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Full Length Research Paper

Phenolic content, radical scavenging activity and cytotoxicity of *Tamarix nilotica* (Ehrenb.) bunge growing in Egypt

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The radical scavenging activity using 1,1-diphenyl, 2-picrylhydrazyl (DPPH) and cytotoxicity using sulphorhodamine B (SRB) assay of the aqueous methanolic extract of *Tamarix nilotica* (Ehrenb.) Bunge (Tamaricaceae) flowers and its subextracts (CHCl₃, EtOAc and Pet.ether) were evaluated. Total phenolic and flavonoid contents were estimated using colorimetric assays. Ethyl acetate (EtOAc) showed the highest free radical scavenging activity with inhibitory concentration (IC₅₀) 7.25 ± 0.86 µg/ml in addition to potential cytotoxic effect on liver cell carcinoma (Huh-7) (IC₅₀ 49.1 ± 0.96 µg/ml) whereas effect on lung cell carcinoma (A-549) was much lower (IC₅₀: 137.9 ± 1.85 µg/ml). EtOAc had the highest flavonoid content (1.75 ± 1.5 mg/g QE) compared to other subextracts. These results indicated that ethyl acetate fraction contains bioactive compounds worthy of more sophisticated studies as free radical scavenger and cytotoxic agent.

Key words: *Tamarix nilotica*, cytotoxic activity, sulphorhodamine B (SRB) assay, scavenging activity, 1,1-diphenyl, 2-picrylhydrazyl (DPPH) assay, flavonoid, phenolic.

INTRODUCTION

Genus *Tamarix* is the largest genus in family Tamaricaceae (Tamarisk). *Tamarix* is represented in Egypt with two indigenous species which are *Tamarix aphylla* (L.) H.Karst and *T. nilotica* (Ehrenb.) Bunge (Boulos, 1999). *T. nilotica* (Ehrenb.) Bunge has its root deep in the Egyptian history where it was mentioned in ancient papyri in pharaonic times to expel fever, relieve headache, to draw out inflammation and as an aphrodisiac, in addition, it was used in Egyptian traditional medicine as an antiseptic agent (Abouzid and Sleem, 2011). In Egypt, different parts of *Tamarix* are used; the leaves and young branches are cooked for oedema of spleen and mixed with ginger for uterus infections, while the bark, when boiled in water with

vinegar is used as lotion against lice (Boulos, 1983).

Flavonoid and phenolic constituents have been reported from the leaves, roots and flowers of *T. nilotica* (Ehrenb.). Roots revealed the presence of gallic acid derivatives, a lignan (syringaresinol) and isoferulic acid, niloticol, and 3-hydroxy-4-methoxycinnamaldehyde (Barakat et al., 1987). Leaves revealed the presence of nilotinins M1, M4, D2, D3, D7, D8, D9, hirtellins B, C, F, tamarixinin A, 1,2,6-tri-O-galloyl-D-glucose, methyl ferulate 3-O-sulphate, coniferyl alcohol 4-O-sulphate, kaempferol 4'-methyl ether, tamarixetin and quercetin 3-O-beta-D-glucopyranuronide (Orabi et al., 2009, 2010; Abouzid et al., 2009). Moreover, flowers revealed isolation of the methyl and ethyl esters of gallic acid, p-methoxygallic acid, kaempferol, quercetin 3-O-glucuronides, the 3-O-sulphated kaempferol 7,4'-dimethyl ether and the free flavonols, besides the digalloylglucose and niloticin (Nawwar et al., 1982, 1984a, b; Nawwar and Souleman, 1984). However, in spite of intensive studies

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on its constituents, few reports were achieved concerning its biological activities.

Antioxidant and hepatoprotective activities were evaluated for total flower extract (Abouzid et al., 2008; Abouzid and Sleem, 2011), moreover, nilotinib D8 and hirtellin A isolated from leaf extract were found active against the human oral tumor cell lines, human squamous cell carcinoma (HSC-2, HSC-3, and HSC-4) and human promyelocytic leukemia (HL-60) cells (Orabi et al., 2010).

In our investigation to discover new drug candidates from natural sources, *T. nilotica* appeared promising. Phenolic compounds are known to have antioxidant activity (Tepe et al., 2006) in addition, cytotoxic drugs play a major role in cancer chemotherapy (Zunino and Capranico, 1997). Through previous studies, *T. nilotica* is a very rich phenolic source but correlation between bioactive components and biological activity was not well studied. In addition, flowers were not intensively studied as leaves. Therefore, the aim of this study was to enlighten two activities of *T. nilotica* (Ehrenb.) Bunge flowers in total aqueous methanolic and its successive subextracts in relation to phenolic and flavonoid contents. Radical scavenging activity was assessed using DPPH method, cytotoxic activity was tested using SRB assay against two human tumor cell lines, liver (huh-7) and lung (A-549) cancer cell lines while total phenolic and flavonoid contents were measured using colorimetric methods.

MATERIAL AND METHODS

Plant

The flowers of *T. nilotica* (Ehrenb.) Bunge were collected from Ismailia road, Egypt, in October, 2011. Authentication of the plant was performed by Dr. Mona M. Marzouk (PhD), Department of Phytochemistry and Plant Chemosystematics, National Research Center (NRC) of Cairo. Voucher specimen (No RS01) was deposited at the herbarium of Pharmacognosy Department, Faculty of pharmacy MSA University, Egypt.

Chemicals

All chemicals used, including solvents, were of analytical grade. DPPH, folin ciocalteu's phenol reagent, quercetin, and gallic acid were purchased from (Sigma-Aldrich Chemie, Steinheim, Germany).

Preparation of plant extract and successive fractions

The powdered air-dried flowers of *T. nilotica* (Ehrenb.) Bunge (1 kg) were exhaustively extracted with 70% methanol. The combined aqueous methanolic extract was concentrated by evaporation under reduced pressure then, the residue was weighed and suspended in water, then exhaustively defatted with petroleum ether (60 to 80°C) (Pet.ether)(300 × 15). Combined Pet.ether subextracts were evaporated under reduced pressure. Methanol was removed from the remaining extract and diluted with distilled H₂O to 400 ml and

successively extracted with chloroform (CHCl₃) (20 × 500 ml) and Ethyl acetate (EtOAc) (20 × 500 ml). Each solvent extract was evaporated to dryness under reduced pressure to give CHCl₃ (yield: 0.47%) and EtOAc (yield: 5.27%), respectively. The remaining aqueous extract was further extracted with n-butanol (BuOH) (20 × 500 ml) and evaporated to dryness to yield BuOH (yield: 9.5%). The final aqueous phase was also evaporated to dryness (yield: 38.09%)

Biological activity

1,1-diphenyl, 2-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of different plant subextracts was measured spectrophotometrically using the stable free radical DPPH (Shimada et al., 1992).

Total extract and all successive subextracts (CHCl₃, EtOAc, BuOH and aqueous) were dissolved in methanol and screened at 100 µg/ml where the most potent active extracts (gave more than 90%) were assayed at 25 to 75 µg/ml. 0.1 mM solution of DPPH in methanol was prepared. Then, 1 ml of this solution was added to 3 ml of extract solution at different concentration (25 to 75 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was measured at 517 nm carried out in triplicate. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was calculated by the following formula: DPPH scavenging effect (%) = 100 - [(A₀ - A₁) / A₀] × 100], where: A₀ was the absorbance of the control reaction and A₁ was the absorbance in the presence of the sample (Oktay et al., 2003). The concentration of sample required to scavenge 50% of DPPH was calculated from a graph plotted for the % inhibition against the concentration in µg/ml.

Cytotoxic activity

The cytotoxicity of the total extract and its successive fractions was tested against two human cancer cell lines of economical importance in Egypt, liver (Huh-7) and lung (A-549) cells by Sulforhodamine (SRB) assay as described by Skehan et al. (1990). Exponentially growing cells were collected using 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) and plated in 96-well plates at 1000 to 2000 cells/well. Cells were exposed to test extracts [concentrations 0.1, 1, 10, 100, 1000 µg/ml, dissolved in Dimethyl sulfoxide (DMSO)] for 72 h and subsequently fixed with trichloroacetic acid (TCA) (10%) for 1 h at 4°C. After several washings, cells were exposed to 0.4% SRB solution for 10 min in dark place and subsequently washed with 1% glacial acetic acid. After drying overnight, Tris-HCl was used to dissolve the SRB-stained cells and color intensity was measured at 540 nm. The dose response curve of different fractions were analyzed using E_{max} model.

$$\% \text{ Cell viability} = (100 - R) \times \left(1 - \frac{[D]^m}{K_d^m + [D]^m} \right) + R$$

[R] is the residual unaffected fraction (the resistance fraction), [D] is the drug concentration used, "K_d" is the dose of the drug that produces a 50% reduction in cell viability and "m" is a Hill-type coefficient. IC₅₀ was defined as the drug concentration required to reduce absorbance to 50% of that of the control (that is, K_d = IC₅₀, when R = 0 and E_{max} = 100 - R) (Al-Abd et al., 2008). The concentration required to reduce cell viability by 50% (IC₅₀) was

determined using the sigmoid E_{max} model.

Statistical analysis

Data are presented as mean \pm standard deviation (SD)

Phytochemical analysis

Phytochemical screening

The Pet.ether, $CHCl_3$, EtOAc, BuOH subextracts and aqueous remaining, obtained from successive fractionation of flowers of *T. nilotica* (Ehrenb.) Bunge, were exposed to preliminary phytochemical analyses to explore the major classes of active constituents responsible for activity using standard procedures of analysis (Harborne, 1993; Sofowora, 1993; Trease and Evans, 2002).

Quantitative estimation of total phenolic and flavonoid contents

The total phenolic content of each fraction was determined by the Folin-Ciocalteu Reagent (FCR) using gallic acid as standard (Sellappan et al., 2002) and measured at maximal absorption 765 nm. Measurements were carried out in triplicate and calculations based on a calibration curve obtained with gallic acid. The total phenolics were expressed as milligram of gallic acid equivalents (GAE) per milligram dry extract. The total flavonoid content was determined by aluminium chloride colorimetric assay (Kosalec et al., 2004). This method is based on the formation of a complex flavonoid aluminium having the absorptivity maxima at 415 nm. Calculations are based on quercetin calibration curve. The total flavonoid content was expressed as milligram of quercetin equivalent per milligram dry extract.

RESULTS

Biological activities

1, 1-diphenyl, 2-picrylhydrazyl (DPPH) radical scavenging activity

DPPH free radical scavenging activity of different *T. nilotica* subextracts has been screened at 100 μ g/ml. EtOAc (100%), BuOH (93%) and total (90%) exhibited potential antioxidant activity while $CHCl_3$ exhibited the lowest effect (26%), followed by aqueous remaining (75%). Comparing the IC_{50} of promising subextracts (> 90%) with ascorbic acid as positive control (IC_{50} 4.8 \pm 0.54 μ g/ml), EtOAc showed the best effect (7.25 \pm 0.86 μ g/ml), with lower IC_{50} followed by BuOH (8.25 \pm 0.65 μ g/ml) and total extract (45 \pm 0.73 μ g/ml) (Figure 1). These results imply the presence of antioxidant principles in the extracts.

Cytotoxic activity

SRB assay was used to assess the cytotoxicity pattern

(dose-response profile) of $CHCl_3$, EtOAc, BuOH, and aqueous subextracts compared to total extract of *T. nilotica* (Ehrenb.) against two human tumor cell lines Huh-7 and A-549 cell lines. Most of the tested samples exerted cytotoxic activity against hepatocellular carcinoma and lung carcinoma cell lines with different concentrations. The EtOAc exhibited the most potent cytotoxic activity against Huh-7 (IC_{50} 49.1 \pm 0.96 μ g/ml), followed by $CHCl_3$ (IC_{50} 84.5 \pm 1.64 μ g/ml). The resistant fraction of Huh-7 was 0%, which denoted the potency of all fractions on liver cell carcinoma (Table 1). Effects of different subextracts on A-549 was lower, still EtOAc was the most potent (IC_{50} 137.9 \pm 1.85 μ g/ml), followed by $CHCl_3$ (IC_{50} 271.1 \pm 3.23 μ g/ml), while IC_{50} of BuOH and aqueous cannot be detected at the used concentrations. Additionally, substantial R-fraction of A-549 ranged from 6.7 to 15.8%, which indicate the partial resistance of lung cell carcinoma (Table 1).

Phytochemical analysis

Phytochemical screening

Phytochemical screening revealed the presence of flavonoids in all fractions except Pet.ether and remaining aqueous. Sterols were only found in Pet.ether and $CHCl_3$ subextracts. Tannins were present only in considerable amount in EtOAc, BuOH and aqueous subextracts. Additionally, alkaloids, saponins and anthraquinones were totally absent in all extracts.

Phenolic and flavonoid contents

Results of total phenolic and flavonoid contents in their chemical equivalents (gallic acid and quercetin, respectively) are shown in Table 2. Contents of total phenols were measured by (FCR) in terms of gallic acid equivalent (standard curve equation: $y = 0.0011x + 0.0009$, $r^2 = 0.9867$). The total phenolic content varied from 7.37 \pm 0.9 to 119.63 \pm 0.9 mg/g GAE. Total extract showed higher concentration (119.63 \pm 0.9 mg/g GAE), followed by n-BuOH (22.12 \pm mg/g GAE), $CHCl_3$, EtOAc and finally aqueous. Flavonoid content (FC) was measured by aluminum chloride colorimetric assay in terms of quercetin equivalent (standard curve equation: $y = 0.005x - 0.0198$, $r^2 = 0.9774$), FC ranged between 0.44 \pm 1.5 and 2.55 \pm 0.19 mg/g QE. Total extract was higher in concentration (2.55 \pm 0.19 mg/g QE) followed by EtOAc (1.75 \pm 1.5 mg/g QE), $CHCl_3$ (0.79 \pm 2.4 mg/g QE), n-BuOH fraction (0.58 \pm 2.3 mg/g QE) while aqueous subextract appeared devoid of flavonoid content.

DISCUSSION

Herbal medicines are the primary form of healthcare

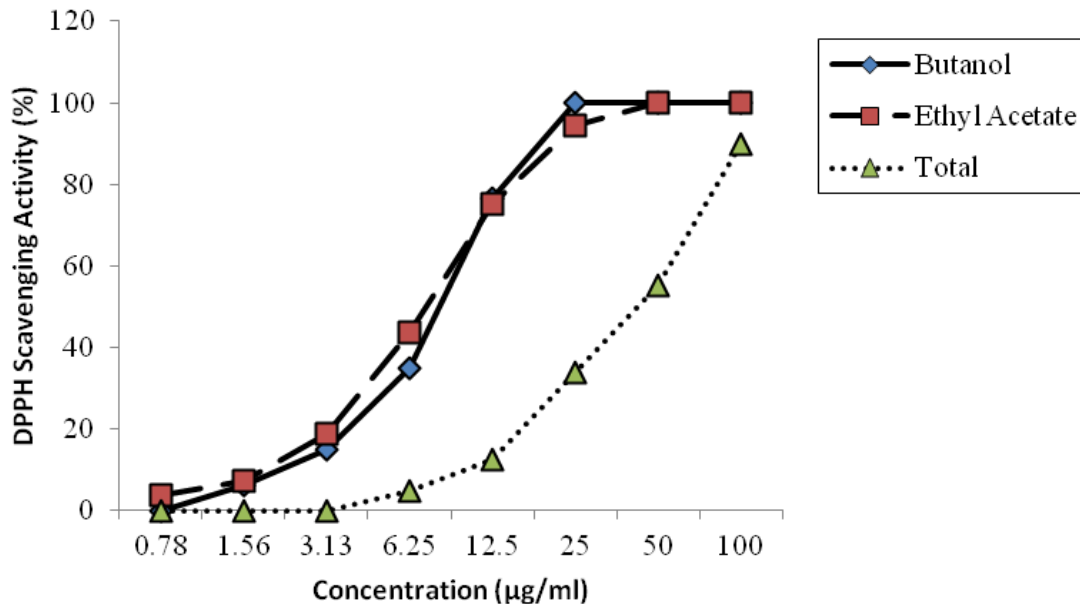


Figure 1. DPPH scavenging activity of butanol, ethyl Acetate and total aqueous methanolic fractions of *Tamarix nilotica* and data are expressed as mean \pm SD (n = 3).

Table 1. Cytotoxicity of *T. nilotica* extracts against different solid tumor cell lines.

Fraction	Cytotoxic effect			
	Huh-7 (liver)		A549 (lung)	
	IC ₅₀ (µg/ml)	R-fraction %	IC ₅₀ (µg/ml)	R-fraction %
Ethyl acetate	49.1 \pm 0.96	0	137.9 \pm 1.85	15.8
Chloroform	84.5 \pm 1.64	0	271.1 \pm 3.23	6.7
Butanol	132.9 \pm 2.23	0	-*	-
Aqueous	285.35 \pm 3.4	0	-	-
Total	162 \pm 2.05	0	727 \pm 3.11	0

*At the maximum used concentration, no cytotoxic effect was observed.

Table 2. Phenolic and flavonoid contents in *Tamarix nilotica* flowers.

Fraction	Total phenolic (mg/g GAE)	Total flavonoid (mg/g QE)
Chloroform	21.67 \pm 2.1	0.79 \pm 2.4
Ethyl acetate	20.6 \pm 1	1.75 \pm 1.5
Butanol	22.12 \pm 2.4	0.58 \pm 2.3
Aqueous	17.2 \pm 1.4	-
Total	119.63 \pm 0.09	2.55 \pm 0.19

Each value in the table was obtained by calculating the average of three experiments \pm standard deviation. GAE = gallic acid equivalent, QE= quercetin equivalent.

known to mankind. Natural products are important sources of antioxidant and anti-cancer lead molecules and this is mainly due to the high degree of diversity and novelty. The increased interest in the measurement and

use of plant antioxidants for scientific research, as well as industrial purposes, is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of

carcinogenesis, in addition to safe and effective use with fewer side effects (Suhaj, 2006; Tadhani et al., 2007). Phenolics are known for their strong reactive oxygen species (ROS) scavenging capacities (Atmani et al., 2009) and inhibition of free radical producing enzymes (Berboucha et al., 2010). In addition, cytotoxic effect of dietary polyphenols has been proved through many studies of tumor cells, therefore these compounds could contribute in the prevention and treatment of cancer (Kampa et al., 2000). DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants (Cao et al., 1997). The degree of its discoloration is attributed to hydrogen donating ability of tested extracts.

In addition, for evaluation of cytotoxic activity, SRB assay offer a simple, rapid, sensitive and inexpensive method for measuring the cellular protein content. It relies on the uptake of the negatively charged pink aminoxanthine dye, SRB, by basic amino acids in the cells. The greater the number of cells, the greater the amount of dye is taken up and after fixing when the cells are lysed, the released dye will give a more intense colour and greater absorbance (Houghton et al., 2007). The types of cancers examined in this study were selected based on epidemiological evidence and high health related problem as representing two main cancer problems present in Egypt. Comparing the efficacy of successive subextracts with the total fraction for their antioxidant and cytotoxic activities, EtOAc showed the best cytotoxic effect against liver cell carcinoma ($49.1 \pm 0.96 \mu\text{g/ml}$) beside the lowest IC_{50} while estimating DPPH scavenging activity ($7.25 \pm 0.86 \mu\text{g/ml}$).

Two colorimetric assays were used to compare the phenolic and flavonoid content extracted by different type of solvents based on Folin-Ciocalteu and AlCl_3 reagents. Although the total extract showed the highest phenolic and flavonoid contents ($119.63 \pm 0.09 \text{ mg/g GAE}$; $2.55 \pm 0.19 \text{ mg/g QE}$) but its effect on hepatocellular cancer cell line (Huh-7) was the least, and its scavenging activity was lower than EtOAc and BuOH. This suggests the presence of other phenolics which did not contribute in this activity. However, the bioactive subextract EtOAc showed the highest flavonoid content ($1.75 \pm 1.5 \text{ mg/g QE}$) representing twice CHCl_3 content and thrice the BuOH.

Findings in this study are in good agreement with previous reports estimating phenolic content of flowers of *T.gallica*, it was $135.35 \pm 7.7 \text{ mg GAE/g}$ with flavonoid $12.33 \pm 2.10 \text{ mg Catechin equivalent/g}$ (Ksouri et al., 2009), while upon fractionation of *T. ramossissima* aqueous acetone extract and estimating the radical scavenging activity, EtOAc showed IC_{50} $8.6 \mu\text{g/ml}$ (Sultanova et al., 2001). In addition, Abouzid et al. (2008) denotes the efficient antioxidant power of the *T. nilotica* flower ($89.34 \pm 0.82\%$ at $500 \mu\text{g/ml}$). This study is the first report concerning cytotoxic activity, phenolic and flavonoid contents of total aqueous methanolic and

successive subextracts of *T. nilotica* flowers.

Conclusion

The investigated species *T. nilotica* (Ehrenb.) Bunge appeared to be a potential good candidate for further phytochemical and chromatographic studies to isolate and identify the bioactive compounds related to the antioxidant and cytotoxic activities.

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