WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 7, 350-357. Research Article ISSN 2277–7105

ANTIOXIDANT AND CYTOTOXIC FRACTIONS OF SELECTED *ALLIUM* SP. GROWING IN GEORGIA

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Article Received on
07 May 2017,

Revised on 26 May 2017,
Accepted on 16 June 2017

DOI:10.20959/wjpr20177-8780

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ABSTRACT

Steroidal saponins are widely distributed among monocots, including the Alliaceae family to which the Allium genus. Apart from sulfur compounds, these are important biologically active molecules that are considered to be responsible for the observed activity of Allium species, including antifungal, cytotoxic, enzyme-inhibitory, and other. In this paper, literature data concerning chemistry and biological activity of steroidal saponins from the Allium genus has been reviewed. Medicinal plants have been investigated for possible anti-cancer effects. The aim of the present study was to examine the cytotoxic and antioxidant activity of several plants of Georgia. 3 selected plant species which have been used in folkloric prescriptions were collected from different sites of Georgia. The fractions and individual steroidal saponins of the plants were prepared and their cytotoxic and antioxidant effects on human cancer cell lines Hela (epithelioid cervix carcinoma) were examined using the MTT and DPPH assay.

KEYWORDS: Allium, cytotoxic and antioxidant action.

INTRODUCTION

The genus Allium are herbaceous geophytes is characterized by bulbs enclosed in membranous tunics, that may become fibrous and may be carried on rhizomes, with tepals that are free or almost free, and a subgynobasic style. Many species are xerophytic and the over 850 species are found almost exclusively in the Northern hemisphere, being particularly diverse in the warm dry summers and cool wet winters of the Mediterranean.[9]

The most characteristic constituents in *Allium* plants are sulfur compounds, which are the most important substances both in terms of chemotaxonomic value and biological

activity.[8,14] However, various researchers tend to attribute the potential pharmacological benefits of *Allium* plants to constituents other than sulfur compounds, such as steroidal saponins. Also, polyphenolic compounds, especially flavonoids, as well as fructans, *N*-cynnamic amides, and antioxidative enzymes are considered to be equally important.[1,10,11,12,16] Steroidal saponins from the genus *Allium* can be divided into three groups on the basis of the sapogenin structure: spirostanols, furostanols, and open-chain saponins. The latter group is often referred to in the literature as "cholestane saponins".[19] *Allium* saponins are mostly mono- or bidesmosides, however a tridesmodic cholestane glycoside has been reported in the bulbs of *A. macleanii*.[18] The sugar residue in *Allium* saponins consists of linear or branched chains made up most often of glucose (Glc), rhamnose (Rha), galactose (Gal), xylose (Xyl), and arabinose (Ara) units.

Allium species, is widely used in Georgian traditional medicine as an antifungal, antiseptic and antibacterial remedy.[4] Various secondary metabolites were identified in genus Allium.[7] Among them, steroidal saponins have been investigated for their antibacterial, antifungal[2] and antioxidant activities.[17] Furthermore, steroidal saponins isolated from different species of onions showed a significant cytotoxic activity against murine fibrosarcoma, lung carcinoma,[3] human melanoma[6] and human leukemia.[13] Most importantly, these compounds are used as substrates in the production of steroid hormones and drugs.

Cancer is one of the main causes of death all over the world. The world health organization (WHO) estimates that 84 million people would die of cancer between 2005 and 2015.[5] Accordingly, much effort has been made to develop various approaches to reduce the threat caused by cancer. Chemotherapy is an important option in modern cancer treatment, and many clinically available anticancer drugs are currently used to treat some types of leukemia, lymphoma and solid tumors.[15]

To find new herbal compounds with anticancer an antioxidant effects, this study focused on selected plants of Georgia those which have been used in folkloric prescriptions, themselves, or other species from this genus. The selection of plants was based on different literature sources, folklore and traditional medicine. Plants were chosen according to their use against sympatology suggestive of cancer including: abscesses, infected wounds, inflammation, skin disorders, ulcers, perforation.

EXPERIMENTAL

Materials: *Allium atroviolaceum Boiss., A. waldsteinii Don.* and *A. fuscoviolaceum Fomin.* were collected in Georgia in june 2013 and identified by Dr. Tsiala Gviniashvili, a botanist from the institute of Botany. Voucher specimens N 7352, N 7387 and N 7418 were deposited in the herbarium at the Department of Pharmacognosy, Faculty of Pharmacy, Tbilisi State Medical University.

Extraction and purification of active fractions: A 200 g above ground of the dried *Allium atroviolaceum Boiss., A. waldsteinii Don.* and *A. fuscoviolaceum Fomin*. powder was mixed separately in 80% methanol (500 ml) and kept in the shaking incubator at 25^{o} C for 2 days and ultered in vacuum using Whatman ulter paper. Later, solvent fractionation of methanol extract (Me-ex) was further fractioned using a liquid-liquid extraction technique with hexane (H-fr), chloroform (Chlo-fr) and ethyl acetate (Ethyl-fr) solvents. After solvent fractionation organic fractions were evaluated for cytotoxic and antioxidant activities.

Isolation and identification of active compounds: The dried bulbs of *Allium atroviolaceum Boiss., A. waldsteinii Don.* and *A. fuscoviolaceum Fomin.* (700 g) were extracted separately twice with 80% methanol for 2 hours each. The alcoholic extracts was concentrated under reduced pressure, suspended in water and then separately passed through Diaion HP-20 column using EtOH-H_20 gradient system (0-100%). The 80% MeOH eluate fraction (10, 11 and 10.5 g), which was subjected separately to silicagel column chromatography with $CHCl_3$-MeOH-H_2O (9:1:0.1; 8:2:0.2; 7:3:0.5; 6:4:0.8) and MeOH finally, gave nine fractions. Fraction 6 was further purfied by ODS column chromatography eluting with MeOH-H_2O (3:7; 4:6; 5:5) and repeated Rp-18 HPLC preparation to yield trilin (10.2 mg), dideglucoeruboside (15.5 mg), aginoside (10.7mg), eruboside B (18.7mg). The compouods were identified of their spectral data (^{13}C, ^{1}H, COSY, HMBC. HSQC) by comparison with an authentic sample.

Acid hydrolysis: Each saponin (5 mg) was heated in an ampoule with 15% HCl (5 mL) at $110^{O}C$ for 2h. The aglycon was extracted with dichloromethane three times and the aqueous residue was evaporated under reduced pressure. Then, pyridine (1 mL) and NH_2OH. HCl (2 mg) were added to the residue, and the mixture was heated at $100^{O}C$ for 1 h. After cooling Ac_2O (0.5 ml.) was added and the mixtures were heated at $100^{O}C$ for 1h. The reaction mixtures were evaporated under reduced pressure, and the resulting aldononitrile peracetates were analyzed by GC-MS using standard aldononitrile peracetates as reference samples.

Cell lines and culture medium: Hela (epithelioid cervix carcinoma, human) Cell lines were obtained from the American Type Culture Collection (Rockville, MD). Hela cells were maintained in continuous culture in DMEM medium (Bio Whittaker) grown at $37^{O}C$ in humidified 5% CO_2 and 95% air atmosphere. Medium was supplemented with 10% heat-inactivated foetal bovine serum (Bio Whitthaker), 1% L-glutamine (200 mm) (Bio Whittaker) and antibiotics: penicillin (100 Ul/ml)-streptomycin (100 Mg/ml) (Penstrep R, Bio Whitthaker).

Cytotoxicity assay: 96-well tissue culture microplates (Micro Test -96 Falcon, Becton-Dickinson) were seeded with 100 Ml medium containing x cells in suspension (x= 7000 cells/well for Hela). Twenty/four hours incubation later, cells were treated with a di1ution of A. rotundum fractions in culture medium. After 48 hours incubation at $37^{O}C$ in presence of compounds, mitochondrial dehydrogenase activity in viable cells was measured by adding MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent and reading absorbance at 450 nm with a scanning multiwell spectrophotometer after one hour delay. The absorbance was directly correlated to the viable cell number. Experiments were performed in triplicate and the results were expressed as cell proliferation in comparison to control.

Calculation of Inhibition Concentrat1on (IC_{50}) and Statistical analysis

The concentration of the extract (mg/ml.) that was required to scavenge 50% of radicals was calculated by using the percent scavenging activities of different extract concentrations. Percent scavenging activity was calculated as $[1- (A_1-A_2)/Ac] \times 100$. Where: A_1 is the absorbance measured with A. rotundum fractions in the particular assay with a DPPH; A_2 is the absorbance measured with different A. atroviolaceum, A. waldsteinii and A. fuscoviolaceum fractions in the particular assay but without a DPPH; Ac is the absorbance of control with particular solvent (without A. atroviolaceum, A. waldsteinii and A. fuscoviolaceum fractions).

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD.

All the experimental results were as mean ± SD of three parallel measurements. The data was entered into a Microsoft Excel© database. The IC_{50} values were obtained by the linear regression analysis. The Extract and fractions giving IC_{50} values lower than 30 µg/mL were

considered to be cytotoxic. The extract and fractions with IC_{50} values lower than 50 µg/mL showed antioxidant activity.

RESULTS AND DISCUSSION

The 80% methanol extract showed significant activities in antioxidant assays and contained a high level of extractives content. The highest DPPH; radical scavenging effect was detected in organic ethyl acetate fraction (IC_{50} 0.54 ± 0.08 mg/ml) followed by chloroform and n-hexane fractions (IC_{50} 0.68 ± 0.02 mg/ml. and 0.77 ± 0.02 mg/ml. respectively (Figure 1). Those activities were higher than that of α- tocopherol (IC_{50} 0.3 ± 0.03 mg/ml). When considering the organic fractions of *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. The DPPH radical scavenging capacities increased towards the ethyl acetate fraction with increasing the polarity of the solvent. Also, DPPH radical scavenging activities were increased with an increased content of extractives compounds in organic fractions.

Table 1: DPPH free radical scavenging activities of *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. fractions.

Plant species	Solvent	Exctractive compounds content (mg/g)	DPPH assay IC_{50} (µg/ml)
Allium atroviolaceum	Me-ex	251.0	18.45±0.76
Allium atroviolaceum	Chlo-fr	54.2	48.07±1.81
Allium atroviolaceum	H-fr	37.8	54.19±2.73
Allium atroviolaceum	Ethyl-fr	201.4	23.92±0.89
Allium waldsteinii	Me-ex	248.3	20.29±0.80
Allium waldsteinii	Chlo-fr	57.9	49.18±1.65
Allium waldsteinii	H-fr	36.5	56.33±1.89
Allium waldsteinii	Ethyl-fr	194.1	26.70±0.95
Allium fuscoviolaceum	Me-ex	255.7	14.12±0.72
Allium fuscoviolaceum	Chlo-fr	59.3	44.23±1.80
Allium fuscoviolaceum	H-fr	40.7	52.06±1.34
Allium fuscoviolaceum	Ethyl-fr	211.6	20.64±0.64
α- tocopherol	-	-	4.32±0.02

The extractives compounds content of different *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. fractions were solvent dependent. Aqueous fractions of *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. showed higher amounts of extractives while their counterparts showed lower extractives content. The content of extractives compounds in aqueous fractions decreased in the order of ethyl acetate (398:4.7 mg/g) > methanol (286 ± 6.7 mg/g) > chloroform (59.3 ± 3.9 mg/g) > n-hexane

(51.3 ± 2.2 mg/g) fraction. As different *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. fractions exhibited free radical scavenging activities, there may be different kinds of extractives compounds in *Allium atroviolaceum Boiss.*, *A. waldsteinii Don*. and *A. fuscoviolaceum Fomin*. fractions.

Table 2 Show the results of the in-vitro cytotoxic testing after 48 hours of exposure to the samples and the positive control Colchicine. The LC50 values of methanol extract, ethyl acetate (Ethyl-fr), hexane (H-fr) and chloroform (Chlo-fr), fractions and were found to be 25.34 ± 1.45, 19.65 ± 1.05, 32.67 ± 2.68 and 38.79 ± 2.98 lg/ml, respectively (Table-2) as compared to 0.25 ± 0.01 lg/ml exhibited by CC. The methanol extract (Me-ex) and Ethyl-fr showed strong cytotoxic activity while H-fr and Chlo-fr demonstrated significant cytotoxic activities.

Table 2: In vitro cytotoxic activities of *Allium atroviolaceum Boiss.*, *A. waldsteinii Don.* And *A. fuscoviolaceum Fomin*, fractions.

Plant species	Solvent	Citotoxic activity IC_{50} (µg/ml)
Allium atroviolaceum	Me-ex	25.34±0.76
Allium atroviolaceum	Chlo-fr	38.79±1.81
Allium atroviolaceum	H-fr	32.67±2.73
Allium atroviolaceum	Ethyl-fr	19.65±0.89
Allium waldsteinii	Me-ex	25.34±0.76
Allium waldsteinii	Chlo-fr	38.79±1.81
Allium waldsteinii	H-fr	32.67±2.73
Allium waldsteinii	Ethyl-fr	19.65±0.89
Allium fuscoviolaceum	Me-ex	25.34±0.76
Allium fuscoviolaceum	Chlo-fr	38.79±1.81
Allium fuscoviolaceum	H-fr	32.67±2.73
Allium fuscoviolaceum	Ethyl-fr	19.65±0.89
Colchicine	-	0.35±0.01

The cytotoxic activities were increased with an increased content of extractives compounds in organic fractions. Further, all fractions showed higher cytotoxic activities and positively correlated with extractives compounds content.

From the above results, it is evident that the methanolic crude extract and ethylacetate soluble faction revealed strong cytotoxicity which also suggest the presence of secondary metabolites in these extractives.

Table 3 show that steroidal saponins possess a relatlvely similar cytotoxicity against tumor cell lines, with IC_{50} values ranging from 2.3±0.08 MM (trilin), 3.9±0.1 MM (dideglucoeruboside), 4.7±0.2 MM (aginoside) 5.9 ± 0.2 MM (eruboside B) for HELA.

Table 3: In vitro cytotoxic activities of steroidal saponins from Allium sp.

Compound	Citotoxic activity IC_{50} (µg/ml)
Trilin	2.3±0.08
Dideglucoeruboside	3.9±0.1
Aginoside	4.7±0.2
Eruboside B	5.9±0.21
Colchicine	0.35±0.01

This study provides only basic data. Further studies are required to determine intracellular pathways involved in the mechanism of cytotoxicity. The plants could be subjected for extensive chromatographic separation and purication processes to isolate bioactive compounds for the discovery of novel therapeutic agents.

Conflict of Interest

All authors of this paper confirm that they have no conflicts of interest.

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