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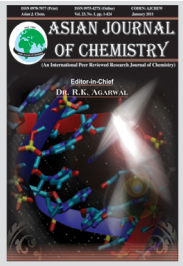

Evaluation of Antitumor, Antioxidant and Antibacterial Activities of the Extracts of *Saussurea atkinsonii*

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The *Saussurea atkinsonii*, a member of family Asteraceae is abundant in the northern areas of Pakistan, traditionally used for the treatment of variety of diseases. The whole plant of *S. atkinsonii* was extracted in hexane, chloroform and methanol by using sohxlet. Antioxidant activity was determined by DPPH, total antioxidant, TBA, FTC and TLC autobiography. Six bacterial strains were used for the evaluation of antibacterial activity. The antitumor activity of the extracts was checked against the BHK-21 cell line. MTT colorimetric assay was used to evaluate the reduction of viability of cell cultures in the presence and absence of the extracts. The 50 % inhibitory concentration (IC_{50}) for each extract was calculated by linear regression analysis and maximum antitumor activity was observed for methanol extract of *S. atkinsonii* with IC_{50} value 1.19×10^{-2} after the incubation period of 72 h.

Key Words: Antitumor, *S. atkinsonii*, MTT, BHK-21.

INTRODUCTION

In Indo-Pak northern areas, some *Saussurea* species are used as traditional medicines to cure various ailments such as asthma, cough, cholera, chronic skin diseases, malaria, rheumatism, leprosy, tooth ache and stomach ache *etc*.[1]. The moderate antiarthritic and antiinflammatory activities of the *S. costus* and its phytochemical constituents, as well as their cytotoxic effects against different tumor cells including HCT-15, SK-MEL-2, SK-OV-3, XF-498 and A-549 are well reported[2-4].

Cancer is a hot issue as public health burden all over the world. Several plant derived antitumor agents including vinblastine, etoposide, taxol, vincristine, irinotecan, topotecan, epipodophyllotoxin and camptothecin and their derivatives are in clinical use[5]. Two plant derived compounds camptothecin and paclitaxel were considered to account for one-third of the total anticancer market at global level[6]. Some phytochemicals can significantly reduce the risk of cancer due to polyphenol antioxidant and antiinflammatory effects[7-9]. Negative cell growth is an important aspect of maintaining normal tissue homeostasis. This regulation involves the suppression of cell proliferation, as well as the induction of cell death[10]. During the last few years it has been established that a large majority of cancer chemotherapy agents affect tumor cell killing *in vivo* and *in vitro* through launching the mechanisms of apoptosis[11]. Numerous drugs are used in cancer chemotherapy but most exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non-tumor cells[12,13]. These side effects limit the use of chemotherapeutic agents despite their high efficacy in treating target malignant cells. Therefore, the search for alternative drugs that are both effective and non-toxic in the treatment of cancers is an important research line[14]. In fact, increased efforts are being made to isolate bioactive products from medicinal plants for their possible utility in cancer treatment[15].

Most of the population in the world predominantly relies on plants for health care[16]. Natural products might be another potent source to introduce potent antitumor agents. *S. atkinsonii* is one of those plants which are still unexplored regarding their efficacy in medicine. Therefore, the present work was designed to evaluate various biological activities of the extracts of *S. atkinsoni*.

EXPERIMENTAL

Fresh whole plant of *S. atkinsonii* was collected from northern areas of Pakistan and identified at the Department of Botonay G.C. University, Lahore, Pakistan where a specimen voucher (GCU-Herb-Bot-849) was deposited.

Preparation of extracts: The crude extracts labeled as SAH, SAC and SAM (where SA; *S. atkinsonii*, H; hexane, C; chloroform, M; methanol) were obtained directly soaking the powdered plant material in respective solvents.

3-[4,5-Dimethyl thiazol-2-yl] 2,5-diphenyl tetrazolium bromide (MTT) (SIGMA), M199 Minimal Essential Medium (MP Biomedicals), phosphate buffer saline (MP biomedicals), Fetal Bovine Serum (PAA Laboratories GmbH), Amphotericin B (Symans Pharmaceuticals), Ampicillin (Symans Pharmaceuticals), Streptomycin (Symans Pharmaceuticals), gentamycin (Symans Pharmaceuticals), Folin-Ciocalteu Reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), α-tocopherol, gallic acid, linoleic acid, ferrous chloride, ammonium thiocyanate, lithium sulphate, ammonium molybdate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Fluka and Sigma-Aldrich. All the Solvents were purchased from Panreac Chemicals.

Orbital Shaker-Timer Control (DIGITEC Instruments), enzyme-linked Immunosorbent Assay (ELISA) reader (Multiscan EX Thermo Electron Corporation), UV-visible spectrophotometer (Cecil CE-7200), Rotary evaporator (Laborota 4000, Heidolph), Hammer mill and Incubator.

Microorganisms: All the samples from both the species tested against panel of bacterial strains including *Streptococcus thermophilus* PTCC-ST, *Bacillus subtilus* PTCC-BS, *Salmonella typhimorium* ATCC-10428, *Nocardia asteroids* PTCC-NA, *Escherichia coli* ATCC-8789, *Bacillus licheniformis* PTCC-BL. All the microorganisms were available at Government College University Biochemistry Laboratory.

Baby hamster kidney cells (BHK-21 cell line): The baby hamster kidney cells were grown as a monolayer culture in M-199 minimum essential medium supplemented with 10 % fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin, 20 mg/mL glutamine, 0.14 % $NaHCO_3$ and MEM non-essential amino acid and vitamins solution. The culture was maintained at 37 °C in a humidified 5 % CO_2 atmosphere. The infected cultures were then subjected to three cycles of freezing-thawing and centrifuged at 2000 rpm for 10 min. The supernatant was collected, titrated and stored at -170 °C in 1 mL aliquots.

Antitumor activity: The dry crude extracts as well as standard, dissolved in dimethyl sulphoxide DMSO (40 mg/mL) were diluted to 1:100 with M199 medium for preparing 400 µg/mL concentration containing 1 % DMSO. Different dilutions were prepared from stock containing DMSO less than 0.5 % of the sample solution. The cryo-preserved form of BHK-21 Baby Hamster Kidney cells, propagated in raux flasks, incubated for 48 h were seeded in 96 well flat bottom plates at the rate of 1×10^5 cells/well in M-199 medium. The media was also supplemented with 10 % fetal bovine serum (FBS), 100 units/mL penicillin,100 mg/mL streptomycin,10 % gentamycin and 100 units/mL amphotericin B was cultured in an atmosphere humidified with 5 % CO_2 and 95 % air at 37 ºC. The cells were grown as a monolayer culture. After 24 h pre-incubation period 200 mL of M-199 media was changed with fresh media containing final concentrations of extracts and standard. After an incubation period of respective intervals of 24, 48 and 72 h, the metabolic activity of each concentration in separate wells were assessed with MTT cytotoxicity assay as reported by Mosmann[17,18]. Supernatants were removed from all wells and 50 µL of MTT solution 2 mg/mL in phosphate buffer saline (PBS) was added to each well and the plates were incubated at 37 ºC for 2 h. Finally 150 µL of DMSO was added to solubilize the MTT crystals. Plates were shaked with a shaker for 15 min. The 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm for absorbance density values to determine the cell viability. The viable cells produced dark blue formazan product, where as no such staining was formed in the dead cells. The viability of untreated cell control was set to 100 %. The dead cell control was considered as 0 % viability .The degree of growth inhibition is expressed as % of the untreated cell control. The concentration of the extract to produce a 50 % reduction of viability of cells (IC_{50}), was determined by MTT assay. Degree of growth inhibition was calculated by the following formula.

$$\%\,\text{Inhibition} = \left[1 - \left(\frac{(\text{Test V} - \text{Min V})}{(\text{Max V} - \text{Min V})}\right)\right] \times 100$$

% Inhibition = % of cell growth inhibition.
Test V = Cell viability by sample and standard compounds.
Max V = Maximum viability of untreated cell control.
Min V = Minimum viability of dead cell control.

Linoleic acid emulsion system (ferric-thiocyanate assay): The antioxidant activity of *S. atkinsonii* extracts on inhibition of linoleic acid per oxidation was assayed by thiocyanate method reported by Yen and Hasieh[19]. 0.1 mL of each of sample solutions (0.5 mg/mL) was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween-20 as emulsifier and 50 mL of phosphate buffer, the mixture was homogenized. The reaction mixture was incubated at 40 ºC to accelerate the oxidation process and was used after 7 days for assessing antioxidant activity. The mixture without added extract was used as control. The mixture (0.1 mL) was taken and mixed with 5.0mL of 75 % ethanol, 0.1mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % HCl and allowed to stand at room temperature. Precisely after 3 min the addition of ferrous chloride to the reaction mixture, the absorbance at 500 nm was taken. The antioxidant activity was expressed as percentage inhibition of per oxidation (IP %):

$$\text{IP}\,(\%) = \left[\frac{1 - (\text{Absorbance of sample})}{(\text{Absorbance of control})}\right] \times 100$$

Thiobarbituric acid (TBA) method: The method of Onay-Uncar[20] was followed to check the antioxidant potential of *S. atkinsonii* extracts under study. 2 mL of 20 % trichloroacetic acid and 2 mL of 0.6 % 2-thiobarbituric acid were added to 0.2 mL of sample solution, prepared for above ferric thiocyanate method. The mixture was incubated at 95 ºC for 15 min and after cooling was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 532 nm.

Scavenging activity on DPPH free radical: The antioxidant activity was measured in terms of DPPH free radical scavenging ability by the method of Erasto[21].1.0 mL of *S. atkinsonii* extracts solutions (0.2 mg/mL) were added to 2 mL of methanolic DPPH solution (0.01 %) and incubated in dark at room temperature for 1 h. The decrease in absorbance at

517 nm was determined and the results were evaluated as % scavenging of radicals:

Scavenging of DPPH (%) =

$$\left[\frac{(\text{Abs. of blank} - \text{Abs. of sample}}{\text{Abs. of blank}}\right]\times 100$$

DPPH TLC autographic assay: Minimum amount of the active *S. atkinsonii* extracts required to scavenge the DPPH radical was determined by applying different concentrations (0.01-1.5 mg/mL) on TLC plates. The plates were sprayed with 0.2 % (2 mg/mL) DPPH solution[22,23] and examined after 0.5 h of spraying. Light yellow colour on purple background indicated the positive result.

Total antioxidant capacity assay: Total antioxidant activity of *S. atkinsonii* extracts was evaluated according to method of Prieto *et al*.[24] and reported by Khan *et al*.[25] 0.1 mL of sample solution in different concentration (0.1, 0.25 and 0.5 mg/mL) was combined with 1.9 mL of reagent solution (0.6M H_2SO_4, 28 mM sodium phosphate and 4 mM ammonium molybdate). The blank solution contained 2 mL reagent solution only. The mixtures were incubated at 95 ºC for 150 min. After the mixture had cooled to room temperature, absorbance was measured at 695 nm. The antioxidant activities were expressed as the absorbance values of the samples.

Antibacterial activity: Antibacterial activity of *S. atkinsonii* extracts was performed using a modified agar well diffusion method[26]. Eighteen hour aged innoculum suspensions of different microbial strains in nutrient broth (2.5 % aq., Fluka) were added to sterilized nutrient agar suspension (≈ 50 ± 5 ºC, 2.8 % aq., Biolab.). To get the final concentration of ≈ 10^5 CFU and 25 mL of mixture was poured in sterilized petri plates. On solidification, 5 mm diameter wells were cut in plates, using a sterile cork borer and 100 µL of sample solutions (20 mg mL^{-1}) were added to the wells. The plates were then incubated for 18 h at 37 ºC.

Determination of minimum inhibitory concentration (MIC): Minimum inhibitory concentration was determined by Muller Hinton Broth (MHB) dilution method[27]. 1 mL microbial suspension was incubated with the same volume of different dilutions of *S. atkinsonii* extracts solution in MHB at 37 ºC for 24 h. The MICs were taken as the lowest concentration of the sample which inhibited the microbial growth. The lack of turbidity was examined visually for the determination of growth inhibition.

Statistical analysis: All the experiments were run as triplicate and presented here as mean ± confidence level (α 0.05 and n = 3).

RESULTS AND DISCUSSION

The phytochemical analysis of *S. atkinsonii* extracts revealed the presence of flavonoides and sesquiterpenes which is generally in accordance with the already reported phytochemical composition of some *Saussurea* species. Some other biologically active compounds have also been isolated from *Saussurea lappa* by different research groups including, costic acid, palmitic acid, linoleic acid, sitosterol, cyclocostunolide, alantolactone, isoalantolactone, isodehydrocostus lactone, saussurea lactone, isozaluzanin, guiainolides and sesquiterpene lactones[28-34].

There are a number of tests for the determination of cytotoxic potency of natural products *via* the indirect measurement of the tumor cell number. The MTT assay is economically cheaper[35] and most frequently used for the assessment of *in vitro* resistance and sensitivity[17] using some viability related features such as dehydrogenase activity of the cells. The MTT assay was performed to determine the antitumor potential of the selected extracts and results are presented in Tables 1 and 2.

TABLE-1
ABSORBANCE VALUES AND % GROWTH INHIBITION OF *S. atkinsonii* EXTRACTS AGAINST BABY HAMSTER KIDNEY CELL LINE (BHK-21) BY MTT ASSAY

Dilutions	Absorbance (at 570 nm)			Growth inhibition (%)		
	24 h	48 h	72 h	24 h	48 h	72 h
			SAH (Hexane)			
D1	0.137 ± 0.002	0.135 ± 0.002	0.126 ± 0.002	25.73	28.87	37.96
D2	0.134 ± 0.001	0.131 ± 0.001	0.122 ± 0.003	29.24	36.69	41.87
D3	0.128 ± 0.003	0.126 ± 0.003	0.118 ± 0.001	35.13	38.24	46.23
D4	0.121 ± 0.002	0.118 ± 0.002	0.109 ± 0.002	44.37	47.49	55.62
			SAC (Chloroform)			
D1	0.142 ± 0.001	0.141 ± 0.002	0.128 ± 0.002	20.30	22.26	35.25
D2	0.137 ± 0.002	0.132 ± 0.003	0.122 ± 0.001	26.11	32.11	41.86
D3	0.123 ± 0.002	0.118 ± 0.002	0.112 ± 0.001	41.73	44.43	54.22
D4	0.104 ± 0.004	0.099 ± 0.002	0.092 ± 0.003	62.95	68.14	75.04
			SAM (Methanol)			
D1	0.121 ± 0.002	0.118 ± 0.002	0.117 ± 0.004	43.12	40.36	47.30
D2	0.110 ± 0.003	0.107 ± 0.003	0.106 ± 0.003	49.64	51.12	60.12
D3	0.105 ± 0.001	0.102 ± 0.001	0.098 ± 0.004	56.31	59.26	68.24
D4	0.096 ± 0.002	0.092 ± 0.001	0.085 ± 0.002	70.39	80.71	88.61
			VINBLASTINE (Standard)			
D1*	1.157 ± 0.001	1.14 ± 0.002	1.11 ± 0.003	24.10	25.66	27.43
D2*	1.099 ± 0.002	1.067 ± 0.003	1.056 ± 0.002	28.62	30.54	31.36
D3*	0.914 ± 0.004	0.869 ± 0.002	0.851 ± 0.003	41.52	44.75	46.03
D4*	0.501 ± 0.003	0.468 ± 0.004	0.430 ± 0.005	71.16	73.52	76.26

D1 = 1.6 (µg/mL) D2 = 8.0 (µg/mL) D3 = 40 (µg/mL) D4 = 200 (µg/mL). D1* = 0.008 (µg/mL) D2* = 0.04 (µg/mL) D3* = 0.2 (µg/mL) D4* = 1.0 (µg/mL) SA; *S. atkinsonii*, H; hexane, C; chloroform, M; methanol. Results are mentioned as mean ± confidence level of triplicates (α 0.05 and n = 3).

TABLE-2
IC_{50} (mg/mL) VALUES FOR % GROWTH INHIBITION OF *S. atkinsonii* EXTRACTS AGAINST BABY HAMSTER KIDNEY CELL LINE (BHK-21) BY MTT ASSAY

Sample code	IC_{50} (mg/mL) values of *S. atkinsonii* extracts		
	24 h	48 h	72 h
SAH	2.60×10^{-1}	2.25×10^{-1}	1.21×10^{-1}
SAC	1.25×10^{-1}	1.03×10^{-1}	5.35×10^{-2}
SAM	2.19×10^{-2}	1.77×10^{-2}	1.19×10^{-2}
Vinblastine	4.13×10^{-5}	4.53×10^{-5}	5.05×10^{-5}

SA; *S. atkinsonii*, H; hexane, C; chloroform, M; methanol.

The methanolic extract has shown maximum potential to inhibit the growth of BHK-21 cells with 1.19×10^{-2} mg/mL IC_{50} value at 72 h incubation period. All the extracts were active with the order of activity SAM > SAC > SAH. A dose dependent behaviour was observed for different extracts in the range of 1.6-200 µg/mL. The results of antitumor activity were correlated with the results of antibacterial and antioxidant activities. Vinblastine was used as standard antitumor agent and showed 76.26 % growth inhibition at 1 µg/mL with remarkable IC_{50} value 5.05×10^{-5} (mg/mL) after 72 h incubation period.

The zone of inhibition values for antibacterial activity of the *S. atkinsonii* extracts against six bacterial strains have been mentioned in Table-3. Almost no bacterial inhibition was observed in case of negative control of DMSO. The ampicillin and streptomycin were used as standards. The methanolic extract proved to be the most significant against all bacterial strains except *Streptococcus thermophilus*. The results of antibacterial were in the order SAM > SAC > SAH.

The minimum inhibitory concentration (MIC) values for most active methanolic extract was in the range of 1.25-2.5 µg/mL against all the bacterial strains under study except *Streptococcus thermophilus* and other MIC values are given in Table-4. All the four bacterial strains *i.e.*, *Bacillus subtilus*, *Salmonella typhimorium*, *Nocardia asteroids* and *Escherichia coli* were significantly susceptible to all the *S. atkinsonii* extracts under investigation during this research work except the hexane extract which was less active as compared to the other extracts. In case of *Nocardia asteroids* the *S. atkinsonii* extracts in the choloroform (SAC) showed moderate results. The antibacterial potential of different species of family Asteraceae has been reported earlier[36-38]. According to the previous literature gram negative bacteria are comparatively more resistant than gram positive bacteria against different medicinally important plants[39].

The antioxidant activity potential of extracts was investigated with thiobarbituric acid assay and ferric thiocyanate assay. Total antioxidant activity and free radical scavenging activity of the *S atkinsonii* extracts were also performed. All results for antioxidant activities with different methods are mentioned in Table-5.

In case of the DPPH free radical scavenging activity the IC_{50} values were in the range of 4.45-233.4 µg/mL. The SAM showed the highest DPPH free radical scavenging potential with IC_{50} value 4.45 µg/mL followed by the SAC and SAH. The results of all other antioxidant assays revealed that the SAM was the most active followed by the SAC. Hexane extract is comparatively less active in all these observations. The sequence of the activity enhancement in case of SAH, SAC and SAM extract was significantly relevant with their antitumor potential, which can be attributed to the presence of flavonoides and sesquiterpenes as indicated in the phytochemical analysis of *S. atkinsonii*.

Conclusion

The present investigation revealed that most of the *S. atkinsonii* extracts were effective against the growth of BHK-21 cells as well as against most of the bacterial strains, therefore, it is concluded as a helpful research work for further isolation of potential antitumor as well as antibacterial and antioxidant natural products from *Saussurea* species.

TABLE-3
ZONES OF INHIBITIONS FOR *S. atkinsonii* EXTRACTS AND STANDARDS

Sample codes	Zones of inhibition excluding well diameter (mm)					
	Streptococcus thermophilus	*Bacillus subtilis*	*Salmonella typhimurium*	*Nocardia asteroids*	*Bacillus licheniformis*	*Escherichia coli*
SAH	15.13 ± 0.21	10.13 ± 0.19	12.18 ± 0.45	13.30 ± 0.13	11.19 ± 0.13	15.54 ± 0.27
SAC	16.36 ± 0.13	18.00 ± 0.47	17.20 ± 0.11	09.15 ± 0.71	19.44 ± 0.16	17.20 ± 0.25
SAM	–	20.88 ± 0.55	20.77 ± 0.83	19.66 ± 0.26	18.44 ± 0.21	17.44 ± 0.33
Amp	23.25 ± 1.44	22.29 ± 0.46	21.76 ± 0.25	19.33 ± 0.33	22.19 ± 0.33	22.59 ± 0.44
Strpt	19.55 ± 0.21	21.17 ± 0.78	19.29 ± 0.17	18.37 ± 0.49	20.42 ± 0.25	20.25 ± 0.35

SA: *S. atkinsonii*, H: hexane, C: chloroform, M: methanol, –: not detected. Results are given as mean ± confidence level of triplicates (α 0.05 and n = 3).

TABLE-4
MIC VALUES OF *S. atkinsonii* EXTRACTS AGAINST BACTERIAL STRAINS

Sample codes	Minimum inhibitory concentration (µg/mL)					
	Streptococcus thermophilus	*Bacillus subtilis*	*Salmonella typhimurium*	*Nocardia asteroids*	*Bacillus licheniformis*	*Escherichia coli*
SAH	2.50	5.00	5.00	5.00	–	2.5
SAC	1.25	1.25	1.25	1.25	1.25	1.25
SAM	–	1.25	1.25	1.25	2.50	1.25

SA: *S. atkinsonii*, H: hexane, C: chloroform, M: methanol, –: not detected. Values are the mean of the three replicates using 1×10^4 CFU of each culture.

TABLE-5
DIFFERENT ANTIOXIDANT ASSAYS FOR *S. atkinsonii* EXTRACTS

Sample code	DPPH assay		Total antioxidant activity (absorbance at 695 nm)			TBA assay (inhibition %)	FTC assay (inhibition %)
	% Scavenging (0.2 mg/mL)	IC_{50} (µg/mL)	500 (µg/mL)	250 (µg/mL)	100 (µg/mL)		
SAH	34.29 ± 0.82	233.4 ± 1.25	0.76 ± 0.02	0.39 ± 0.06	0.24 ± 0.02	34.12 ± 0.68	46.43 ± 2.22
SAC	62.17 ± 1.09	5.45 ± 0.04	0.98 ± 0.03	0.64 ± 0.47	0.33 ± 0.04	54.24 ± 1.23	69.32 ± 1.14
SAM	87.94 ± 0.23	04.45 ± 0.59	1.45 ± 0.07	1.13 ± 0.11	0.95 ± 0.01	58.56 ± 0.43	69.44 ± 0.82
Gallic acid	97.07 ± 1.67	04.59 ± 0.94	1.60 ± 0.09	1.29 ± 0.02	0.56 ± 0.03	35.74 ± 0.93	49.52 ± 0.70
α-Tocopherol	95.91 ± 1.19	36.53 ± 1.85	1.30 ± 0.06	0.93 ± 0.02	0.26 ± 0.01	53.23 ± 0.18	67.87 ± 0.57
BHT	95.53 ± 2.04	12.72 ± 1.38	0.37 ± 0.02	0.27 ± 0.02	0.14 ± 0.02	51.33 ± 1.84	47.69 ± 0.42

DPPH: 2,2-diphenyl-1-picrylhydrazyl, TBA: Thiobarbituric acid, FTC: Ferric-thiocyanate, SA: *S. atkinsonii*, H: hexane, C: chloroform, M: methanol. Results are mentioned as mean ± confidence level of triplicates (α 0.05 and n = 3).

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