

Biological and Chemical Study of *Astragalus gombiformis*

Hassen Teyeb^{a,b,*}, Olfa Houta^b, Hanen Najjaa^b, Ali Lamari^c, Mohamed Neffati^b, Wahiba Douki^a, and Mohamed Fadhel Najjar^a

^a Biochemistry and Toxicology Laboratory, University Hospital Fattouma Bourguiba, Monastir 5000, Tunisia. Fax: (+216)73460678. E-mail: teyeb.hassen@gmail.com

^b Range Ecology Laboratory, Arid Land Institute of Medenine, Medenine 4119, Tunisia

^c Genetic Laboratory, Faculty of Medicine of Monastir, University of Monastir, Monastir 5000, Tunisia

* Author for correspondence and reprint requests

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Extracts of aerial parts and roots of wild *Astragalus gombiformis* Pomel were tested for their antibacterial, antioxidant, and insecticidal activities and contents of phenolic compounds. Antibacterial activity was tested by the paper disk agar diffusion method and determination of the minimal inhibitor concentration. Among the tested extracts, three extracts (methanol, chloroform, and ethyl acetate) from aerial parts and two extracts (water, methanol) from roots exhibited diameters of inhibition zone equal or above 12 mm (at 150 µg/disk) and minimal inhibitor concentrations ranging between 233 and 1250 µg/ml. Spectrophotometric and HPLC analyses showed that contents of both total polyphenols and flavonoids, as well as antioxidant activity were higher in the methanolic extract of aerial parts as compared to roots. No insecticidal activity of the extracts of the aerial parts was found against *Culex pipiens*.

Key words: Antibacterial, Insecticidal, Antioxidant, *Astragalus gombiformis*

Introduction

In recent years increasing interest has focused on biological activities of crude extracts or isolated compounds from plants. Several species have an important biological potential as sources of bioactive compounds of use in medicine and others fields such as food industries and agriculture. Natural products play a highly significant role in the drug discovery and development process, particularly in the areas of cancer and infectious diseases. In fact, over 60% and 75% of these drugs, respectively, were shown to be of natural origin (Newman *et al.*, 2003). Oxidative stress is implicated in the pathogenesis of many diseases such as macular degeneration and Alzheimer's disease. Research on natural antioxidant compounds has received substantial attention. Many species were investigated for their antioxidant activities (Albayrak *et al.*, 2010; Braca *et al.*, 2003), and some of them showed high antioxidant potential which seems to be correlated mainly with the phenolic contents of these plants (Lizcano *et al.*, 2010). In agriculture, extracts from plants present a considerable opportunity to produce ecofriendly insecticides. In fact, with the increased resistance

of insects to synthetic insecticides, diseases transmitted by these vectors remain a serious problem for public health.

Astragalus, a genus of the Fabaceae family, is commonly used as forage for livestock and by wild animals. But several species of this genus are used in foods, medicines, and cosmetics (Ríos and Waterman, 1997; Zarre-Mobarakeh, 2000). In Anatolia (southeast of Turkey), an aqueous extract of the roots of *A. trojanus* is traditionally used against leukemia (Bedir *et al.*, 2001). Roots of *A. membranaceus* are widely used as herbal drug in traditional Chinese medicine (Yin *et al.*, 2006). *Astragalus* species are also of economical importance. In Turkey, *A. microcephalus* is used for the production of the gum tragacant (Bedir *et al.*, 1998). Several studies related to *Astragalus* species have been conducted and many secondary metabolites have been identified in *Astragalus* species such as *A. microcephalus*, *A. trojanus*, and *A. zahlbruckneri* (Bedir *et al.*, 1998, 1999; Calis *et al.*, 2001). On the other hand, some bioactivities, such as antibacterial, antifungal, and antioxidant, have been demonstrated in extracts of *Astragalus* plants (Abbas and Zayed, 2005; Adigüzel *et*

al., 2009; Jassbi *et al.*, 2002; Godevac *et al.*, 2008; Tawaha *et al.*, 2007; Türker *et al.*, 2009).

The Tunisian flora contains several *Astragalus* species, among them *A. gombiformis* Pomel which grows in desert areas, where plants synthesize several metabolites to adapt to different forms of stress and are thus a promising source of bioactive molecules. In Morocco, this plant is traditionally used to cure bites of snakes and scorpions (El Rhaffari and Zaid, 2002). *A. gombiformis* is under consideration in the program of valorization of Tunisian plants conducted by the Range Ecology Laboratory (Arid Land Institute of Medenine, Medenine, Tunisia). For the purpose of its valorization, in previous works we studied the chemical composition of essential oils of *A. gombiformis* (Teyeb *et al.*, 2011) and the antibacterial and cytotoxic activities of leaf extracts (Teyeb *et al.*, 2012). In the present study, we evaluated the antibacterial, insecticidal, and antioxidant activities of aerial parts and roots extracts of *A. gombiformis*. Phenolic contents of methanolic extracts were also investigated.

Material and Methods

Plant collection and preparation of extracts

Aerial parts and roots of wild *A. gombiformis* were collected at Bir Soltane (33° 28' 10" N, 009° 23' 50" E, 107 m above sea level) in the south of Tunisia in the flowering season. The plant was identified by Professor Mohamed Neffati, and voucher specimens were deposited in the laboratory.

The samples were air-dried protected from direct sun light, then powdered and stored until use. Different extracts were prepared, as shown in Table I, using a Soxhlet apparatus for organic solvents and direct maceration for water. Organic solvents were evaporated by a rotavapor and aqueous extracts were lyophilized. For antibacterial and antioxidant tests, all residues were dissolved in the respective extraction solvent at concentrations of 10 mg/ml. For determination of the insecticidal activity, residues were dissolved in dimethyl sulfoxide (DMSO). The extracts for high-performance liquid chromatography (HPLC) analysis were prepared by maceration of 10 g of plant powder in 100 ml of pure methanol. After 1 h in an ultrasonic bath, the mixtures were allowed to stand at room temperature for 48 h.

Table I. Plant parts, used solvents, and tested activities.

Activity	Solvents	Plant parts
Antibacterial	Methanol, ethyl acetate, chloroform, water	Aerial parts and roots
Insecticidal	Methanol, ethyl acetate, dichloromethane, petroleum ether	Aerial parts
Antioxidant	Methanol	Aerial parts and roots

Antioxidant activity and phenolic content

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant activity was evaluated using DPPH (95%) according to Braca *et al.* (2002), with minor modifications. The methanolic extract (0.5 ml) was mixed with 0.5 ml of 0.004% methanolic DPPH solution. After 30 min of incubation in the dark, the absorbance was measured at 517 nm. Ascorbic acid and methanol were used, respectively, as positive and negative controls and treated under the same conditions. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate, and IC_{50} values are reported as means \pm SD.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

The method of Re *et al.* (1999) was adopted for the ABTS test with slight modifications. The ABTS radical was generated in an aqueous ABTS solution with 2.45 mM potassium persulfate. This mixture was allowed to stand for 12 to 16 h at room temperature. Before use, this solution was diluted with 20 mM sodium acetate to an absorbance of 0.70 ± 0.02 at 734 nm. One ml was mixed with 20 μ l of methanolic extract. After 6 min of incubation in the dark, the absorbance was determined at 734 nm. Trolox and methanol were used, respectively, as standard and blank. Antioxidant activity was expressed as μ mol of Trolox equivalents (TE) per g of plant dry weight (DW), and all measurements were performed in triplicate.

Total polyphenols

The total polyphenols content was determined by the Folin-Ciocalteu procedure (Dewanto *et al.*, 2002). An aliquot (125 μ l) of the methanolic ex-

tract was added to 500 μ l of distilled water in a test tube, then 125 μ l of Folin-Ciocalteu reagent were added. After the mixture had been vortexed and allowed to stand for 3 min, 1.25 ml of 7% Na_2CO_3 were added and the volume adjusted to 3 ml by distilled water. After 90 min of incubation in the dark, the absorbance was recorded at 760 nm against a blank containing 125 μ l of methanol. The amount of total polyphenols was calculated as gallic acid equivalents (GAE) and expressed as mg of GAE/g of DW. All measurements were done in triplicate.

Flavonoids

The total flavonoids content was determined by the procedure of Dewanto *et al.* (2002). The methanolic extract (250 μ l) was added to 1.25 ml of distilled water in a test tube, then 75 μ l of 5% NaNO_2 solution were added. After 6 min, 150 μ l of freshly prepared 10% AlCl_3 solution were added. After another 5 min, 500 μ l of 1 M NaOH were added to the mixture which was adjusted to 2.5 ml by distilled water. Absorbance was measured at 510 nm against the blank methanol. Flavonoids in extracts were expressed as mg catechin equivalents per g of DW (mg CE/g DW). All measurements were performed in triplicate.

Statistical analysis

For flavonoids, total polyphenols, and antioxidant activity, comparisons between aerial parts and roots extracts were performed with the Student *t*-test ($p < 0.05$ as significance level).

HPLC analysis

After filtration, methanolic extracts were analysed by an Agilent (Palo Alto, CA, USA) 1100 series HPLC system using a C18 SymmetryShield™ (2.1 mm x 150 mm; Waters, Milford, MA, USA) column. The mobile phase was a gradient mixture of two solutions. Solution A was constituted of filtered and degassed distilled water and 0.05% trifluoroacetic acid. Solution B was a mixture of acetonitrile (MicroSolv®) and 0.05% trifluoroacetic acid. The injected volume was 20 μ l and the flow rate was adjusted to 0.250 ml/min. Run time was 55 min (0 min 100% A, 55 min 30% A). Detection of flavonoids was at 254 and 350 nm. The methanolic extract of aerial parts was also analysed by semipreparative HPLC using a μ Bondapak™ C18 (7.8 x 300 mm, 15–20 μ m) column under the same conditions, starting with 100% A and finishing with 20% A after 55 min.

Antibacterial activity

Bacterial strains

Six bacterial strains, stored on Mueller-Hinton agar (Bio-Rad, SA, Marnes-La-Coquette, France) at 4 °C, were used: *Staphylococcus epidermidis* CIP 106510, *Salmonella typhimurium* NRRLB 4420, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19115, and *Bacillus subtilis* ATCC 168. Nutrient broth (Bio-Rad) and the Mueller-Hinton agar were used, respectively, for growing and diluting the microbial suspensions for the antibacterial assays.

Disk diffusion method

The antibacterial activity was tested by the paper disk agar diffusion method according to Najjaa *et al.* (2007). Pure bacterial strains were suspended in molten nutrient agar, and the optical density was adjusted to 0.5 at 570 nm (Jenway 6405 UV/Vis spectrophotometer; Dunmon, UK). Mueller-Hinton agar plates (90 mm) were inoculated with this bacterial suspension and Whatman paper disks (6 mm in diameter) were deposited. Each disk was impregnated with 15 μ l of extract or extraction solvent for negative control, and the plates were then incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the clear inhibitory zone around each disk including its diameter. Absence of inhibition was expressed by the value 0 mm. Extraction solvent and gentamicin (10 UI) were used, respectively, as negative and positive controls. Each extract was tested in duplicate at 10 mg/ml. For each extract exhibiting a diameter equal to or above 12 mm, the minimal inhibitor concentration (MIC) was determined.

Determination of MIC values

The method described by Lim *et al.* (2007) was used, with slight modifications, for the determination of MIC values. Different concentrations of each extract were prepared by serial dilutions in sterile nutrient broth. Each well of an ELISA plate contained 100 μ l of extract (or 100 μ l of extraction solvent for negative control), 95 μ l of nutrient broth, and 5 μ l of bacterial suspension. A growth control, containing 200 μ l/well, was performed for each tested microorganism. After 24 h of incubation, the MIC value was recorded as the lowest concentration at which no bacterial growth was observed.

Insecticidal activity

A local strain of *Culex pipiens* larvae was collected from a river in Tazarka (Tunisia). The species were reared at a 12 h/12 h light/dark photoperiod, $(60 \pm 10)\%$ relative humidity, and $(26 \pm 2)^\circ\text{C}$, in an insectary in the Genetic Laboratory, Faculty of Medicine, University of Monastir, Monastir, Tunisia.

One ml of each extract was added to 99 ml of tap water in plastic cups containing 20 *C. pipiens* larvae. Five replicates were maintained for each extract. The number of dead larvae, after 24 h and 48 h, was noted and compared to the control. Positive control (permethrin at 1 mg/ml) and negative controls (DMSO and water) were treated in the same way. The lethal dose 50 (LD_{50}) is the concentration that can cause death in 50% of the larvae group.

Results and Discussion

The biological activities of extracts of the aerial parts and roots of *A. gombiformis* were evaluated. The tested extracts were found to have especially antioxidant and antibacterial activities.

Phenolic content and antioxidant activity

Total polyphenols and flavonoids in the methanolic extracts of both roots and aerial parts of *A. gombiformis* were determined by colorimetric methods. Two test methods, DPPH and ABTS assays, were used to evaluate the antioxidant effect of these extracts (Table II). The antioxidant activity of the methanolic extract from aerial parts (yield: 20.08%) in the DPPH assay showed an IC_{50} value of $(473.33 \pm 64.29) \mu\text{g/ml}$, compared to $(7.36 \pm 0.70) \mu\text{g/ml}$ of ascorbic acid used as

positive control. The corresponding value of the roots extract (yield: 10.10%) was $(626.66 \pm 64.29) \mu\text{g/ml}$. The highest level of antioxidant potential expressed in Trolox equivalents was also observed for the aerial parts extract ($p < 0.05$). Similarly, the phenolic content was significantly higher in aerial parts than in roots. These data were in agreement with those of Lizcano *et al.* (2010), who showed a positive correlation between phenolic contents and antioxidant activity in extracts from various plants. HPLC analysis revealed that the methanolic extract from aerial parts is more enriched in flavonoids than that from roots. In Fig. 1 the elution profile of the preparative HPLC of the methanolic extract of aerial parts at 254 and 350 nm is shown, with more abundant signals at 254 nm.

Antioxidant activities of various *Astragalus* species have been studied by many authors. In the DPPH assay, methanolic extracts of aerial parts of some *Astragalus* species had IC_{50} values ranging from 68.8 to 400.4 $\mu\text{g/ml}$. Root extracts of these species exhibited antioxidant activity with IC_{50} values varying from 115.1 to 328.5 $\mu\text{g/ml}$ (Adigüzel *et al.*, 2009). Total flavonoids, total saponins, and total polysaccharides of *A. mongholicus* exhibited antioxidant activity (Bian and Li, 2009). Flavonoids from *A. complanatus* protected against radiation-induced damages in mice (Qi *et al.*, 2011).

The antioxidants scavenge free radicals, which are associated with the pathogenesis of various disorders such as diabetes, cardiovascular diseases, and cancer. Polyphenols play a role in the prevention of these diseases (Manach *et al.*, 2004; Ratnam *et al.*, 2006). While this work showed that *A. gombiformis* has an interesting phenol content and antioxidant activity, one must realize that these properties depend on a number of biotic

Table II. Polyphenol and flavonoid contents of methanolic *Astragalus gombiformis* extracts and their antioxidant activities.

Parameter	Roots	Aerial parts	Ascorbic acid	<i>p</i>
Yield (% of DW)	10.10	20.08	-	-
Polyphenols (mg GAE/g DW) ^a	3.340 ± 0.491	9.194 ± 0.273	-	0.0002
Flavonoids (mg CE/g DW) ^b	0.767 ± 0.051	3.133 ± 0.344	-	0.006
DPPH (IC_{50} , $\mu\text{g/ml}$)	626.66 ± 64.29	473.33 ± 64.29	7.36 ± 0.70	0.043
ABTS ($\mu\text{mol TE/g DW}$) ^c	47.13 ± 0.05	79.81 ± 1.31	-	0.003

All data are shown as mean \pm SD from three replicates.

^a mg of gallic acid equivalents (GAE) per g of plant dry weight (DW).

^b mg of catechin equivalents (CE) per g DW.

^c μmol of Trolox equivalents (TE) per g DW.

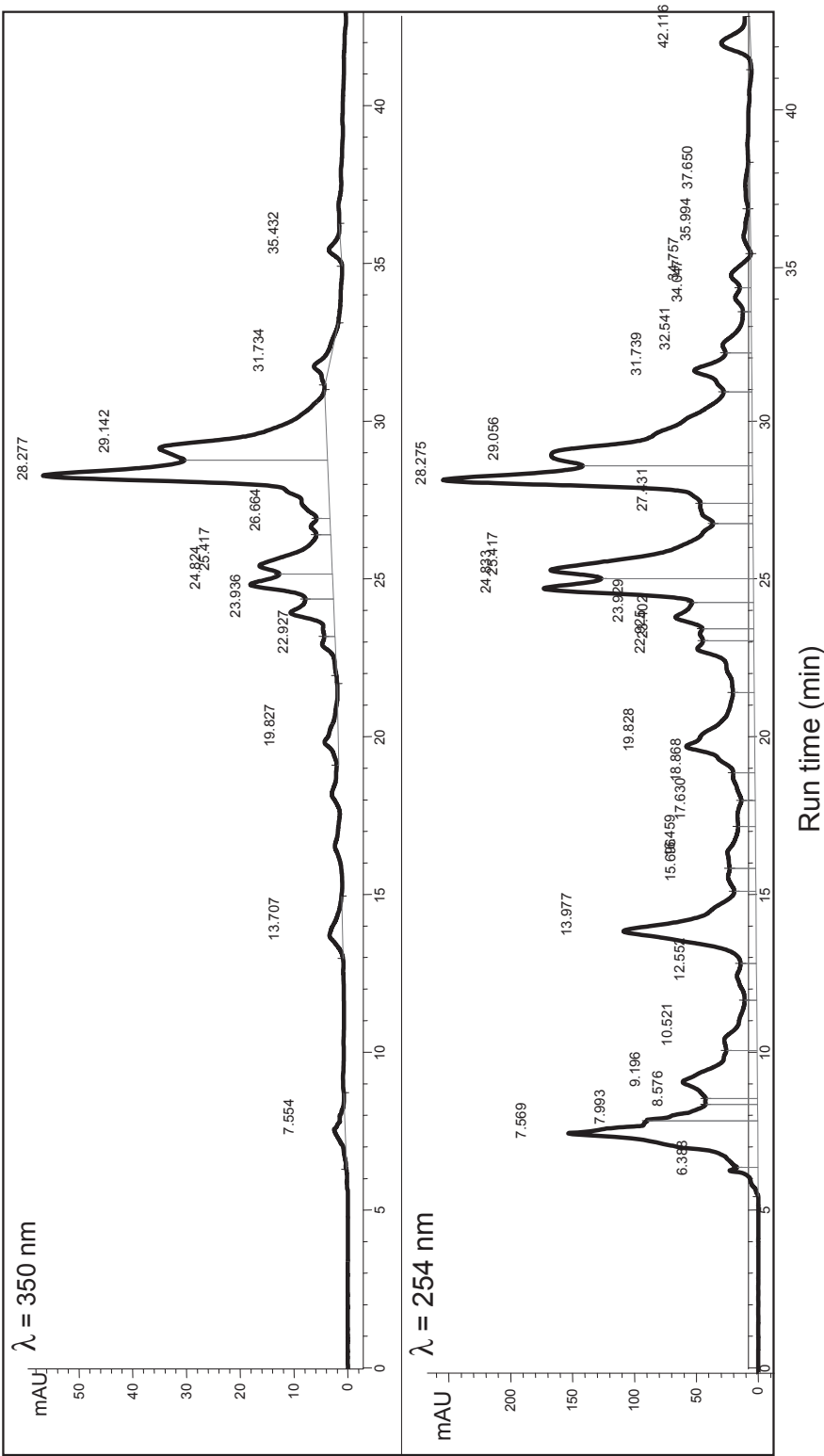


Fig. 1. HPLC profile ($\lambda = 254$ and 350 nm) of the methanolic extract of *Astragalus gombiformis* aerial parts.

Table III. Antibacterial activity of *Astragalus gombiformis* extracts (150 µg/disk) expressed as diameter of inhibition zones.

Plant part	Solvent	Inhibition zone [mm]					
		<i>L. monocytogenes</i>	<i>S. epidermidis</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>S. typhimurium</i>
Aerial parts	Methanol	12	10	10	11	9	10
	Chloroform	10	0	8	9	0	15
	Ethyl acetate	10	10	0	8	10	12
	Water	9	9	7	11	0	8
Roots	Methanol	10	9	8	9	9	14
	Chloroform	9	8	9	11	9	0
	Ethyl acetate	11	11	0	10	10	11
	Water	10	12	0	13	0	8
Gentamicin (15 µg/disk)		20	12	16	20	20	20

and abiotic factors such as environmental conditions, especially light conditions, and phenological stage (Ksouri *et al.*, 2008). Niknam and Ebrahimzadeh (2002) found the phenolic contents of some *Astragalus* species to vary between 0.25 and 0.91% for roots and between 0.52 and 3.75% for leaves.

Antibacterial activity

In vitro antibacterial activities of *A. gombiformis* against some infectious bacteria were tested by the paper disk agar diffusion method at the concentration of 10 mg/ml. Data are summarized in Table III. The chloroform extract of the aerial parts exhibited the highest antibacterial effect. Against *Listeria monocytogenes*, the MIC value of the methanolic aerial parts extract was 310 µg/ml. Chloroform and ethyl acetate extracts from aerial parts had the same MIC value (1250 µg/ml) against *Salmonella typhimurium*. The aqueous roots extract exhibited MIC values of 233, 310, and 1250 µg/ml, respectively, against *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Salmonella typhimurium*. The methanolic extract from roots showed also a MIC value of 1250 µg/ml against the last bacteria.

A. gombiformis seems to have an antibacterial activity potentially useful for the development of new antibiotics. Among the Fabaceae family, methanolic extracts of aerial parts of other *Astragalus* species such as *A. ponticus*, *A. microcephalus*, *A. macrocephalus*, *A. erinaceus*, and *A. argyroides* have previously been tested for their antibacterial activity but, at 300 µg/disk, were found inactive against the tested bacteria (Adigüzel *et al.*, 2009).

Antimicrobial resistance has steadily increased (Stahl, 2006). To discover new therapeutic opportunities, many studies on antibacterial activities of plants have been carried out (Kudi *et al.*, 1999; Mothana and Lindequist, 2005; Palombo and Semple, 2001). In fact, several clinically used antibiotics, such as daptomycin (Cubicin®) and teicoplanin (Targocid®), were derived from plants (Newman and Cragg, 2007). In this context, our findings can be a contribution to the efforts focusing on the development of natural antibacterial drugs.

Insecticidal effect

While the LD₅₀ value of permethrin in the positive control was (0.51 ± 0.10) µg/ml, the *A. gombiformis* extracts had little, if any, insecticidal effect on the *C. pipiens* larvae at 100 mg/l (data not shown).

Shaalan *et al.* (2005) declared that only if at 10 mg/l (crude extract) 100% mortality is observed, further evaluation of an insecticidal activity is indicated. Thus, we can conclude that the tested *A. gombiformis* extracts were inactive against *C. pipiens*.

Conclusion

This work reveals that the tested extracts possess interesting antioxidant and antibacterial activities, while they were inactive against *C. pipiens* larvae. Our results show that *A. gombiformis* could be a candidate as a source for antibacterial and antioxidant compounds. Currently, our studies are focused on the isolation and identification of phenolic compounds from the methanolic extracts.

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