Moroccan Journal of Chemistry
ISSN: 2351-812X
http://revues.imist.ma/?journal=morjchem&page=login
A. Zeroual / Mor. J. Chem. 4N°4 (2016) 987-995

Antioxidant and antimicrobial activities of *Tamarix aphylla* (L.) Karst. growing in Tunisia

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Received 27 July 2016, Revised 10 Aug 2016, Accepted 08 Jan 2017

Abstract

This study was carried out with the objective to investigate antioxidant, antibacterial and antifungal activities of *Tamarix aphylla* (L.) Karst. The antioxidant activities were evaluated by determining total phenolic content and IC_{50} values. The obtained results allowed us to note that total phenolic content of the different extracts varied from 5.13 to 22.02 mg GAE/g DW. Regarding the IC_{50} values, they varied from 0.81 to 19.92 mg/ml for $DPPH^{\cdot}$ and from 12.16 to 21.23 mmol TE/g DW for $ABTS^{+\cdot}$. All the tested extracts showed remarkable antibacterial activities with MIC values ranged from 0.078 to 2.5mg/ml. The strongest inhibitions were observed for the ethyl acetate stems extract with MIC values of 0.156; 0.117; 0.117; 0.078 and 0.235 mg/ml against *E. coli*; *P. aeruginosa*; *S. aureus*; *E. feacalis* and *Acinetobacter baumannii* respectively. *T. aphylla* extracts have not antifungal activities against *Candida albicans*, *Candida krusei*, *Candida glabrata* and *Candida parapsilosis*. These findings suggest that *T. aphylla* may be considered as a potential sources of new antioxidant and antibacterial drugs.

Keywords: *Tamarix aphylla (L.) Karst.; DPPH; $ABTS^{+\cdot}$; MIC; MBC; Inhibition zone.*

1. Introduction

Free radicals are produced in the body in small amounts, the excess of these radicals is called "oxidative stress" [1]. This stress is involved in many diseases such as diabetes, Alzheimer, rheumatism and cardiovascular ones [2].

Infectious diseases are still one of the leading causes of death in the world. Human fungal infections have increased at an alarming rate in the last 20 years, mainly among immunocompromised individuals [3]. Among human gastrointestinal tract isolates, 50-70% of total yeast isolates were identified as *Candida albicans*. Further frequent isolates are *C. tropicalis*, *C. parapsilosis*, and *C. glabrata*, while *C. kefyr* and *C. guilliermondii* are found occasionally [4, 5]. Nowadays, the indiscriminate use of commercial antimicrobial drugs has caused multiple drug resistance in human pathogenic microorganisms [6]. Therefore, the search for new antimicrobial agents is imperative. It has been recognized that natural compounds play an important role in modern pharmaceutical care. In particular, natural drugs originating from plant tissues have received attention as a source of novel molecules. In this context, our team has undertaken many research studies to discover new natural extracts, extract fractions and pure natural substances having antioxidant and antimicrobial activities [7-17]. *Tamarix* species are employed in traditional medicine as astringent, aperitif, stimulus of perspiration and diuretic [18]. They are useful again in leucoderma, spleen trouble and eye diseases [19]. Furthermore, the *Tamarix* species are found to be rich in polyphenolic compounds such as flavonoids, phenolic acids, tannins and coumarins [20, 21]. Several researches proved antioxidant and antimicrobial activities of some Tamarix species such as *T. aphylla* [22], *T. ramosissima* [23], *T. hispida* [24] and *T. boveana* [25].

According to our previous results [26], the analysis of *T. aphylla* polyphenols, performed by means of HPLC-UV/DAD, HPLC-ESI-MS and MS^2, showed the presence of the following compounds: gallic acid, caffeic acid, *p*-coumaric acid, ellagic acid, syringic acidisomer, luteolin, quercetin, apigenin, kaempferol, isorhamnetin, tamarixetin, quercetin dimethyl-ether, kaempferide and kaempferol-7,4'-dimethyl-ether. The aims of the present work were: (i) to determine the total phenolic content of *T. aphylla* leaves extracts, (ii) to examine the antioxidant potential and the antimicrobial activities of *T. aphylla* extracts on selected bacterial and fungal pathogens.

2. Materials and methods

2.1. Plant material

Leaves and stems of *T. aphylla* were collected during the month of December 2012 from Skanès area (latitude: 35°75', longitude: 10°82'), Governorate of Monastir, TUNISIA. The plant material was authenticated by Dr. Béchir Ben Tiba (Higher Institute of Agronomic Sciences of Chott-Mariem, BP 47, 4042 Chott Mariem, Sousse, TUNISIA). Voucher specimen, FSM-TA 2012, was deposited in the herbarium of the Faculty of Sciences of Monastir, TUNISIA.

After the collection, leaves and stems were manually separated, air-dried under shade at room temperature, and grounded. Then the powder was stored for subsequent analysis.

2.2. Preparation of extracts having different polarities from the plant T. aphylla

Extraction at room temperature: 300 g of dried and powdered leaves of *T. aphylla* were extracted by maceration, for 72 h at room temperature, using one liter of each of the following solvents: cyclohexane, dichloromethane, butanol and ethanol.

Extraction by soxhlet apparatus: Leaves and stems extracts from *T. aphylla* were prepared in a soxhlet apparatus using five solvents having different polarities: petroleum ether, dichloromethane, ethyl acetate, ethanol and methanol. For each solvent and for leaves and stems, 100 g of the powder were extracted with 750 ml of solvent. The obtained macerates and extracts were filtered through Whatman paper No.1 then evaporated under vacuum.

2.3. Antioxidant activities

DPPH assay: A dilution series was prepared for each macerate, and then an aliquot of 25 µl was added to 975 µl of DPPH methanolic solution (6 10^{-5} M). The mixture was shaken and incubated at room temperature for 30 min in darkness. The absorbance against blank (methanol) was measured at 515 nm. Vitamin C was used for comparison. The ability of the macerates to scavenge the DPPH radical was calculated using the following equation: Inhibition percentage of DPPH (%) = $(A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the DPPH solution, and A_1 is the absorbance of the sample after 30 min of incubation with DPPH [27].

$ABTS^{+\cdot}$ assay: 25 µl of each macerate were added to 975µl of $ABTS^{\cdot+}$ ethanolic solution (7 mmol/L) having an absorbance of 0.7 ± 0.02 at 734 nm. The absorbance of the mixture at 734 nm was recorded after 6 minutes. The ability of the macerates to scavenge the $ABTS^{+\cdot}$ cation radicals was calculated using the following equation:
Inhibition percentage of $ABTS^{+\cdot} = (A_0 - A_1/A_0) \times 100$, where A_0 is the absorbance of the $ABTS^{+\cdot}$ solution and A_1 is the absorbance of the sample after 6 min of incubation with the $ABTS^{+\cdot}$ solution. Antioxidant activity was expressed as mmol ET/g DW [28, 29].

2.4. Phytochemical composition

Determination of total polyphenols contents: 50 µl of the studied extract were mixed with 1ml of distilled water, 0.5 ml of Folin–Ciocalteu reagent (F-C) and 2.5 ml of 20% Na_2CO_3. The absorbance of the resulting blue complex was measured at 735 nm after 20 min of incubation in dark at room temperature. Total polyphenols contents were expressed as mg GAE/g DW. All samples were analyzed in triplicate [30].

2.5. Antimicrobial activities

Antibacterial activities: Petroleum ether, dichloromethane, ethyl acetate, ethanolic and methanolic leaves and stems extracts from *T. aphylla* were tested against five bacterial strains. The Gram-positive bacteria were: *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212); for the Gram negative, the species tested were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumannii* (Multidrug-Resistant Clinical Strain). To evaluate the antibacterial activity, the values of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. MIC is the lowest concentration of an antimicrobial agent that inhibited the visible bacterial growth after 24 h of incubation at 37 °C. MBC is the lowest concentration that kills at least 99.99% of the initial number of bacteria.

a-Determination of Minimal inhibitory concentrations (MIC): Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well plates [31, 32]. The investigated dried extracts were dissolved in 10% Dimethyl Sulfoxide (DMSO) to achieve the desired concentrations (10 mg/ml). Each bacterial inoculum was prepared in broth medium and suspension was adjusted to 0.5 McFarland standard turbidity using a densitometer (*Densimat®, BioMérieux).* Sterile plates (8 rows of 12 wells) were prepared under aseptic conditions as follow: A volume of 200 µL of the studied extract (10 mg/mL) was pipetted into the first well of the plates. Wells 2 to well 9 were filled by 100 µL of DMSO (10%). Serial dilutions were performed from well 1 to well 9 to achieve the desired concentrations (10- 5- 2.5- 1.25- 0.625- 0.312- 0.156- 0.078- 0.039 mg/mL). Then, 50 µL of the bacterial suspension were added to these wells (each row corresponds to one bacterial strain). Well 10 was filled by 150 µL of the bacterial inoculum. Well 11 contains 100 µL of DMSO (10%) add with 50 µL of the bacterial suspension. Finally, well 12 contains 100 µL of DMSO (10%) add with 50 µL of the broth medium. Each plate was

wrapped with cling film to ensure that bacteria did not become dehydrated. The plates were prepared in triplicate, and placed in an incubator set at 37°C for 18-24 h. The average of three values was calculated and that was the MIC.

b-Determination of Minimum bactericidal concentrations (MBC): To determine the MBC values, wells solutions with an extract concentration equal or higher to the MIC values were used. 10 µL from each well were subcultured on nutrient agar in Petri dishes. The bacterial cultures were incubated at 37°C for 24 h. MBC was determined as the lowest concentration that showed no bacterial growth in the subcultures [33, 34].

Antifungal activity: The antifungal assays were carried out by disc-diffusion method in Sabouraud chloramphenicol agar medium. Four strains from the American Type Culture Collection (ATCC), namely *Candida albicans* (ATCC 90028), *Candida krusei* (ATCC 6258), *Candida glabrata* (ATCC 2001) and *Candida parapsilosis* (ATCC 22019) were used. Before each experiment, all reference species were grown on Sabouraud Dextrose Agar (SDA; Merck, Germany) for 24 h at 37◦C. Yeast cells were suspended in 5 ml of sterile saline solution (0.9%). The resulting yeast suspension was mixed and adjusted to 1 McFarland turbidity standard using a densitometer (*Densimat®, BioMérieux)*. The dried surfaces of Sabouraud chloramphenicol agar in square Petri dishes were inoculated by 2-3 ml of each fungal suspension. Petri dishes were rotated approximately 60° to ensure a uniform distribution of inoculum. Next, the Petri dishes were incubated at 37°C for 15 min. *T. aphylla* leaves and stems dried extracts (100 mg) were dissolved in 1 ml of methanol. Sterile filter discs (diameter 6 mm, Whatman paper N° 1) were impregnated with 10 µl of *T. aphylla* leaves and stems extracts (100 mg/ml). Discs impregnated with 10 µL methanol (used solvent) were included as negative controls and discs impregnated with fluconazole (40 µg/disc) were included as positive controls. All discs were placed aseptically on the inoculated Petri dishes that are subsequently incubated at 37°C for 24h. The inhibition zones around the discs were measured in millimeters after 24h of incubation at 37ºC. Each microorganism was tested in triplicate [35].

3. Results and Discussions

Table 1 shows the yield corresponding to each solvent extraction process. Results showed that the highest yields were obtained with the soxhlet apparatus compared to maceration. Methanolic extracts obtained by soxhlet extraction have the highest yields. However, dichloromethane leaves and ethyl acetate stems extracts were obtained with low yields.

Table 1. *Yields of T. aphylla extracts: maceration (EF1-EF4) and soxhlet apparatus extraction (EF5-ET5)*

	Solvents	Yields (%)
Leaves	Cyclohexane EF1	1.59
	Dichloromethane EF2	1.30
	Butanol EF3	1.60
	Ethanol EF4	0.75
	Methanol EF5	20.5
	Petroleum ether EF6	8.3
Leaves	Dichloromethane EF7	0.9
	Ethyl acetate EF8	1.6
	Ethanol EF9	3.5
	Methanol ET1	17.7
	Petroleum ether ET2	3
Stems	Dichloromethane ET3	2.2
	Ethyl acetate ET4	0.9
	Ethanol ET5	8.8

3.1. Determination of total polyphenols contents of EF1, EF2, EF3 and EF4 extracts

Results in table 2 show that ethanolic, butanolic and dichloromethane extracts have total polyphenols contents of 22.02, 21.51 and 19.38 mg GAE/g DW respectively. Cyclohexanoic extract has the lowest contents 5.13 mg GAE/g DW.

Table 2. *Total polyphenols contents (mg GAE/g DW) of T. aphylla extracts*

Extracts	Total polyphenols contents (mg GAE/g DW) ± SD
Cyclohexanoic	5.13 ±1.29b
Dichloromethane	19.38 ±1.26a
Butanolic	21.51 ±0.06a
Ethanolic	22.02 ±2.44a

SD : standard deviation

A few researchers have found highest rates of Total polyphenols contents for extracts prepared from *T. aphylla* growing in Algeria [22]. Theses contents were 262.26; 199.54; 165.12 and 115.37 mg GAE/g DW for methanolic (70%), ethanolic (70%), acetonic (70%) and aqueous extracts respectively.

3.2. Antioxidant activities

DPPH assay: Compared with Vitamin C (EC_{50} = 2.01 ± 0.02 mg/ml), only the ethanolic leaves extract of *T. aphylla* showed significant antiradicalar activity with EC_{50} of 0,81 mg/ml. butanolic, dichloromethane and cyclohexanoic extracts presented week antioxidant activities illustrated by the EC_{50} values of 6.01 ; 10.22 and 19.92 mg/ml respectively (Table 3). The Algerian *T. aphylla* leaves extracts have interesting antioxidant activities against DPPH radicals shown by EC_{50} values of 0.91 ; 0.14 ; 0.08 and 0.17 mg/ml for methanolic (70%), ethanolic (70%), acetonic (70%) and aqueous extracts respectively [22].

Table 3. *EC_{50} values (mg/ml) of T. aphylla leaves extracts against DPPH radicals*

Extracts	EC_{50} (mg/ml) ± SD
Cyclohexanoic	19.92±0.219d
Dichlorométhane	10.22±1.99c
Butanolic	6.01±0.36b
Ethanolic	0.81 ±0.65a

SD : standard deviation

$ABTS^{+\cdot}$ assay: Antioxidant activities against $ABTS^{+\cdot}$ cation radicals are interesting for ethanolic and butanolic extracts with EC_{50} values of 12.15 and 16.56 mmol TE/g DW respectively. Cyclohexanoic and dichloromethane extracts have less important activities with EC_{50} values of 21.23 and 21.02 mmol TE/g DW respectively (table 4).

Table 4. *EC_{50} values (mmol TE/g DW) of T. aphylla leaves extracts against $ABTS^{+\cdot}$ cation radicals*

Extracts	EC_{50} (mmol TE/g DW) ± SD
Cyclohexanoic	21.23 ± 0.12^{d}
Dichlorométhane	21.02 ± 0.25^{c}
Butanolic	16.56 ± 0.28^{b}
Ethanolic	12.16 ± 0.70^{a}

SD : standard deviation

Leaves and flowers extracts of *T. gallica* showed significant antioxidant activities against $ABTS^{+\cdot}$ cation radicals illustrated by EC_{50} values of 1040 and 316.7 µg/ml [36]. Compounds with antioxidant activity are mainly phenolic acids, flavonoids and polyphenols [37]. Phenolic acids have repeatedly been implicated as natural antioxidants in fruits, vegetables, and other plants. For example, gallic acid, caffeic acid, ferulic acid, and vanillic acid [38]. The most widespread and diverse phenolics are the flavonoids which have the same C_{15} (C_6-C_3-C_6) skeleton and retard oxidation of a variety of easily oxidizable compounds [39]. Flavonoids are ubiquitous in plants, since almost all plant tissues are able to synthesize flavonoids. They include catechins, proanthocyanins, anthocyanidins, flavones, flavonols and their glycosides [40]. Among the most widely distributed are the flavonols quercetin and rutin. The main of these compounds were identified in T. aphylla extracts. Phytochemical studies have shown that *T. aphylla* extracts contains phenolic antioxidant compounds, such as flavonoids (luteolin, quercetin, apigenin, kaempferol, isorhamnetin, tamarixetin, quercetin dimethyl-ether, kaempferide and kaempferol-7,4'-dimethyl-ether) and phenolic acids (gallic acid, caffeic acid, *p*-coumaric acid and ellagic acid) [26].

3.3. Antimicrobial activities

We choose to submit the entire *T. aphylla* extracts to antimicrobial activity studies for several reasons. On the first hand and in a general way, the antimicrobial capacity of phenolic compounds is well known. On the other hand, extracts may be more beneficial than isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extracts [41].

Antibacterial activities: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) against the pathogenic bacteria *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Acinetobacter baumannii* are summarized in table 5. All the tested extracts from Tunisian *T. aphylla* showed antibacterial activity against all tested strains. The minimum inhibitory concentration (MIC) varied according to the nature of the extract. Moreover, the MIC value of the same extract has changed according to the tested organism. The plant organs with the highest antibacterial properties were stems with MIC ranging from 0.078 to 0.625 mg/ml. The strongest inhibitions were obtained for the ethyl acetate stems extracts with MIC values of 0.156; 0.117; 0.117; 0.078 and 0.235 mg/ml against *E. coli*; *P. aeruginosa*; *S. aureus*; *E. feacalis* and *Acinetobacter baumannii* respectively. Our reported results showed that *T. aphylla* extracts have a stronger antibacterial activity towards Gram-positive bacteria than towards Gram-negative, even if some exceptions could be observed. The MIC values of the ethanolic extract of the Saudi *T. aphylla* against *A. baumanni*, *E. coli* and *S. aureus* were the same and equal at 2 mg/ml [42]. It should be noticed that the differences among the results could be related to the use of strains with different resistance profiles, but also to different methodologies used including different solvents for extracts preparation or different techniques to determine MICs.

Table 5. *Antibacterial activities of T. aphylla extracts*

	Leaves extracts				
	PE	DCM	EA	ET	ME
	MIC (mg/mL)				
E. coli (ATCC25922)	0,469	0,235	0,235 MBC=0.156	0,391	0,313
P. aeruginosa (ATCC27853)	0,625	0,235	0,235 MBC=0.156	0,469 MBC=0.625	0,196
S. aureus (ATCC25923)	0,235	0,391	0,235 MBC=0.156	0,625	0,352
E. feacalis (ATCC29212)	2,500	0,313	0,235 MBC=0.156	0,313	0,664
Acinetobacter baumannii	0,625	0,703	0,352 MBC=0.078	0,313 MBC=0.313	0,313
	Stems extracts				
	PE	DCM	EA	ET	ME
	MIC (mg/mL)				
E. coli (ATCC25922)	0,313	0,156	0,156	0,235	0,391
P. aeruginosa (ATCC27853)	0,313	0,235	0,117	0,313 MBC=0.313	0,313
S. aureus (ATCC25923)	0,235	0,313	0,117	0,313	0,469
E. feacalis (ATCC29212)	0,235	0,078	0,078	0,235	0,235
Acinetobacter baumannii	0,313	0,156	0,235	0,469 MBC=0.313	0,625

PE: Petroleum ether, DCM: Dichloromethane, EA: Ethyl acetate, ET: Ethanol, ME: Methanol

Gram-negative bacteria, such as *E. coli*, are characterized by an outer membrane that provides the cell with a hydrophilic surface that is able to exclude certain hydrophobic molecules, therefore imparting an intrinsic resistance of these bacteria to antimicrobial compounds [43]. Small phenolic compounds, such as phenolic acids, would easily cross the membrane and exert their antimicrobial activity [44].

Antifungal activity: *Candida albicans* is the most common cause of human candidal infections. However, several non-albicans species such as *C. tropicalis*, *C. parapsilosis* and *C. glabrata*, have been increasingly implicated in human disease [45, 46]. These non-albicans species are often resistant to conventional therapy [47].

The *T. aphylla* extracts which we have tested at the concentrations of 100 mg/ml against *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis* have not antifungal activities. We have noted that Fluconazole (2 mg/ml) showed inhibition zones of 10, 10, 15 and 25 mm towards *C. albicans*, *C. krusei*, *C. glabrata* and *C. parapsilosis* respectively.

The antifungal activities of *T. aphylla* growing in Saudi Arabian desert against several fungal strains: *Candida albicans*, *Saccharomyces cerevisiae*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Penicillium chrysogenum* were investigated. The methanolic leaves extract showed a MIC value of 8 mg/ml against *Candida albicans* [42]. The study of antifungal activities of petroleum ether, dichloromethane and methanolic extracts prepared from the aerial part of *T. aphylla* at the concentration of 25 µg/ml against *C. albicans* (Fungal Culture Bank of Pakistan (FCBP)#478) showed inhibition zones of 23, 23 and 17 mm respectively [48].

Antimicrobial activity of the total extract may be due to the presence of some aglycones, unstable flavonoid glycosides, or some other bioactive secondary metabolites [49]. Although the mechanism of action of such compounds against fungi is still unknown, their efficacy, availability at low cost, and low toxicity to humans give the phenolic acids and flavonoids potential as natural fungicides [50]. The antimicrobial properties of pure phenolic compounds and polyphenols of different plant extracts against pathogens were investigated. Rashed et al. (2014) [49] reported the antimicrobial potential of m-gallate, gallic acid, luteolin, quercetin, myricetin, myricetin 3-O-α-rhamnoside, myricetin 3-O-β-glucoside, and myricetin 3-O-β-glucuronide against bacteria (*E. coli*, *P. aeruginosa* and *S. aureus*) and fungi (*A. fumigatus, A. versicolor, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron*).

4. Conclusion

The present study investigated antioxidant, antibacterial and antifungal activities of *Tamarix aphylla* (L.) Karst growing wild in TUNISIA. Results showed that *T. aphylla* has interesting antioxidant and antibacterial activities. Further work on phytochemical constituents and purification of individual groups of bioactive components can reveal the exact potential of the plant to inhibit free radicals and several pathogenic microbes.

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