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# Effect of crude saponins from *Gaultheria trichophylla* extract on growth inhibition in human colorectal cancer cells

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## Abstract

The genus *Gaultheria* also comprised of species with reported cytotoxic activities. Current research work was carried out to evaluate *G. trichophylla* crude extract and respective saponins fraction against human colorectal cancer cell line (Caco-2) based on cell viability assays. Caco-2 cells treated with the crude extract showed significant growth inhibition ($p < 0.001$) in a dose dependent manner with apparent $IC_{50}$ value of 200 µg/mL and 100 µg/mL in MTT and NRU assays respectively. The fractioned crude saponins showed an enhanced response and inhibited the growth of Caco-2 by 93.6 and 97.4% in MTT and NRU assays respectively, with compared to actinomycin-D (65%). The DAPI staining of cell treated with crude saponins observed under confocal microscope showed shrunken nuclei with apparent nuclear fragmentation and chromatin condensation indicating apoptosis mode of cell death. The study exhibited that the *G. Trichophylla* saponins induced apoptosis of Caco-2 cell lines. This study provides new evidences to further explore this plant for the novel targets in anticancer drug development.

## Introduction

Colorectal cancer is the third most common malignant neoplasm worldwide and has become one of the major causes of cancer mortality (Siegel et al. , 2013). Several studies revealed that loss of control of apoptosis results in initiation of cancer and therefore, many new treatment procedures targeting apoptosis are feasible and may be used to treat various types of cancers (Wong, 2011).

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). However, most of these chemotherapeutic agents showed untoward effects like mutagenicity and teratogenicity. Therefore, as always, there is need to find alternative drugs with low toxicity, more effective and accessible to common man.

The present study was carried out to investigate the anticancer activity of crude extract and respective saponins fraction of *Gaultheria trichophylla* in cultured human colorectal cancer cells (Caco-2).

*G. trichophylla* belongs to family Ericaceae which consists of 90 genera and over 1,700 species. It is native to Himalayas and commonly known as Himalayan snowberry (Huang, 2003). The flowers range in colour from red, to pink; fruits are blue-coloured berries; and small green leaves are approximately 3-7 mm in length. This plant is an inhabitant of cold and lofty locations of the mountains and like many plants of such locations, it is furnished with setae. The calyx is adherent to the lower part of the capsule, becomes succulent, and forms an edible fruit in the

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month of September (Royle, 1839). In China, India, USA and Canada, *G. yunnanensis*, *G. fragrantissima and G. procumbens* are used in traditional medicine to treat arthritis (Liu et al., 2013; Simon et al., 1984). Research work on other species of this plant indicates that it has anti-inflammatory (Zhang et al., 2011), antibacterial (Cybulska et al., 2011) and antiarthritis activities (Xiong et al., 2009). The plants of this genus including *G. itoana* Hayata and *G. yunnanensis* are reported to possess cytotoxic activities against the selected cancer cell lines (Chen et al., 2009; Li et al., 2010). Gaultherin a natural salicylate isolated from *G. yunnanensis* possess analgesic and anti-inflammatory activity (Zhang et al., 2006). The phytochemical investigation of species investigated reported to contain methyl salicylate, diterpenoids, acids, dilactone, alkaloids and other glycosides (Alauddin et al., 1965; Cambie et al., 1961; El-Basyouni et al., 1964; Grisebach and Vollmer, 1964; Ibrahim and Towers, 1960; Zhang et al., 1999; Zhang et al., 1998).

## Materials and Methods

### *Chemicals*

The Caco-2 (human colon adenocarcinoma) cell line was obtained from the ECCC (European Collection of Cell Cultures) through Health Protection Agency, Salisbury, UK (Catalogue No. 86010202). Neutral red solution, fetal bovine serum (FBS), actinomycin-D, Dulbecco's modified Eagle medium (DMEM) and 4,6-diamidineo-2-phenylinldole (DAPI) were obtained from Sigma Chemical Co. (St Lois, MO, USA).

### *Plant material*

*G. trichophylla* plant (5 kg) was collected 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, 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 was 21.9%.

Extraction of saponins from powdered materials (200 g) of whole plant of *G. trichophylla* was done first with petroleum ether, followed by extraction with methanol in Soxhlet apparatus. The solvent was reduced on rotary evaporator under vacuum to obtain dry semi solid extracts. 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 saponin extract (Dande et al., 2010).

### *Cell culture*

Caco-2 Cells were grown in complete growth medium: Dulbecco's modified Eagle medium (DMEM) containing 10% v/v FBS, 2 mM L-glutamine, gentamycin (40 µg/mL), penicillin (100 units/mL) and streptomycin (1,040 µg/mL). When cells were in a logarithmic phase of growth during passages, cells were seeded into 96-well cell culture plates at a density of 1 x $10^4$ cells per well in 100 µL aliquots of medium. The cells were allowed to incubate for 24 hours at 37°C, 5% $CO_2$ in air in a humidified atmosphere.

### *Preparation of test samples for the experiment*

Crude extract of the medicinal plant *G. trichophylla* was tested for cell cytotoxicity against cancer cell line Caco-2. A series of eight dilutions (10, 25, 50, 100, 200, 300, 400 and 500 µg of final concentration) of plant 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 assay and 100 µg for neutral red uptake assay was set for saponins extract based on apparent $IC_{50}$ of methanol extract.

### *Cytotoxicity assays*

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

### *MTT assay*

Mitochondria are the cell's main energy producers and are therefore essential for cellular life, however, recent research has shown that these organelles play a key role in cell death when their membranes become permeable (Green and Kroemer, 2004). The MTT assay measures the mitochondrial function activity of mitochondrial dehydrogenases. MTT is a yellow colored dye [3-(4,5-dimethyl- 2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], which converts to a purple insoluble product formazan as a result of reduction reaction with concomitant oxidation of NADH (nicotinamide adenine dinucleotide-reduced) and NADPH (nicotinamide adenine dinucleotide phosphate-reduced) through the ability of the mitochondrial succinic dehydrogenase enzyme in living cells (Twentyman and Luscombe, 1987).

According to the method described by (Borenfreund et al., 1988) 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 microplate reader (LabtechLT-4000MS, Labtechm International Ltd., East Sussex, and UK). For each treatment, five replicate wells were examined, and each experiment was repeated three times (n=3). Standard error of mean (SEM) was calculated between three experiments. The results were calculated as percentage growth inhibition, untreated (control) cells versus treated cells according to the following formula (Qadir, 2014):

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

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

***Neutral red uptake assay***

The neutral red assay is basically a metabolic impairment assay, which works simply on the principle that this dye accumulates in the lysosomes of viable cells by a combination of active endocytosis and pinocytosis until a stable equilibrium is reached. Alterations of the cell surface or the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible. Such changes produced by toxic substances cause decreased uptake and binding of neutral red, making it possible to distinguish between viable, healthy and damaged or dead cells. Metabolically inactive cells lose their ability to accumulate and retain neutral red dye (Borenfreund and Puerner, 1985). However, this loss does not occur until late in the apoptotic process, when membrane integrity is compromised.

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 (n=3). 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)***

DAPI (4', 6-diamidino-2-phenylindole) is a DNA-specific probe, which forms a fluorescent complex by attaching in the minor groove of A-T rich sequences of DNA. Binding of DAPI to DNA produces a ~20-fold fluorescence enhan-cement, apparently due to the displacement of water molecules from both DAPI and the minor groove (Kapuscinski, 1995). The blue-fluorescent DAPI nucleic acid stain preferentially stains DNA and is used to study the morphological condition of cancer cells, espe-cially as a marker for apoptosis in the cells. DAPI produces a blue fluorescence when bound to DNA with excitation at about 360 nm and emission at 460 nm.

In the present study, DAPI stain was used to assess the morphological changes in nuclei of control and treated cells. Cells were seeded at density of 1 x $10^4$ cells/well in 500 µL of medium on sterilized glass cover slips in well plates for 24 hours. Caco-2 cells were treated with the negative control (culture medium), positive control (actinomycin-D, 4 µM). The plates were incubated at 37°C, 5% $CO_2$ in air in a humidified atmosphere for 24 hours. After treatment, cells were briefly equilibrated with PBS, fixed with 4% paraformaldehyde for 15 min, permeabilised 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.

***Data presentation and statistical analysis***

All data were compiled from a minimum of three experiments. Data for statistical analysis were expressed as the standard error mean, n (number of experiments). 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 present study was conducted to determine the ability of *G. trichophylla* crude extract and isolated saponins fraction against the proliferation of human colorectal cancer cells. Previous studies conducted showed that various isolated phytoconstituents provi-

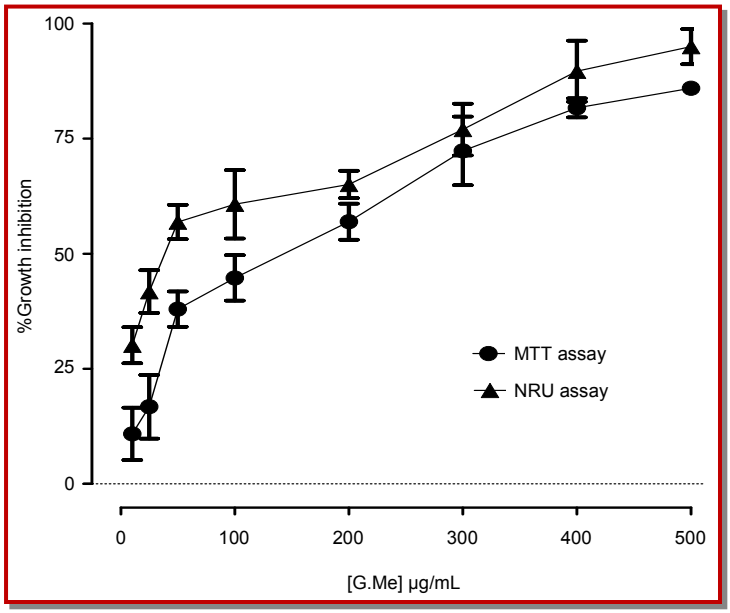


Figure 1: Dose-dependent growth inhibitory activity of *G. trichophylla* methanol extract (G.Me) against Caco-2 human cancer cells. The graph also provides a comparison between MTT and NRU assay sensitivities

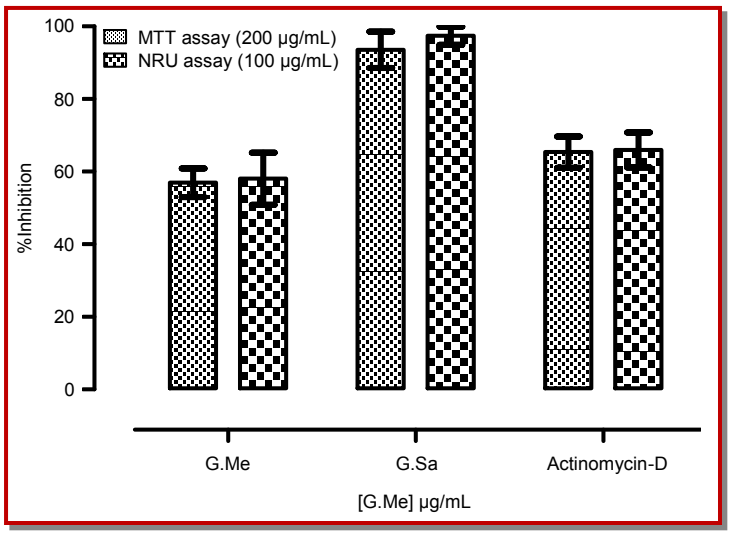


Figure 2: A comparison of *G. trichophylla* methanol and saponins extract and standard drug actinomycin D growth inhibitory activities against colorectal cancer cells (Caco-2). All the results are represented as mean ± SEM (n =3)

ded a great potential for new strategies in exploring anticancer drugs (Brown and Attardi, 2005).

The crude methanol extract of *G. trichophylla* showed a dose dependent growth inhibition of Caco-2 cancer cells with an apparent $IC_{50}$ value of 200 µg/mL in MTT assay. The very high significance inhibition ($p<0.001$) of the growth was observed above the dose of 200 µg/mL (Figure 1). The effect of saponins extract against Caco-2 cancer cells was tested at dose of 200 µg/mL for MTT assays. The crude saponins showed maximum effect and inhibited the growth of Caco-2 by 93.6 (± 5.0) % with respect to 65.4 (± 4.3)% inhibition of actinomycin-D (4 µM).

The methanol extract showed the dose dependent growth inhibition of the Caco-2 cancer cell lines with apparent $IC_{50}$ value of 100 µg/mL in NRU assay. The highly significance ($p<0.001$) response was observed with a dose of 50 µg/mL and above (Figure 1). The saponins showed maximum effect and inhibited the growth of Caco-2 by 97.4 (± 3.3)% with respect to 66.0 (± 4.8)% inhibition of actinomycin-D (4 µM)(Figure 2).

The DAPI staining of crude saponins treated cells when observed under confocal microscope. The nuclei were shrunken and signs of marked nuclear fragmentation

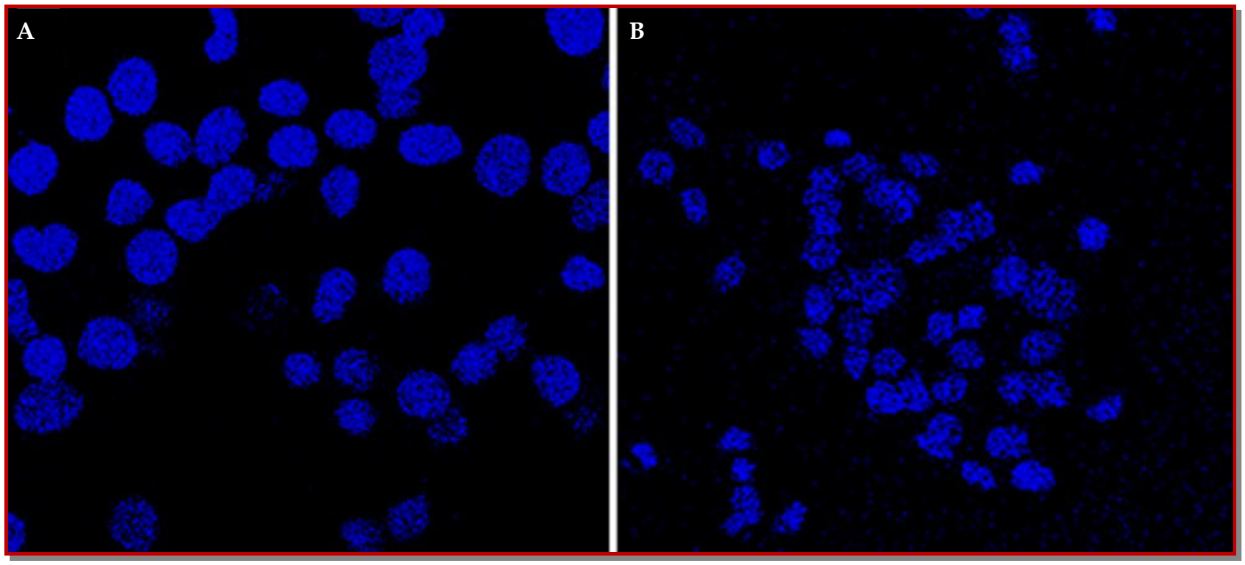


Figure 3: A) Control (untreated Caco-2 cells); B) Cells treated with *G. trichophylla* saponins (10 µg/mL, final concentration) for 24 hours and visualized under confocal microscope for DAPI stain

and chromatin condensation were observed. It was very clear that the numbers of apoptotic body fragments were more as compared to untreated cells (control) where nuclei is intact and there was no apparent signs of DNA fragmentation. Based on these observations we may suggest that Caco-2 cells, after treating with saponins fraction followed a programmed cell death pathway, an indication of the apoptosis mode of cell death, that is more obvious than necrosis (Figure 3).

## Discussion

The plants belonging to genus *Gaultheria* have also been reported to inhibit the human lung, prostate and liver cancer cell lines (Chen et al., 2009; Li et al., 2010). It is reported that saponins exert its cytotoxic activity through apoptosis through signalling pathways to prevent the tumors (Han et al., 2013). DAPI staining is most commonly used assay for observing the apoptosis at DNA level (Saha et al., 2013). In this study, saponins extract induced morphological changes in apoptotic cells which were observed in DAPI staining. This demonstrates that the treatment with saponins extract resulted in apoptotic body formation, chromatin condensation and nuclear fragmentation. It clearly indicates the potential of saponins extract to induce apoptosis against colon cancer cell lines i.e. Caco-2.

In conclusion, the present study shows that *G. trichophylla* methanol extract and its saponins fractions possess dose dependent growth inhibitory and potentially an apoptotic pathway against the human colon carcinoma Caco-2 cells.

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