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Editor's choice

Water Extract of *Tamarix gallica* L. as effective Agents against C6 and HeLa tumor cell lines

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Salah Akkal received his PhD. in Organic Chemistry from University of Constantine, Algeria in 2001. He was supervised 4 M.Sc. Theses and 10 Ph.Ds. He is professor in natural product chemistry since 2007 in Chemistry Department, Faculty of science. He has published more than 99 papers in reputed journals and has been serving as an editorial board member of repute and has been serving as a reviewer for many articles in her specialization. He attended more than 30 International Conferences and five workshops. He teaches the general chemistry and natural product courses for the students of Faculty of Science and Faculty of Technology at University of Constantine, Algeria.

Main fields of activity: Natural product isolation (Research of flavonoids and Sesquiterpene Lactones from medicinal plants growing in Algeria. Study of biological activity, Chemotaxonomy, NMR spectroscopic technique

Abstract: *The aerial parts of Tamarix gallica L. taken from three different climatic stages in Algeria (Oum El Bouaghi: Semi-arid, ElTaref: Humid, and Ouargla: Arid) were extracted using boiling distilled water. The crude extracts were subjected to total phenolics and flavonoids quantifications in addition to anti-proliferative assessment against two tumor cell lines namely rat brain tumor (C6) and human cervix carcinoma (HeLa) using BrdU (bromo-deoxyuridine) ELISA(Enzyme-linked immune-sorbent assay) and xCELLigence assay. The total phenolics yield was found to range between 16.14 and 39.32 mg GAE (Gallic acid equivalent)/g of extract and a flavonoids yield ranging between 16.51 and 20.35 mg QE(quercetin equivalent)/g of extract. The various phenolics were identified using HPLC-TOF/MS to highlight hesperidin and rosmarinic Acid as major components. Moreover, the extracts exhibited different levels of antitumor potency against C6 and HeLa cell lines depending upon the climatic stage and the concentration. A good cytotoxic effect was recorded with the species collected from the humid region at 250 µg/mL. On the contrary, the other extracts revealed a weak activity for both tests.*

Keywords*: Tamarixgallica; phenolics; flavonoids; antiproliferative activity; C6; HeLa.*

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Introduction

Cancer is considered as the second foremost cause for death worldwide. It can be caused by both carcinogenic and genetic factors. It is considered as an avoidable malady since the majority of the cancer was caused by the environmental factors [1].

The use of plant extracts has a long-term history in traditions in many countries. Many traditional herbs are well known for remediation of simple illnesses to grave and dangerous diseases, including different types of cancer owing to their efficacy as potential sources of natural antioxidants [2-5].

Recently, various herbal preparations have fascinated many researchers' consideration for cancer treatments. A number of medicinal plants and their bioactive compounds have shown antitumor effects on cancer cells. Currently, over 60% anti-cancer agents are derived from natural sources, including plants, marine organisms, and micro-organisms. A variety of reports have shown the ability of phytochemicals to offer protection against free radical induced disorders due mainly to their content of flavonoids and phenolics in free or complex forms. These compounds have been identified and quantified in several fruits and vegetables and showed a high correlation with antioxidant activity [6-9].

The genus *Tamarix* (Tamaricaceae) is represented in Algeria by ten species. *Tamarix gallica* has been reported to be useful in leucoderma, spleen trouble and eye diseases. Antimicrobial and antioxidant activity of *Tamarix gallica* have also been reported. It can be used as prophylactic and therapeutic remedies to cure malaria. Moreover, it is reported to have anticancer effect on human colon cancer cells involving Erk1/2 and p38 action on G_2/M cell cycle arrest [10-14].

In our study, we have carried out the phenolics assessment and the antitumor activity of water extracts of *Tamarix gallica* collected from three different climatic stages in Algeria.

Materials and Methods

Soil and climatic data

The geographic areas covered in this study are Oum El Bouaghi (semi-arid), ElTaref (humid) and Ouargla (~~3.~~ arid). The climate data during 2015 were obtained from the available climate database of the National office of Meteorology, Algeria.

Table 1.: Geographical and Soil Characteristics of Different Collection Sites

parameters	semi-arid	humid	arid

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Latitude and Longitude	35°52′31″ N, 7°06′48″ E	36°46′01″ N, 8°18′49″ E	31°56′57″ N, 5°19′30″ E
Altitude (meters above sea level)	925	24	138
Soil			
Clay %	27.94	19.82	0.83
Silt %	48.36	33.51	25.94
Sand %	23.7	46.66	73.23
Texture	clay loam	clay loam	Silty loam
EC(electric conductivity)	0.54	0.34	13.73
pH	7.81	7.06	7.64
$CaCO_3$ T %	23.22	0.00	3.44
$CaCO_3$ A %	16.38	0.00	0.00
OM(organic matter)	2.53	4.65	2.11

Table 2: Analyses Methods

Analysis parameter	**Method**
Particle size (%),	pipette method ROBINSON ISO, 11277
pH Rapport ½.5 at 25°c	Soil referential; 1995
EC (dS/m) rapport 1/5 at 25°c	FAO, 1985
$CaCo_3$ T (%)	G.E.P.P.A
OM (%)	Brochure OLIB

Table 3: Climatic data of the three selected sites in 2015

Month	**Average Mean Temperature °C**	**Average Humidity, rate %**	**Relative precipitation, in mm**	**Relative Evaporation, in mm**

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	semi-arid	humid	arid	semi-arid	humid	arid	semi-arid	humid	arid	semi-arid	humid	arid
Jan	5.9	11.3	11.4	81	85	52	39	124	1	49	27	93
Feb	5.7	11.8	14.2	80	83	56	52	82	24	41	19	91
Mar	9.6	13.7	17.4	71	73	47	22	68	4	86	40	158
Apr	14.3	15.5	24.4	60	74	36	3	53	0.0	114	31	256
May	19.1	19	28.9	54	70	38	16	39	0.0	189	38	334
June	22.4	23	32.3	52	68	30	10	16	0.0	250	48	365
July	26	25.7	34.3	46	63	30	27	3	0.0	259	52	346
August	25.9	26.5	36	51	65	38	68	5	0.0	208	51	366
Sept	22.2	24.5	30.9	56	67	45	6	37	1	162	41	233
Oct	17.4	20.2	24.8	58	73	45	46	72	0.0	184	31	158
Nov	11.2	16	17.7	77	80	56	44	90	0.0	51	19	98
Dec	7.8	12.4	12.5	69	82	54	43	105	0.0	48	10	95
average	**15.62**	**18.30**	**23.73**	**62.91**	**73.58**	**43.91**	**31.33**	**57.83**	**2.5**	**136.75**	**33.91**	**216.08**

Plant material

The aerial parts of *Tamarix gallica* were collected from Oum El Bouaghi (semi-arid), El Taref (humid) and Ouargla (arid). The plant was identified by Pr. A. Zellagui, Oum El Bouaghi University, Algeria. Voucher specimens (TG1, TG2, and TG3) were deposited in the Laboratory of Natural Resources and Management of Sensitive Environments, University of Oum El Bouaghi, Algeria

Water extract:

500 mL of boiling distilled water were added to 100 g of finely ground dry plant material. After 60 min the solution was filtered and dried under vacuum and kept at 4°C in the dark until further analysis.

Total phenolics content :TPC

The Total phenolics content of each extract was determined using the folin-ciocalteau reagent (FCR) method according to the method of Singleton *et al* [15]. Each extract diluted with methanol (0.5 mL) was added to 2.5 mL of FCR (diluted 1/10 with distilled water) and mixed. After 5 min of agitation, 2 mL of sodium carbonate water solution Na_2CO_3 (75g/L) was added to the mixture and incubated at 40 °C for 30 min. Results were expressed as mg of Gallic acid equivalent (GAE/g of dry extracts). All samples were analyzed in triplicates.

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Total flavonoids content:TFC

The total flavonoids content of each extract was estimated according to the colorimetric method using aluminum chloride [16]. This method based on the formation of a complex flavonoid-aluminum having the maximum absorbance at 430 nm. The extracts (1 mL) were mixed with 2% $AlCl_3$methanol solution (1 mL) and the absorbance at 430 nm was determined using UV-VIS spectrophotometer. The total flavonoids content was expressed as mg quercetin equivalent/g dry extract. All samples were analyzed in triplicates.

HPLC-TOF/MS Analysis

Phenolic contents were identified using Agilent Technology of 1260 Infinity HPLC (high performance liquid chromatography-) System (Stevens creek, Santa-Clara, USA) joined with 6210 Time of Flight (TOF) LC/MS (liquid chromatography Mass Spectrometry detector (same company) and ZORBAX SB-C18 (4.6 × 100mm, 3.5μm) column (same company). Mobile phases were ultra-pure water with 0.1% formic acid (A) and acetonitrile (B), respectively. Flow rate, column temperature and injection volume were 0.6 mL min^{-1}, 35°C and 10 μL, respectively. The solvent rates were 10% B (0–1 min), 50% B (1–20 min), 80% B (20–23 min), 10% B (23–25 min), and 10% B (25–30 min). The phenolic compounds were determined using the standards with the samples in terms of retention times and *m*/*z* values. Ionization mode of HPLC-TOF/MS instrument was negative and operated with a nitrogen gas temperature of 325 °C, nitrogen gas flow of 10.0 L min^{-1}, nebulizer of 40 psi, capillary voltage of 4000 V, and, finally, fragmentor voltage of 175 V, 200 ppm of dried crude extracts were dissolved in methanol at room temperature for sample analysis and were filtered passing through a PTFE (0.45 μm) filter by an injector to remove particulates.

Determination of in vitro anti-proliferative activity

Anti-proliferative activities of the three extracts were evaluated *in vitro* by estimation of the inhibitory effect on the growth of rat brain tumor (C6) and human cervix carcinoma (HeLa) cell lines using BrdU ELISA and xCELLigence assays [17].

Cell culture

The cells were grown in DMEM (Dulbecco's modified eagle's medium) (Sigma, Munich, Germany), supplemented with 10% (v/v) fetal bovine serum (Sigma, Munich, Germany) and PenStrep solution (Sigma, Munich, Germany) at 37°C in a 5% CO_2 humidified atmosphere [18].

Cell proliferation assays

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- ELISA (Enzyme-linked immune-sorbent assay):

The cells were plated in 96-well culture plates (COSTAR, Corning, NY. USA) at a density of 30000 cells per well. The activities were investigated at250, 100 and 50 µg/mL of extracts in methanol. The cells were then incubated overnight before applying the BrdU Cell Proliferation ELISA assay reagent (Roche, Berlin, Germany) according to the manufacturer's procedure. The amount of cell proliferation was determined at 450 nm by using a microplate reader (Awareness Chromate Minnesota, USA). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. The stock solution of the extracts was prepared in dimethyl sulfoxide (DMSO) and diluted with DMEM. DMSO final concentration is below 0.1% in all tests. 5-FU was used as standard compound. Percentage of inhibition of cell proliferation was calculated as follows [19].

$[1-(A_{samples} / A_{control})] \times 100$

- xCELLigence assay

The ability of extracts was analyzed to induce cell growth of HeLa cell line using a real-time cell analyzer–single plate (RTCA-SP) instrument (Roche Applied Science, Basel, Switzerland). The electronic cell sensor array was newly developed as xCELLigence RTCA and performed with a recently published literature method at the concentrations of 250, 100 and 50 µg/mL [20]**.**

The disposable E-plate was employed for 96 xCELLigence system with a control and medium. The measurements base on the impedance difference was caused by the cells attached to the plates.

The medium was added to each well and placed into the incubator. E-plate 96 was inserted to the xCELLigence station, and the background impedance was measured during 1 min. Then, 50 µL of each cell suspension was placed in medium containing wells and adjusted to 20000HeLa cells mL^{-1}. The plate was held in a sterile cabinet at room temperature for 30 min for attaching the cells to the E-Plate 96 wells. Finally, the cells were monitored every 10 min for adhesion, growth and proliferation in a period of up to 3 h *via* the incorporated sensor electrode arrays. The extracts were added to wells and adjusted to the concentrations of 250, 100, and 50µg/mL. The plates were placed in the incubator immediately and monitored every 10 min during 48 h. The tests were replicated and repeated three times given with standard deviation bars.

Statistical analysis

The results of *in vitro* investigation of anti-proliferative activity are means ± SD of six measurements. Differences between groups were tested with ANOVA. p values of <0.01 were

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considered as significant and analyzed by SPSS (version 11.5 for Windows 2000, SPSS Inc.. Chicago, USA).

The results of total phenolics and flavonoids were performed from the averages of samples reading mean ±SD (standard deviation) using excel 2003. All analyses were carried out in triplicates.

Results and Discussion

Total phenolics and flavonoids

As evaluated by the appropriate methods, the total phenolics and flavonoids are presented as mg GAE/g DW and QE/ g DW of extracts and displayed in table 4.

Table 4: Total phenolics and flavonoids for the three samples

site	Total phenolics(mg GAE/g DW)	flavonoids (QE/g DW)
Semi-arid	039.32±0.15	16.51±0.18
Humid	133.84±0.22	20.35±0.13
Arid	16.14±0.01	17.20±0.05

It is worth noting that the amount of phenolics in the plant collected from humid region is higher than other samples which depicted the important impact of climatic factors on this class of secondary metabolites.

High Performance Liquid Chromatography Analysis

The phenolics composition analyses of the two extracts were performed using HPLC-TOF/MS analysis. eleven compounds were detected in the three extracts including Hesperidin and Rosmarinic Acid as major components. The compounds were identified by comparing their chromatographic characteristics (retention time (t_R), mass spectra) with reference standards (Table 5).

Table 5: Composition of water extracts determined by HPLC-TOF/MS (mg of phenolic compound/kg plant).

Compounds	(Semi-arid)	(Humid)	(Arid)
Gentisic Acid		5.451	3.082
Chlorogenic Acid		136.058	55.251
4-Hydroxy Benzoic Acid		57.027	43.791
Protocatechuic Acid	16.898	16.627	16.454
Caffeic Acid	8.343	21.355	9.575
Vanilic Acid		25.723	
Rutin		14.080	0.371
Ferulic Acid		8.998	2.042
Hesperidin	**3752.284**	**6369.896**	**11194.851**

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Rosmarinic Acid	8.298	**1778.545**	**1943.1755**
Cinnamic Acid	70.272	66.999	191.415

Anti-proliferative activity

C6 cell lines.

The antiproliferative activity of the three extracts and 5-FU were determined against C6 cells and showed almost no impact the samples collected in arid and semi-arid regions. Nonetheless a good potency was observed for the species collected in the humid region which involved a relative abundance in term of phenolics (table 2). Some reports stated the antitumor effect of some detected phenolics such as Hesperidin and Cinnamic Acid which support further the present findings [21-23].

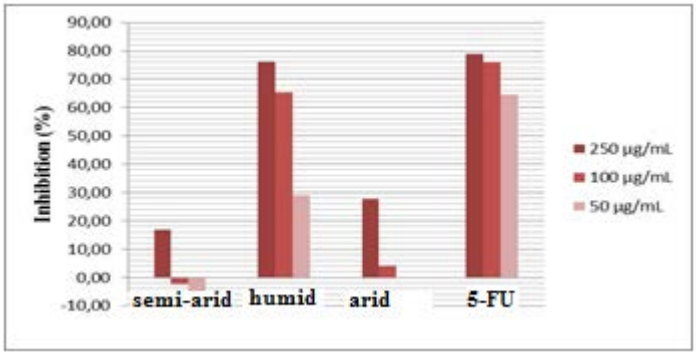

Figure 1. Antiproliferative activities of the three extracts and the standard 5-FU against C6 cell lines at three concentrations. Each extract was tested twice in triplicate against cell lines. The data show the average of two individual experiments ($p < 0.01$).

HeLa cells

The antiproliferative effect of the three extracts was studied on HeLa cell lines at 50, 100 and 250 µg/mL by using real time cell analyzer xCELLigence technique. The system quantifies the impedance variation in order to determine the Cell Index (CI)values at time points whose periods can be set by the operator. These impedance variations and thus the CI values depend on the cell activity at the base of the wells [24]. CI is a dimensionless parameter resulting as a relative change in measured electrical impedance to represent cell status. If it is decreasing, it shows that the tumor cells are dying. The results showed that all extracts displayed variable effect against HeLa cell lines. Meanwhile the plant extract from the humid region averred to be more potent especially at 250 µg/mL.

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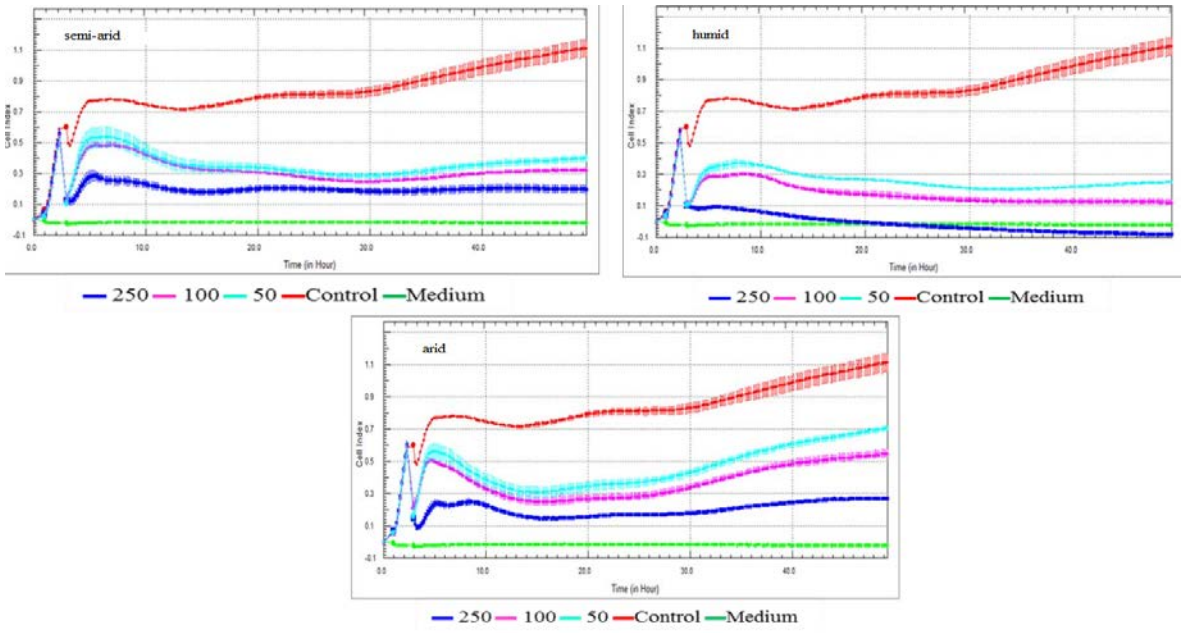

Figure 3. Antiproliferative activity of the water extracts of *T. gallica* using xCELLigence RTCA instrument

Only a few studies were reported about the antiproliferative effect of this plant. A report stated the anticancer effect of *T. gallica* on human colon cancer cells and the possible primary mechanisms. At 50 and 100 µg/mL, the extracts of different parts greatly inhibited Caco-2 cell growth. It was found that, roughly all plant part extracts inhibited cell growth by 62 % at the concentration 100 µg/mL. DAPI staining results showed that these extracts decrease DNA synthesis and confirm their effect on Caco-2 cells proliferation, mostly at 100 µg/mL. More prominently, cell mitosis was arrested at G2/M phase. The changes in the cell-cycle-associated proteins (cyclin B1, p38, Erk1/2, Chk1, and Chk2) are correlated with the changes in cell cycle distribution [14]. Another study reported the potential of Methyl ferulate from *Tamarix aucheriana* to control cell growth, cell cycle, apoptosis, generation of reactive oxygen species (ROS), cancer cell invasion, nuclear factor kappa B (NFkB) DNA-binding activity and proteasomal activities, as well as the enhancement of chemo-sensitivity in human colorectal cancer cells, were evaluated. The possible molecular mechanism of MF's therapeutic efficacy was also assessed [25].

Conclusion

The present study is a modest contribution to deepen the knowledge about this widespread plant and its therapeutic uses. The amount of total phenolics in plant extracts affirmed to be well correlated

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with the biological activity. It was shown through two antiproliferative tests that the water extract of *Tamarix gallica* collected from the humid climatic stage exhibited a good potency when evaluated by BrdU ELISA and xCELLigence. Based on these results the plants can be a potential source of natural cytotoxic agents and *in vivo* studies are needed to further confirm the advantageous quality of these species.

Conflict of Interest: The authors declare no conflict of interest.

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