00	13.00	13.00
	Ethanol	13.00	13.00	13.00	13.00	13.00	13.00	13.00
	Methanol	13.50	12.50	12.50	12.50	12.50	12.50	12.50
	1/1 ethanol/methanol	14.50	14.50	14.50	14.50	14.50	14.50	14.50
	1/1 ethanol/acetone	13.00	13.00	13.00	13.00	13.00	13.00	13.00
	1/1 acetone/ methanol	12.5	12.5	12.5	12.5	12.5	12.5	12.5
	Aqueous (hot)	17.5	17.5	17.5	17.5	17.5	17.5	17.5

mg/ml; methanol extract (20.6 mm) and a MIC of 13.5 mg/ml; methanol extract (20.6 mm) and a MIC of 13.5 mg/ml, 1/1 ethanol/methanol mix extract (19.5 mm) and a MIC of 14.5 mg/ml and the hot water extract (19.3 mm) and a MIC of 17.5 mg/ml all against *Micrococcus* sp. The results presented in Table 1, indicates that the selected plant extracts have a variable inhibitory effect against all bacterial strains tested. In 1/1 ethanol/acetone mix extracted; the inhibition values were in the range of (35.0 to 11.2 mm) and showed significant antibacterial activity against *Micrococcus* sp., and moderate activity against *S. aureus, E. coli, K. pneumonia* and *S. epidermidis.* Ethanol extract showed highest activity against *Micrococcus* sp. and *S. epidermidis* and moderate activity against *P. aeruginosa, E. coli* and *S. aureus* and least activity against *Proteus* sp. and *K. pneumonia*. In 1/1 acetone/methanol mix extract showed effective antibacterial activity against *Micrococcus* sp., while 1/1 ethanol/methanol mix exhibited high activity against *Micrococcus* sp., *S. aureus* and *E. coli.* Acetone extract was active against *Micrococcus* sp. and *S. epidermidis* and methanol extract was active only against *Micrococcus* sp. The hot aqueous extract showed the lowest activity on the tested organisms while the cold aqueous extract did not inhibit the growth of any of tested microorganisms.

Table 3. Antifungal activity of different extracts of *Astragalus atropilosulus* subsp. *abyssinicus* against some pathogenic fungi at 10 mg of extract. (Zone of inhibition measured in mm).

Zone of inhibition (mm)										
Pathogenic fungi	**Acetone**	**Ethanol**	**Methanol**	**1/1 ethanol/ methanol**	**1/1 ethanol/ acetone**	**1/1 acetone/ methanol**	**Aqueous**		**DMSO**	**Fluconazole (30 µg/disc)**
							Cold	**Hot**		
Alternaria alternatra (PI)	NI	NI	NI	NI	NI	NI	NI	NI	NI	33.3±0.76
Candida sp. (HI)	8.66±0.51	14.5±0.45	8.83±0.25	6.56±0.33	8.73±0.41	10.9±0.58	NI	7.57±0.26	NI	35.3±0.69
Drechslera halodes (PI)	11.6±0.45	11.3±0.58	13.6±0.42	13.6±0.53	13.7±0.59	13.8±0.46	NI	10.3±0.67	NI	30.9±0.57
Fusarium oxysporum (PI)	9.60±0.75	9.70±0.10	13.0±0.17	12.3±0.22	10.6±0.27	11.6±0.91	NI	9.56±0.18	NI	32.3±0.51
Macrophomina phaseolina (PI)	NI	NI	NI	NI	NI	NI	NI	NI	NI	30.2±0.84
Pythium ultimum (PI)	19.6±0.36	20.0±0.26	20.3±0.40	19.3±0.18	20.0±0.44	18.5±0.14	NI	14.0±0.44	NI	32.3±0.98
Rhizoctoina solani(PI)	NI	NI	NI	NI	NI	NI	NI	NI	NI	34.3±0.46

NI (no zone of inhibition); DMSO, dimethyl sulfoxide; Data are means (n = 3) ± standard deviation of three replicates; (PI) plant isolate; (HI) human isolate.

The results presented in Table 3 show that among the fungal pathogens selected for this study, highest sensitivity was exhibited by *Pythium ultimum* by all eight extracts. The inhibition zones for the different extracts were as follows: methanol (20.3 mm), 1/1 ethanol/acetone and ethanol (20.0 mm), acetone (19.6 mm), 1/1 ethanol/methanol mix (19.3 mm), 1/1 acetone/methanol mix (18.5 mm), hot water (14.0 mm). The cold water extract was not able to inhibit any of the fungi tested. For *Candida* sp. the zone of inhibition was between 14.5 to 7.6 mm whereas for *Drechslera halodes* the inhibition zones were 13.8 to 10.3 mm and *Fusarium oxysporum* the range was 13.0 to 9.6 mm. *Alternaria alternatra, Macrophomina phaseolina* and *Rhizoctoina solani* showed resistance to all extracts tested.

DISCUSSION

Medicinal plants have been used to treat a variety of ailments for generations, and are still in use, especially in developing countries. With the increase of the incidence of microbes resistant to antibiotics, the natural products from herbal plants can represent an interesting alternative (Lu et al., 2007; Mbwambo et al., 2007). It is of interest to search for new and hopefully more potent antimicrobial drugs of natural origin to complement the existing antibiotics that are gradually becoming less potent against human pathogenic microorganisms. To reach this goal, one of the first steps is the *in vitro* antimicrobial activity assay (Akinsulire et al., 2007; Tona et al., 1988).

Many studies have been done on the antiviral,antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory effects of plant extracts (Adesanya., 2005; Behera and Misra, 2005; Bylka et al., 2004; Ellof, 1998; Kumaraswamy et al., 2002; Spector et al., 1981; Stepanovic et al., 2003). It was reported that the appropriate method to extract plant materials is to use a variety of solvents such as methanol, ethanol and acetone as extracting solvents, whereas the methods and the types of solvents affect greatly antimicrobial activity of plants (Tortora et al., 2001; Vukovic et al., 2008). On the basis of this background, *in-vitro* antimicrobial activities of the extracts of *A. atropilosulus* subsp. *abyssinicus* from pure or mixed solvents were screened using a variety of isolated pathogens.

Compounds present in leaves extracts of *A. atropilosulus* subsp. *abyssinicus* have antibacterial effect against Gram-positive and Gram-negative bacteria, with *Micrococcus* sp. being the most sensitive organism. 1/1 ethanol/methanol extracts exhibited significant antimicrobial activity against certain Gram-positive bacteria (*S. aureus, S. epidermidis* and *Micrococcus* sp.) and Gram-negative bacteria (*E. coli, P. aeruginosa* and *K. pneumonia*). The acetone extract showed significant antimicrobial activity against certain Gram-positive bacteria (*S. aureus,* and *Micrococcus* sp.) and *P. aeruginosa* as a Gram-negative bacteria, while ethanol extracts were active against the Gram-positive bacteria *S. aureus* and *Micrococcus* sp. Extracts of methanol, 1/1 ethanol/acetone and 1/1 acetone/ methanol were found to be active against Gram-positive bacteria (*Micrococcus* sp.).

It was found that distinctive pure or mixed solvents had different effects on the growth of microorganism, and therefore these extracts should be further tested for their potential use in treatment of microbial infections in man. *Micrococcus* sp. was the most susceptible bacteria to the different plant extracts of *A. atropilosulus* subsp. *abyssinicus* and this bacteria is a good candidate for the use of this plant to treat *Micrococcus* sp. infections. Except for the acetone extract *P. aeruginosa* showed a lack of susceptibility, and this might be attributed to the fact that this bacteria is naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane (Spector et al., 1981). Differences in sensitivity between Gram-positive and Gram-negative bacteria to these extracts could be due to the morphological differences between these microorganisms since the Gram-negative bacterial cell wall outer membrane appears to act as a barrier to many substances, including antibiotics (Tortora et al., 2001).

A. atropilosulus subsp. *abyssinicus* extracts were more active against bacteria compared to fungi. All extracts were effective towards *Pythium ultimum*, and the hot water extract was least active on all fungi tested. The ethanol extract was effective on *Candida* sp., the 1/1 acetone/ methanol extract on *Drechslera halodes* and the methanol extract on *Fusarium oxysporum.* None of the eight extracts have an effect on *Alternaria alternatra* and *Rhizoctonia solani* which may be due to a failure of these solvents to extract active ingredients against these fungi.

In conclusion, from the present investigation, it is evident that *A. atropilosulus* subsp. *abyssinicus* possess compounds with antibacterial andantifungal properties. Further characterization and isolation of the specific antimicrobial chemicals and their purification is necessary to find novel treatments to tackle the emergence of drug resistant pathogens. Toxicity tests of the active plants compounds is required to evaluate their safety.

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