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ANTIPROLIFERATIVE ACTIVITY OF THREE WILD GROWING SPECIES IN TUNISIA: *NICOTIANA GLAUCA, ARTEMISIA CAMPESTRIS* AND *ASTRAGALUS GOMBO*

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ABSTRACT: Tunisian flora contains several wild plants with diverse therapeutic uses. As a contribution to the efforts to select natural sources of antitumor compounds, we investigated in this study the antiproliferative activity of several extracts of the Tunisian species: *Astragalus gombo*, *Nicotiana glauca* and *Artemisia campestris*. Dried aerial part (*A. gombo* and *A. campestris*) and leaves (*N. glauca*) were extracted with ethyl acetate, dichloromethane, and methanol. In the vitro anti-proliferative activity of the extracts was tested against the human solid tumor cell lines: HBL-100, T-47D, and WiDr. Tests were performed using the sulforhodamine B (SRB) assay. All extracts of *A. campestris* and *A. gombo* were active against all tested cell lines, with GI_{50} values between 12 and 93 µg/ml. Dichloromethane extract of *A. campestris* was the most active extract with GI_{50} of 12 µg/ml against HBL-100 cell line. This kind of cell seems to be the most sensitive regarding all tested extracts. Our results showed that *A. gombo*, *A. campestris* and *N. glauca* are a promising source of natural antitumor compounds. More detailed studies should be conducted especially for most active extracts.

INTRODUCTION: Cancer remains a serious problem for public health. Natural products play a highly significant role in the drug discovery and development process, particularly in the areas of cancer. With the increased resistance to the used anticancer drugs, increasing interest has been shown in research of new compounds from plants. Over 60% of these drugs were shown to be of natural origin [1].

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Several species represent a significant biological potential and have been a source of active compounds for antitumor effects and cancer therapy adjuvant. Among species of Tunisian flora, several were traditionally used for various diseases.

To adapt to different stresses especially in desert zones, plants synthesize several metabolites and are thus a promising source of active molecules. These metabolites confer to these species biological activity responsible for their therapeutic use, like for *Artemisia*, *Nicotiana*, and *Astragalus* genus. Our previous studies about *Astragalus gombiformis* Pomel showed that this plant has cytotoxic, antibacterial and anticholinesterasic activities [2, 3]. Many other investigations suggest that *Artemisia*

campestris could be beneficial for protection against diabetes and its complications, and showed that this plant exhibited antioxidant and anti-bacterial properties [4-7]. Other species of this genus, *Artemisia princeps*, is a potential anti-endometriotic agent that induces apoptosis of endometrial cells [8].

Nicotiana glauca R.A. Graham, also called wild tobacco or tree tobacco, can be a source of benefit molecules such as 7-dehydrocholesterol, vitamin D_3, and other vitamin D_3-related compounds [9, 10]. *N. glauca* was used as medicinal smoke for the treatment of the ear and general skin diseases [11]. It has a hepatoprotective effect [12]. This species is toxic for animals and human can be accidentally poisoned [13, 14]. Main toxic principles of *N. glauca* are the two alkaloids nicotine and anabasine, which possess a similar structure. Others alkaloids have been identified in *N. glauca* such as anatabine, metanicotine and myosmine [15-17]. The anti-oxidant, antimicrobial and antiacetylcholinesterase activities of Tunsisian *N. glauca* was recently studied by Sellem *et al*., (2016) [18].

With many of the studies of the biological activities of plants, they remain dependent to several factors such as the natural environment of growth, the season when the plant was collected, the solvent used for extraction and the experimental conditions. We evaluate in this study the antitumor activity of three Tunisian species, *Astragalus gombo*, *N. glauca*, and *A. campestris* as a contribution to the efforts to select natural sources of anticancer compounds.

MATERIAL AND METHODS:

Plant Collection and Extracts Preparation

Samples: Aerial parts of *A. gombo* (February) have been collected on flowering stage from Djerba island in Southeast of Tunisia. Leaves of *N. glauca* and total aerial part of *A. campestris* have been collected on October from the region of Ben Gardane in Southeast of Tunisia. After air-drying in shadow, the samples have been powdered and conserved until use.

Solvents: Methanol and ethyl acetate were both from Lab-Scan. Dichloromethane and dimethyl sulphoxide (DMSO) were respectively from Carlo Erba and Sigma (St Louis, MO).

Extract Preparation: Different extracts were prepared by direct overnight maceration with organic solvents **Table 1**. Then, solvents were evaporated by rotavapor. All residues were dissolved in DMSO at 100 mg/mL concentrations as the stock solution. Both cisplatin and etoposide were used as positive control.

TABLE 1: PREPARATION OF TESTED EXTRACTS WITH DIFFERENT SOLVENT

Plant	Solvent (200 ml)	A quantity of sample (g)	Yield (%)
A. campestris	Methanol	10	7.18
	Dichloromethane	16	6.18
	Ethyl acetate	20	6.5
A. gombo	Methanol	10	7.17
	Dichloromethane	20	0.99
	Ethyl acetate	20	2.82
N. glauca	Methanol	7.5	11.38
	Dichloromethane	12.5	5.08
	Ethyl acetate	20	4.89

Antitumor Activity: All starting materials were commercially available research-grade chemicals and used without further purification. RPMI 1640 medium was purchased from Flow Laboratories (Irvine, UK), fetal calf serum (FCS) was from Gibco (Grand Island, NY), trichloroacetic acid (TCA) and glutamine were from Merck (Darmstadt, Germany), and penicillin G, streptomycin, DMSO and sulforhodamine B (SRB) were from Sigma (St Louis, MO).

Cells, Culture and Plating: The human solid tumor cell lines HBL-100 (breast), T-47D (breast) and WiDr (colon) were used in this study. These cell lines were a kind gift from Prof. Godefridus J. Peters (VU Medical Center, Amsterdam, The Netherlands). Cells were maintained in 25 cm^2 culture flasks in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine at 37 ºC, 5% CO_2, 95% humidified air incubator. Exponentially growing cells were trypsinized and resuspended in antibiotic containing medium (100 unit's penicillin G and 0.1 mg of streptomycin per ml). Single cell suspensions displaying >97% viability by trypan blue dye exclusion were subsequently counted. After counting, dilutions were made to establish the appropriate cell densities for inoculation onto 96-well microtiter plates. Cells were inoculated in a volume of 100 µl per well at densities of 10 000 (HBL-100), 15 000 (T-47D), and 20 000 (WiDr) cells per well, based on their doubling times.

Chemosensitivity Testing: Chemosensitivity tests were performed using the SRB [19] assay of the NCI with slight modifications. Briefly, plant extracts were initially dissolved in DMSO at 400 times the desired final maximum test concentration of 250 µg/mL. Control cells were exposed to an equivalent concentration of DMSO (0.25% v/v, negative control). Each agent was tested in triplicate at different dilutions in the range of 2.5-250 µg/mL. The drug treatment was started on day 1 after plating.

Drug incubation times were 48 h, after which cells were precipitated with 25 µL ice-cold 50% (w/v) trichloroacetic acid and fixed for 60 min at 4 ºC. The SRB assay was then performed. The optical density (OD) of each well was measured at 492 nm, using BioTek's Power Wave XS Absorbance Microplate Reader. Values were corrected for background OD from wells only containing a medium. The percentage of growth (PG) was calculated concerning untreated control cells (C) at each drug concentration level based on the difference in OD at the beginning (T_0) and end of drug exposure (T), according to NCI formulas [20].

Therefore, if T is greater than or equal to T_0, the calculation is $100 \times [(T–T_0)/(C–T_0)]$. If T is lower than T_0 denoting cell killing, the calculation is $100 \times [(T–T_0) / (T_0)]$. The effect is defined as the percentage of growth, where 50% growth inhibition (GI_{50}) represents the concentration at which PG is +50. With these calculations, a PG value of 0 corresponds to the amount of cells present at the beginning of drug exposure, while negative PG values denote net cell kill.

RESULTS AND DISCUSSION: In total, nine extracts from *N. glauca*, *A. campestris*, and *A. gombo* species were assayed for their anti-proliferative activity against three human solid tumor cell lines: HBL-100 (breast), T-47D (breast), and WiDr (colon).The results are summarized in **Table 2**. All extracts of *A. campestris* and *A. gombo* were active against all cell lines. The GI_{50} values were in the range 12-93 µg/ml. Dichloromethane extract of aerial part of *A. Campestris* showed GI_{50} of 12 µg/mL against HBL-100 cells, and it was the most active. In fact, HBL-100 seems to be the most sensitive regarding all tested extracts. *N. glauca* exhibited activity only against this cell line. For T-47D and WiDr, this species showed GI_{50} above 250 µg/mL. It is the first time, that *N. glauca* and *A. gombo* Tunisian species were tested for anti-proliferative activity against human solid tumor cell lines. However, *A. campestris* was previously studied for this activity by [21].

TABLE 2: *IN-VITRO* ANTIPROLIFERATIVE ACTIVITY OF PLANT EXTRACTS AGAINST HUMAN SOLID TUMOR CELL LINES EXPRESSED AS GI_{50} VALUES GIVEN IN µg/mL

Plant	Extract	HBL-100	T-47D	WiDr
A. campestris	Methanol	24	45	45
	Dichloromethane	12	49	43
	Ethyl acetate	13	46	46
A. gombo	Methanol	49	57	42
	Dichloromethane	40	53	57
	Ethyl acetate	74	87	93
N. glauca	Methanol	82	>250	>250
	Dichloromethane	83	>250	>250
	Ethyle acetate	164	>250	>250
Control 1	Cisplatin	0.6	4.5	7.9
Control 2	Etoposide	0.8	12	14

In their study, the plant was collected from Beni-Khedache, a mountainous region in the Southeast of Tunisia at around 150 km from our region (Ben Gardane), which is a few km from the sea. Also, there is also a difference between tested extracts and used cells. These authors showed that *A. campestris* possess antitumor activity, with a positive correlation between this activity and the antioxidant activity. Using HT-29 human adenocarcinoma cell line, GI_{50}values were more than 100 and 1920 µg/mL for ethanol-water and infusion extracts of aerial part, respectively. Because WiDr is considered a derivative of HT-29 [22], our results indicate that dichloromethane seems better solvent to extract the anti-proliferative compounds present in the aerial parts of *A.*

campestris. The essential oil was found the most active with GI_{50} of 46.8 µg/mL [21]. Our results, in agreement with previous data, show the antiproliferative potential of *A. campestris* [4, 5]. Ethanolic leaf extracts of *Artemisia campestris* var. *glutinosa* and *A. molinieri*, showed activity against mosquito *Culex pipiens* larvae, with low calculated lethal concentrations 50%, after 48 h of exposure [23].

Concerning *N. glauca*, few studies investigating the biological activity of this species were reported. Another Tunisian group studied the antifungal activity of *N. glauca* leaf and flower collected from the region of Monastir. Their results revealed an important antifungal effect of aqueous extracts at all concentrations tested (1, 2, 3 and 4%) [24]. A recent study investigating *N. glauca* from Egyptian flora showed that cytotoxicity of methanol extract of flowers and leaves was 25.5, 34.2, 24.3, and 1.6 % against MCF-7, HCT-116, HepG 2, and A-549 cell lines respectively [25].

In our knowledge, it is the second study about the biological properties of *A. gombo* after our previous work concerning essential oil of this plant [26]. This genus is widely studied. *Astragalus*-based herbs were known in Chinese Pharmacopoeia. This study focuses on another *Astragalus* species with antitumor effect. Recent reports were oriented to investigate the mechanism of action of *Astragalus* metabolites. Kim *et al.*, (2013) [8] showed that *Astragalus* polysaccharides are a potential anti-endometriotic agent. It induces apoptosis of endometrial cells by the modulation of the p38 and NF-kB pathways. *Astragalus* polysaccharides also improved experimental TNBS-induced colitis in rats through regulation of TNF-a, IL-1b and NFATc4 expression [27].

CONCLUSION: In the frame of selection of interesting wild Tunisian species, this study was focused on the evaluation of the antitumor potential of three wild growing plants. Extracts of *A. campestris* and *A. gombo* were active against all tested cell lines, with GI_{50} under 100 µg/mL. Dichloromethane extract of *A. campestris* was the most active extract, with GI_{50} equal to 12 µg/mL against HBL-100 cell line. Our results show that *A. gombo*, *A. campestris* and less *N. glauca* are a promising sources of anticancer natural compounds. Further studies should be performed to investigate the most active extracts.

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CONFLICT OF INTEREST: Nil

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