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Fiaz Alam¹, Qazi Najam us Saqib¹ and Abdul Waheed²

¹Department of Pharmacy, COMSATS Institute of Information Technology, Abbottabad, Pakistan; ²Wolfson Cell Science Centre, Faculty of Health & Medical Sciences, University of Surrey, Guildford, UK.

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Abstract

This study was conducted to evaluate *Gaultheria trichophylla* crude extract and respective saponins fraction against human breast cancer cell lines. In MTT assay, cell viability was inhibited by *G. trichophylla* crude extract (500 µg/mL) and saponins (200 µg/mL) in a dose dependent manner with maximum inhibition of (82 and 85%) and (71 and 42%) against MCF-7 and MDA MB-468, respectively. In neutral red uptake assay, the cell viability was inhibited by crude extract and saponins (100 µg/mL) in a similar manner with maximum inhibitions of (96 and 93%) and (87 and 61%) against MCF-7 and MDA MB-468, respectively, with respect to 91 and 93% inhibition by actinomycin-D (4 µM). The DAPI (4',6-diamidino-2-phenylindole) (10 µg/mL) staining of MCF-7 cells treated with crude saponins showed shrunken nuclei with apparent nuclear fragmentation indicating apoptosis and in contrast, MDA MB-468 showed necrosis mode of cell death. The study exhibited that the *G. trichophylla* provides new evidences to further explore this plant for the novel targets in anti-cancer drug development.

Introduction

With more than 10 million new cases every year, cancer has become one of the most devastating diseases worldwide. Natural products have been the source of most of the active ingredients of medicines. According to a report half of the drugs approved since 1994 are based on natural products. Thirteen natural-product-related drugs were approved from 2005 to 2007. They cover a range of therapeutic indications, mainly the anti-cancer (Cragg et al., 1997). It is well known that in the past some medicinally important plants like *Catharanthus roseus*, *Podophyllum peltatum* and *Taxus brevifolia* and others provided therapeutically active metabolites which were used to control the advance stages of malignancies (Farnsworth and Soejarto, 1991). Therefore, as always, there is need to find alternative drugs with low toxicity, more effective and accessible to common man. Therefore, the present study was carried out to investigate the anti-cancer activity of extract and crude saponins fraction from *Gaultheria trichophylla* in cultured

MCF-7 and MDA MB-468 human breast cancer cells.

G. trichophylla Royle (Family Ericaceae) is native to the Himalayas and therefore common name is Himalayan Snowberry. Plants belonging to genus *Gaultheria* like *G. yunnanensis*, *G. fragrantissima* and *G. Procumbens* are reported to have anti-inflammatory activity, antibacterial activity, and is used to treat arthritis. Gaultherin a natural salicylate isolated from *G. yunnanensis* possesses analgesic and anti-inflammatory activities. The phytochemical investigation of the species investigated reported to contain methyl salicylate, diterpenoids, acids, dilactone, alkaloids and other glycosides. Cytotoxic activities against the selected cancer cell lines are also reported in plants like *G. itoana* and *G. yunnanensis* (Liu et al., 2013).

Materials and Methods

Chemicals



Neutral red solution, fetal bovine serum (FBS), actinomycin-D, Dulbecco's modified Eagle medium (DMEM) and 4,6-diamidino-2-phenylindole (DAPI) were obtained from Sigma Chemical Co. (St Louis, MO, USA). MCF-7 and MDA-MB-468 cells were purchased from ATCC (American Type Culture Collection, USA) through an authorized distributor, LGC Standards, Teddington, UK.

Plant material

G. trichophylla plant (5 kg) was collected by the author from Kaghan valley, District Mansehra, KPK, Pakistan, in November, 2013. After authentication of the plant, its voucher specimen (CTPHM-GT01, 13) was deposited in the herbarium of the Department of Pharmacy, the COMSATS Institute of Information Technology, Abbottabad. The whole plant was washed under running water and dried in shade at room temperature and was ground to a coarse powder. The powder drug was stored in air tight and light resistant container before extraction.

Preparation of extract and crude saponins

The dried, powdered plant material (200 g) was extracted with methanol using soxhlet extractor for 20 hours. It was filtered through a Whatman Grade-I filter paper. The filtrate was evaporated on a vacuum rotary evaporator under reduced pressure at 40°C. Extracted percent yield of the methanol fraction (G. Me) was 21.9%. For the extraction of saponins from powdered materials (200 g) of whole plant of *G. trichophylla*, was extracted first with petroleum ether, and followed by extraction with methanol in soxhlet apparatus. The solvent was reduced on a rotary evaporator under vacuum to obtain dry semisolid extract. The methanol extract of the plant was further fractionated with *n*-butanol and water, in equal proportion. The *n*-butanol fraction was separated. The crude saponins were precipitated with petroleum ether; yield was approximately 4.5 g of crude saponins (G. Sa) (Dande et al., 2010).

Cell culture

Cells were grown in complete growth medium: Dulbecco's modified Eagle medium (DMEM) containing 10% v/v FBS, 2 mM L-glutamine, gentami-cin (40 µg/mL), penicillin (100 units/mL) and strepto-mycin (1040 µg/mL). Cells were seeded into 96-well cell culture plates at a density of 1×10^4 cells per well in 100 µL aliquots of the medium. The cells were allowed to attach for 24 hours at 37°C, 5% CO₂ in air in a humidified atmosphere in an incubator.

Preparation of the drug for the experiment

Crude extract of *G. trichophylla* was tested for cell cytotoxicity against breast cancer cell lines. A series of eight dilutions (10, 25, 50, 100, 200, 300, 400 and 500 µg of final concentration) of crude extract were prepared in DMEM (100 µL) containing DMSO (dimethyl sulfoxide,

maximum: 0.01%). After the preliminary screening results a test dose of 200 µg for MTT (methylthiazolyltertrazolium) assay and 100 µg for NRU (neutral red uptake) assay was set for G. Sa based on apparent IC₅₀ of G. Me.

Cytotoxicity assays

After a 24 hours exposure test period, the toxic end-points were determined using two colorimetric assays; namely the MTT assay and neutral red uptake (NRU) assay.

MTT assay

According to the method described else-where (Borenfreund et al., 1988; Green and Kroemer, 2004; Qadir et al., 2014) growth of cancer cells was quantified. Following a 24 hours exposure test period of drugs, cells were washed twice with phosphate buffer saline (PBS), and a 10 µL of MTT reagent (5 mg/mL in PBS) was added to each well including the blanks, which contained medium only. The plates were returned to the incubator for 4 hours at 37°C. Subsequently, cells were washed twice with PBS, and 100 µL/well DMSO was added in each well as solvent to dissolve the insoluble crystalline formazan products. The effect of plant extracts on cancer cells was quantified as the percentage of control absorbance of reduced dye at 550 nm on a microplate reader (LabtechLT-4000MS, Labtech International Ltd., East Sussex, and UK). For each treatment, five replicates wells were examined, and each experiment was repeated three times. Standard error of mean was calculated between three experiments. The results were calculated as percentage growth inhibition, untreated (control) cells versus treated cells according to the following formula:

$$\% \text{ Growth inhibition} = \frac{\text{Control} - \text{actual absorbance}}{\text{Control}} \times 100$$

Absorbance of the media was subtracted, both from control and treated cells.

NRU assay

The neutral red uptake assay was performed according to the method of (Borenfreund and Puerner, 1985) by removal of the medium after dosing cells and 200 µL of neutral red solution (40 µg/mL) was added to each well (including the blanks, which contained medium only). After incubation for two and half hours, the neutral red was removed, cells were carefully rinsed with pre-warmed PBS, and 200 µL of ethanol/acetic acid (1% glacial acetic acid in 5% ethanol) was added to all wells.

The plates were covered in foil and placed on a plate shaker for 30 min to extract neutral red dye from the cells to form a homogeneous solution. Absorbance of the wells was measured at 540 nm in a microplate reader within 60 min. For each treatment, five replicates

wells were examined and each experiment was repeated three times. The results were calculated as percentage growth inhibition, untreated (control) cells versus treated cells, according to the formula as described previously for MTT assay. Standard error means were calculated between three experiments.

Cytomorphological alterations (DAPI staining)

In the present study, DAPI (4',6-diamidino-2-phenylindole) stain was used to assess the morphological changes in nuclei of control and treated cells. Cells were seeded at density of 1×10^4 cells/well in 500 μ L of medium on sterilized glass cover slips in well plates for 24 hours. The cells were treated with the negative control (culture medium) and the positive control (actinomycin-D, 4 μ M). The plates were incubated at 37°C, 5% CO₂ in air in a humidified atmosphere for 24 hours. After treatment, cells were briefly equilibrated with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilized with methanol for 5 min, and mounted in a DAPI-containing medium (Vector Shield, Vector Labs, Peterborough, UK). The morphology of the nuclei was observed using a confocal fluorescence microscope, Leica SP2 AOBS confocal microscope (Leica Microsystems, Mannheim, Germany) with excitation at 350 nm and emission 460 nm under a x40 oil objective (Saha et al., 2013).

Data presentation and statistical analysis

All data were compiled from a minimum of three experiments. Data for statistical analysis were expressed as the mean \pm SEM. One-way ANOVA with Dunnett's test, as specified, was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego California, USA.

Results

The results from the MTT and NRU assays of cellular viability in various concentrations of methanolic crude extract (G.Me) of *G. trichophylla* are listed in Table I. MCF-7 and MDA MB-468 cells were inhibited by crude extract with a rate increasing with increasing concentration. The inhibition rate of G. Me (500 μ g/mL) against MCF-7 and MDA MB-468 cells was about 82 and 85% respectively in MTT assay. The inhibition rate of G. Me (500 μ g/mL) against MCF-7 and MDA MB-468 cells was about 96 and 93% respectively in the NRU assay (Table I).

The inhibition of crude saponins of *G. trichophylla* (G. Sa) against MCF-7 and MDA MB-468 cancer cell lines were tested in dose of 200 μ g/mL for MTT assays and in dose of 100 μ g/mL for NRU assay. In MTT assay the saponins inhibited the growth of MCF-7 and MDA MB-468 by about 71 and 42%, respectively, with respect to actinomycin-D (4 μ M) which showed the growth inhibition of about 62%. The response to G. Sa was more pronounced in NRU assay, and a growth inhibition of about 87 and 61% was observed against MCF-7 and MDA MB-468, respectively, with respect to actinomycin-D (4 μ M) which showed the growth inhibition of 91 and 93%, respectively (Table II and III).

Both the untreated (control) and G. Sa (10 μ g/mL) treated cells were stained with DAPI and visualized by confocal microscope (Figure 1). The control cells of MCF-7 were found with normal morphology and intact nuclei, while the treated cells were exhibiting the apoptotic characteristics, with condensed and fragmented nuclei, along with marked shrinkage in the cell membranes. This was a clear indication of apoptosis

Table I

Dose-dependent growth inhibition of cancer cell lines by crude extract of *Gaultheria trichophylla*

Concentration (μ g/mL)	MTT assay		NRU assay	
	%Growth inhibition		%Growth inhibition	
	MCF-7 cells	MDA MB-468 cells	MCF-7 cells	MDA MB-468 cells
10	22.1 \pm 5.9 ^a	9.4 \pm 4.9	29.0 \pm 6.1 ^a	30.1 \pm 3.2 ^b
25	24.7 \pm 7.1 ^a	15.8 \pm 5.4 ^a	33.0 \pm 5.3 ^b	43.9 \pm 3.4 ^b
50	41.6 \pm 3.8 ^b	38.4 \pm 6.3 ^b	44.1 \pm 3.6 ^b	46.0 \pm 4.2 ^b
100	40.0 \pm 3.4 ^b	46.2 \pm 1.8 ^b	50.7 \pm 4.2 ^c	53.3 \pm 3.4 ^c
200	48.4 \pm 4.2 ^b	61.6 \pm 7.3 ^c	51.5 \pm 5.1 ^c	52.6 \pm 3.9 ^c
300	67.6 \pm 3.0 ^c	70.0 \pm 4.7 ^c	58.0 \pm 3.2 ^c	73.7 \pm 4.0 ^c
400	70.0 \pm 6.5 ^c	74.8 \pm 3.7 ^c	81.6 \pm 6.3 ^c	87.2 \pm 6.4 ^c
500	82.3 \pm 3.9 ^c	85.0 \pm 6.4 ^c	96.1 \pm 7.0 ^c	93.8 \pm 7.3 ^c
Vehicle control	1.1 \pm 4.4	-1.6 \pm 5.2	-1.6 \pm 6.1	4.0 \pm 3.0

Statistically (Dunnett's multiple comparison test) ^ap<0.05; ^bp<0.01; ^cp<0.001

Table II

Cytotoxic activity of saponins fractions from *Gaultheria trichophylla* using MTT assay

Experiment	Absorbance (cells + fraction + media)			Actual absorbance (cells + fraction + media)			% Growth inhibition (mean ± SD)
	1	2	3	1	2	3	100
<i>Saponins fraction (200 µg/mL)</i>							
Media	0.1	0.1	0.1	0.0	0.0	0.0	-
Media + Cells	0.1	0.1	0.1	0.1	0.1	0.09	-
MCF-7 cells	0.1	0.1	0.1	0.0	0.0	0.02	71.5 ± 3.7
MDA MB-468	0.1	0.1	0.1	0.1	0.1	0.05	42.6 ± 4.4
Vehicle Control	0.1	0.1	0.1	0.1	0.1	0.08	2.8 ± 3.1
<i>Actinomycin-D (4 µM)</i>							
MCF-7 cells	1.2	1.2	1.1	0.9	1.0	0.9	62.0 ± 1.4
MDA MB-468	1.5	1.5	1.5	1.0	0.8	0.7	62.8 ± 5.3
Vehicle Control	0.3	0.3	0.2	0.1	0.1	0.0	1.1 ± 1.1

Table III

Cytotoxic activity of saponins fractions from *Gaultheria trichophylla* using NRU assay

Experiment	Absorbance (cells + fraction + media)			Actual absorbance (cells + fraction + media)			% Growth inhibition (mean ± SD)
	1	2	3	1	2	3	100
<i>Saponins fraction (100 µg/mL)</i>							
Media	0.1	0.1	0.1	0.0	0.0	0.0	-
Media + Cells	0.2	0.2	0.2	0.1	0.1	0.1	-
MCF-7 cells	0.1	0.1	0.1	0.0	0.0	0.0	87.2 ± 2.8
MDA MB-468	0.1	0.1	0.1	0.0	0.0	0.0	61.4 ± 14.8
Vehicle Control	0.2	0.2	0.2	0.1	0.1	0.1	-2.01 ± 4.7
<i>Actinomycin-D (4 µM)</i>							
MCF-7 cells	0.2	0.2	0.2	0.0	0.0	0.0	91.8 ± 3.7
MDA MB-468	0.5	0.6	0.6	0.0	0.0	0.0	93.9 ± 5.0
Vehicle Control	0.3	0.3	0.3	0.1	0.1	0.1	1.6 ± 1.0

induced in MCF-7 cancer cells. DAPI staining of G. Sa treated MDA MB-468 cells showed necrotic cells characterized by swelling and increasing in size before the disruption.

Discussion

In the recent past, there has been unforeseen interest in the clinical utilization of saponins as chemotherapeutic agents and over 400 studies have been reported regarding the saponins and their ability to treat cancer or induce apoptosis. It is reported that saponins exert its cytotoxic activity through apoptosis through signaling pathways to prevent the tumor. Numerous steroid and triterpenoid saponins exhibit cytotoxic properties (Thakur et al., 2011). The plants of genus *Gaultheria* have been reported to suppress the malignant neoplastic cells (Li et al., 2010). Our previous study also showed that the crude saponins caused the inhibition of colon cancer cells lines through mechanism involving apoptosis (Alam et al., 2015). MTT assay is used to determine viable cell number in

cytotoxicity studies. Mitochondrial dehydrogenase produced by normal cells reduce MTT (Yellow) to blue formazan product, which reflects the normal function of mitochondria and cell viability (Lau et al., 2004). MTT assay results showed that G. Me and G. Sa showed dose dependent anti-proliferative activity and is effective in inhibiting the growth of MCF-7 and MDA MB-468 cells. The neutral red (NR) metabolic impairment assay also showed that G. Me and G. Sa inhibited the growth of MCF-7 and MDA MB-468 cells.

Apoptosis is a programmed cell death process that occurs in physiological and pathological conditions and a defect in apoptotic pathways has an important role in carcinogenesis. It is characterized by cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. The induction of apoptosis for the prevention of cancer is therefore desirable. Necrotic cell death, in contrast, is characterized by the loss of metabolic functions, and of the integrity of the cell membrane. Structurally, the cells' organelles swell and become nonfunctional during the initial stages of necrosis (Nanji and Hiller-

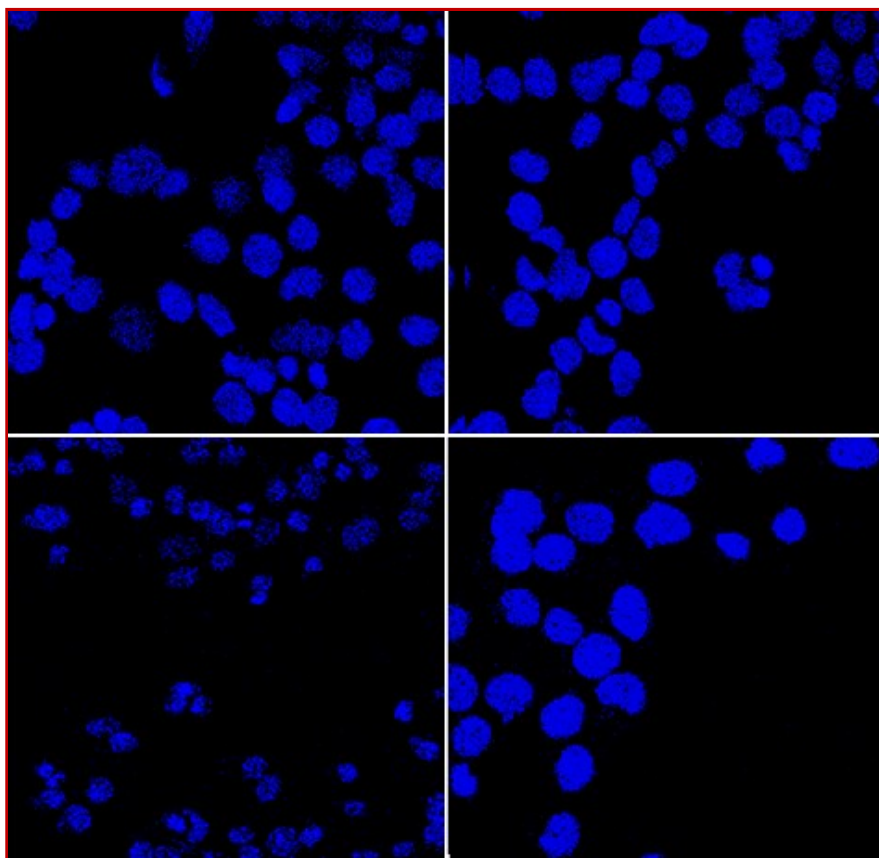


Figure 1: Morphological assessment of control (A and B) and *Gaultheria trichophylla* saponins (G. Sa) (10 µg/mL, final concentration) treated MCF-7 cells (A2) and MDA MB-468 (B2) for 24 hours. The nuclei were stained with DAPI and observed under confocal microscope

Sturmhöfel, 1997). DAPI staining is most commonly used assay for observing the apoptosis at the DNA level (Saha et al., 2013). In this study G. Sa induced morphological changes in apoptotic cells, which were observed in DAPI staining of MCF-7 cells.

Conclusion

This study demonstrates that the treatment with G. Sa resulted in the apoptotic body formation, chromatin condensation and nuclear fragmentation. It clearly shows the potential of G. Sa to induce apoptosis against MCF-7 cancer cell lines. While, on the other hand DAPI staining of MDA MB-468 cells indicated necrosis mode of cell death.

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Conflict of Interest

Authors declare no conflict of interest

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Author Info

Fiaz Alam (Principal contact)

e-mail: alamfiaz@ciit.net.pk; Tel: 0092 992 383591-5; Fax: 0092 992 383441; cell: +03005275200