***In vitro* cytotoxicity of *Gymnema montanum* in human leukaemia HL-60 cells; induction of apoptosis by mitochondrial membrane potential collapse**

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Abstract

Objectives: *Gymnema montanum* Hook, an Indian Ayurvedic medicinal plant, is used traditionally to treat a variety of ailments. Here, we report anti-cancer effects and molecular mechanisms of ethanolic extract of *G. montanum* (GLEt) on human leukaemia HL-60 cells, compared to peripheral blood mononuclear cells.

Materials and methods: HL-60 cells were treated with different concentrations of GLEt (10–50 µg/ml) and cytotoxicity was assessed by MTT assay. Levels of lipid peroxidation, antioxidants, mitochondrial membrane potential and caspase-3 were measured. Further, apoptosis was studied using annexin-V staining and the cell cycle was analyzed by flow cytometry.

Results: GLEt had a potent cytotoxic effect on HL-60 cells (IC₅₀ 20 µg/ml), yet was not toxic to normal peripheral blood mononuclear cells. Exposure of HL-60 cells to GLEt led to elevated levels of malonaldehyde formation, but to reduced glutathione, superoxide dismutase, catalase and glutathione peroxidase activities ($P < 0.05$). Induction of apoptosis was confirmed by observing annexin-V positive cells, associated with loss of mitochondrial membrane potential. Cell cycle arrest at G0/G1 was observed in GLEt-treated HL-60 cells, indicating its potential at inducing their apoptosis.

Conclusions: Findings of the present study suggest that *G. montanum* induced apoptosis in the human leukaemic cancer cells, mediated by collapse of

mitochondrial membrane potential, generation of reactive oxygen species and depletion of intracellular antioxidant potential.

Introduction

Phytochemical herbal medicines for treatment of a variety of diseases, and ancient Indian science of healthy living, are based on natural products. Isolation and characterization of novel bioactive compounds from medicinal plants, that might serve to lead development of new and effective drugs, has become an area of much interest worldwide (1,2). Experimental evidence has demonstrated that anti-cancer successes of medicinal plant-derived compounds may result from a number of mechanisms, including effects on inducing apoptosis and cell differentiation, enhancing the immune system and of DNA repair enzymes, antioxidant activity, inhibition of angiogenesis and reversal of multidrug resistance (3). Globally, medicinal plants continue to be subjective to extensive screening, in an attempt to develop still more effective anti-cancer treatments. Plant-derived natural products such as flavanoids, terpenes and alkaloids have received considerable attention in recent years, due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (4,5). In anti-tumour drug discovery or drug assessment, researchers have made considerable efforts to discover compounds that trigger apoptosis in cancer cells, only (6,7). Approximately 60% of anti-tumour active agents, clinically used or in various stages of development, are of natural origin (8). Phytoconstituents derived from varieties of herbs including *Vinca rosea*, *Allium sativum*, *Aloe vera*, *Angelica sinensis*, *Glycyrrhiza glabra*, *Hordeum vulgare*, *Hydrocotyle asiatica*, *Medicago sativa*, *Morinda citrifolia*, *Withania somnifera*, *Zingiber officinale* and more, have been demonstrated to have anti-cancer potential, albeit by diverse means (9).

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Recently, antioxidant and anti-proliferative potentials of medicinal plants used in traditional Indian medicine such as *Asclepias curassavica*, *Ophiorrhiza mungos*, *Cynodon dactylon*, *Costus speciosus*, *Achyranthes aspera*, *Amaranthus tristis*, *Blepharis maderaspatensis*, *Merremia emarginata*, *Aegle marmelos*, and *Tabernaemontana heyneana* have been reported (10) and active compounds from widely varied groups of plants are still to be explored scientifically. One approach pursued in this study, as part of ongoing investigation aimed at anti-cancer agents from medicinal plants, was to study *Gymnema montanum* using *in vitro* assays for both cytotoxic and apoptotic activities, seeking to identify agents capable of retarding the cell cycle and/or activating the apoptotic response, in malignant cells.

Gymnema montanum Hook, belonging to the family Asclepiadaceae, is endemic to India and is found mainly in the Shola forests of the Western Ghats, Nilgiris. *Gymnema montanum* is traditionally used to treat disorders such as diabetes, high cholesterol, wounds, inflammation and gastrointestinal ailments. In earlier studies we have explored a variety of its pharmacological properties including its anti-diabetic, anti-hyperlipidaemic, antioxidant and anti-microbial activities (11–14). As anti-proliferative and apoptosis-inducing effects of *G. montanum* on cancer cells have not previously been explored, this study was undertaken to investigate whether its ethanolic extracts would have anti-proliferative consequences and also to determine mechanisms of cell death elicited by the extract, in HL-60 cell line.

Materials and methods

Plant material and preparation of extract

Fresh leaves of *G. montanum* Hook were collected in November 2008 from the Shola forests of the Western Ghats, Gudalur, The Nilgiri Biosphere Reserve, at an altitude of 900–1500 MSL. Plants were identified using the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (accession no. 32561–65) and specimens were deposited in the Department of Biotechnology, Anna University of Technology Tiruchirappalli.

Fresh leaves of *G. montanum* (500 g) were air-dried in the shade, and dried material was crushed to a fine powder (particle size ~0.25 mm) using a laboratory mill; this was defatted with 750 ml petroleum ether (60 °C–80 °C) for 2–3 h in a Soxhlet apparatus. Resulting residues were dried and extracted further with 95% ethanol. Ethanolic extract was filtered and concentrated on a rotary evaporator at 40 °C–50 °C, under reduced pressure, and was lyophilized. Remarkable free radical scavenging activity of GLEt was observed in the ethanolic fraction (14);

hence, in this study, we used only ethanolic fractions of GLEt rather than other solvent fractions. A greenish-black powder, ethanolic extract of *G. montanum* leaves (GLEt) was obtained (20–30 g) and the residue was suspended in DMSO (final concentration <0.01%) for further use.

Cell culture conditions

The HL-60 cell line was obtained from the National Centre for Cell Sciences, Pune, India and cells were cultured at 37 °C in humidified, 5% CO₂ atmosphere in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) and 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml of amphotericin B.

Assessment of *G. montanum*-induced cytotoxicity

Cell viability was assessed using the MTT assay. HL-60 cells (1×10^4 cells/well) were cultured in 96-well plates at 37 °C for 24 h, treated with chosen concentrations of GLEt (5–50 µg/ml) in DMSO, and further incubated for another 24 h. Spent medium was removed and MTT solution was added (10 µg/100 µl medium), then plates were incubated at 37 °C for 4 h in humidified 5% CO₂ atmosphere. After incubation, 100 µl of DMSO was added to each well, and mixed thoroughly to dissolve stain crystals, and absorbance was read at 570 nm using a microplate ELISA reader.

Isolation and treatment of lymphocytes

Peripheral blood samples (5 ml) were obtained from four healthy non-smoking, male volunteers not exceeding the age of 35 years. Lymphocytes were isolated from blood samples by centrifugation using Ficoll-based density gradient, washed twice with serum-free RPMI-1640 medium and treated independently. To assess cytotoxicity of GLEt, lymphocytes ($\sim 1 \times 10^4$ cells/well) were resuspended in 1 ml fresh serum-free RPMI-1640 to which 10 µl aliquots of the chosen concentrations of GLEt (5–50 µg/ml) prepared in DMSO, were added. After 24 h incubation at 37 °C in the dark, cells were separated by centrifugation and immediately were analysed using a cytotoxicity assay with trypan blue.

Estimation of lipid peroxidation and antioxidants

For determination of malonaldehyde (MDA), cells ($\sim 1 \times 10^6$ cells/well) were seeded in 6-well culture plates and allowed to proliferate for 24 h; then they were treated with GLEt (5, 10 and 20 µg/ml) for 24 h at 37 °C; at the end of the experimental period, cells were centrifuged at 1000g for 5 min (15). Cell pellets

were homogenized according to recommendations of the manufacturer, and assayed for MDA using the MDA Assay kit (Oxis Research, CA, USA).

For determination of antioxidants [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH)], 24 h cultures of HL-60 cells ($\sim 1 \times 10^6$ /well in 6-well culture plates) were treated with GLEt (5, 10 and 20 $\mu\text{g/ml}$) for 24 h at 37 °C; after incubation, they were centrifuged and supernatant was removed. Cell pellets were resuspended in PBS, and sonicated.

Total SOD activity was assayed according to the method of Kakkar *et al.* (16) based on inhibition of formation of nicotinamide adenine dinucleotide (NAD), phenazine methosulphate (PMS) and nitro blue tetrazolium (NBT) formazan. One unit was taken as quantity of enzyme that produced 50% inhibition of nitro blue tetrazolium reduction per milligram of protein.

CAT was assayed colorimetrically at 620 nm and expressed in units as described by Sinha (17). This method is based on determination of H_2O_2 decomposed and remained after ending enzyme reaction on substrate (with a mixture of potassium dichromate and glacial acetic acid). One unit of enzyme activity equals enzyme quantity to decompose one H_2O_2 micromole (0.034 mg) for one minute at pH 7. Results were expressed in units per milligram of protein.

GPx activity was assayed by measuring H_2O_2 consumed in presence of reduced GSH for a specified time period (18). Remaining reduced GSH was measured by the method of Ellman (19), using 5, 5-dithiobisnitro benzoic acid (DTNB) as substrate in 1% sodium citrate buffer; yellow colouration developed was read at 412 nm and GPx activity was expressed as nanomoles reduced GSH consumed per minute, per milligram of protein.

GSH measurement is based on development of yellow colouration due to reaction of DTNB with compounds containing sulphhydryl groups, and extent of this was read at 412 nm. A calibration curve based on the standards was used to calculate quantities of reduced GSH, and expressed as micromoles per milligram of protein.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Changes in $\Delta\Psi_m$ during apoptosis were measured using 3,3'-dihexyloxacarbocyanine (DiOC6(3); Molecular Probes, Eugene, OR, USA) (20). HL-60 cells (5×10^5) in 500 μl RPMI-1640, were exposed to 100 nM DiOC6 (3) for the last 30 min of GLEt treatment. Cells were then pelleted at 1000g for 5 min. Pellets were resuspended and washed twice in PBS then this was lysed by addition of 600 μl of PBS followed by homogenization. Concentration

of retained DiOC6(3) was read on a spectrofluorometer (Hitachi F-2500, Tokyo, Japan) with excitation wavelength of 488 nm and emission wavelength of 500 nm.

Caspase-3 activity assay

HL-60 cells (3×10^3 /well) were cultured on glass coverslips in 24-well plates followed by 24 h treatment with GLEt ($\mu\text{g/ml}$) at the chosen concentrations. After incubation, cells were lysed in cell lysis buffer (100 mM of HEPES, pH 7.4, 0.5 mM of phenylmethylsulphonyl fluoride, 5 $\mu\text{g/ml}$ aprotinin, 5 $\mu\text{g/ml}$ pepstatin and 10 $\mu\text{g/ml}$ leupeptin), then centrifuged at 15 000g for 20 min at 4 °C. Activity of caspase-3 in the supernatant was estimated by colorimetric method using DEVD-p-nitroanilide as substrate, according to the manufacturer's instructions (ApoAlert Caspase-3 Colorimetric Assay Kits; Clontech, Palo Alto, CA, USA).

Cell cycle phase distribution

Quantitative cell cycle analysis was performed using cycl-eTEST PLUS DNA Reagent kit plus FACS Vantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For determination of cell cycle phases, peak area of FL2-H was recorded on a linear scale. Percentage of cells in sub-G1, G1, S and G2/M phases was determined using CELLQuest Software (Becton Dickinson, NJ, USA.). Briefly, HL-60 cells (1×10^6 /ml) in the exponential phase of growth were seeded in 6-well plates and allowed to adhere for 24 h. Old medium was replaced by fresh medium without and with GLEt at the chosen concentrations (5, 10 and 20 $\mu\text{g/ml}$) and further incubated for 24 h. After the experimental period, cells were trypsinized, centrifuged and washed in PBS. Cells were fixed in 70% ethanol, washed in PBS, subjected to proteinase and RNase digestion (37 °C for 30 min), followed by staining of clean nuclear material (nuclei) with PI (25 $\mu\text{g/ml}$) using procedures and reagents as described by manufacturers. Cells incubated in RPMI-1640 only, were used as controls. Duplicate samples were prepared for each treatment, unless otherwise indicated, and each experiment was repeated at least three times; approximately 10 000 cells were evaluated for each sample. In all flow cytometric determinations, suitable gating was employed to exclude cell debris and cell clumps from the analysis.

Measurement of annexin-V binding

Phosphatidylserine redistribution in membranes was measured by binding of annexin-V fluorescein isothiocyanate (FITC), according to the manufacturer's protocol (Miltenyi Biotec, CA, USA). Cells ($\sim 1 \times 10^3$) were treated

with GLEt (5, 10 and 20 µg/ml) for 24 h at 37 °C. Then medium was removed and replaced with fresh medium, incubated for 24 h and subjected to analysis; control cells were incubated in RPMI-1640 only. In all flow cytometric determinations, cell debris and cell clumps were excluded from the analysis by suitable gating. Effects of GLEt on apoptosis were determined by changes in proportion of sub-G1 (hypodiploid) cells. After treatment and subsequent incubation, cells were washed and resuspended in 100 µl HEPES buffer (10 mM HEPES (pH 7.4), 140 mM NaCl and 5 mM CaCl₂) containing annexin-V FITC (10 µl); samples were mixed gently and incubated at room temperature in dark for 15 min. Immediately before analysis by flow cytometry (FACSVantage SE; BD Biosciences, San Jose, CA, USA), 5 µl of propidium iodide (PI) (100 µg/ml) was added to each sample. For each experiment, 10 000 events were collected and analysed. Each experiment was repeated three times.

Statistical analysis

All data were expressed as mean ± SD of 3 independent experiments and tested using ANOVA using SPSS version 7.5 (SPSS Inc., Cary, NC, USA). Group means were compared by Duncan's multiple range test (DMRT); values were considered statistically significant at $P < 0.05$.

Results

Cytotoxicity of GLEt to human HL-60 cells and to human lymphocytes

MTT assay was performed to determine cytotoxic effects of GLEt on HL-60 cells. As shown in Fig. 1, GLEt treatment induced their death in a concentration-dependent manner. GLEt treatment at 5 and 20 µg/ml concentrations for 24 h resulted in $20.7 \pm 3.2\%$ ($P < 0.05$) and $45.4 \pm 4.3\%$ ($P < 0.05$) inhibition of cell viability, respectively. Thus, all further studies were carried out using these dose levels.

Cytotoxicity of GLEt on human lymphocytes was evaluated by trypan blue exclusion assay. Exposure of cells up to 50 µg/ml of GLEt for 24 h at 37 °C did not induce significant cytotoxicity and cell viability was found to be more than 90%. When the dose was increased to 500 µg/ml, viability slightly reduced, but still remained more than 85% (data not shown).

Effect of GLEt on lipid peroxidation and antioxidant levels

Oxidative stress represents one of the molecular mechanisms by which bioactive compounds induce cytotoxicity

Figure 1. The cytotoxic profile of GLEt on HL-60 cells and peripheral blood mononuclear cells. Cells were treated with GLEt extract for 24 h. Data were presented as mean ± SD of three independent experiments. Significant compared to control, * $P < 0.05$, ** $P < 0.01$ as determined by ANOVA followed by Duncan's multiple range test.

and apoptosis. To determine extent of oxidative cell damage in HL-60 cells exposed to GLEt, we performed the malondialdehyde assay. Our results revealed that GLEt treatment dose-dependently induced significant ($P < 0.05$) increase in malondialdehyde (a by-product of lipid peroxidation and biomarker of oxidative stress), which lead to increase in concentration of reactive oxygen species (ROS) in HL-60 cells compared to controls (Fig. 2). Assays for effects of GLEt on enzymic antioxidants, SOD, CAT, GPx, and non-enzymic antioxidant GSH, in HL-60 cells, revealed dose-dependent reduction in activities of these endogenous antioxidants ($P < 0.05$) (Fig. 3a–d).

Effect of GLEt on mitochondrial membrane potential

As reduction in mitochondrial membrane potential initiates the apoptotic cascade, we assessed effects of GLEt

Figure 2. Effect of GLEt on the levels of malonadehyde in HL-60 cells. The cells were cultured with or without extract for 24 h. The level of lipid peroxidation was assessed by malonadehyde using colorimetric assay. Data were presented as mean ± SD of three independent experiments. Significant compared to control, * $P < 0.05$, ** $P < 0.01$ as determined by ANOVA followed by Duncan's multiple range test.

Figure 3. Effect of GLEt on the (a) superoxide dismutase (SOD), (b) catalase (CAT), (c) glutathione peroxidase (GPx) activity and the level of (d) glutathione (GSH) in HL-60 cells. Data were presented as mean $\pm$ SD of three independent experiments. Significant compared to control, * P < 0.05, ** P < 0.01 as determined by ANOVA followed by Duncan's multiple range test.

on mitochondrial membrane potential in HL-60 cells and observed dose-dependent decrease in mitochondrial membrane potential in them (Fig. 4a).

Effect of GLEt on caspase-3 activity

Caspase-3 is the major effector caspase involved in apoptotic pathways. We investigated the role of caspase-3 in HL-60 cell responses to GLEt treatment. Following 24 h treatment of cells with a chosen range of GLEt concentrations, levels of caspase-3 were found to be elevated compared to those of control cells (Fig. 4b).

Cell cycle phase distribution

To further determine and confirm whether cell population growth inhibitory effects of GLEt were related to induction of apoptosis, subdiploid fractions (a character of apoptosis) were measured by flow cytometry. Percentages of cells in sub-G1, G1, S and G2/M phases

were determined, using CELLQuest Software (Becton Dickinson). After exposure of cells to GLEt (5, 10 and 20 µg/ml) for 24 h, a continuous increase in the sub-G1 fraction (comprising the apoptotic population) was observed, implying extent of cell death (Table 1). This cell damage was more apparent at higher concentrations of GLEt, over the period of study.

Measurement of annexin-V binding

To further quantify apoptotic effects of GLEt, HL-60 cells were stained with annexin-V FITC and PI, and subsequently analysed by flow cytometry. Annexin V assay monitors turnover of phospholipids from inner to outer layers of plasma membranes, an event typically associated with apoptosis. Significant dose-dependent elevation in numbers of apoptotic HL-60 cells were detected for cells treated with GLEt. Proportions of annexin V-stained cells were higher in 24 h GLEt-treated cells. Quantitative analysis of phosphatidylserine externalization through

Figure 4. Effect of GLEt on mitochondrial membrane potential (a) and caspase-3 activity (b) in HL-60 cells. Data were presented as mean $\pm$ SD of three independent experiments. Significant compared to control, * P < 0.05, ** P < 0.01 as determined by ANOVA followed by Duncan's multiple range test.

Table 1. Cell cycle profile of HL-60 cells after 24-h GLEt treatment

Treatment	Sub G1 (%)	G1 (%)	S (%)	G2/M (%)
Control	1.52 ± 0.18	67.26 ± 3.54	20.32 ± 1.62	10.44 ± 0.84
GLEt (5 µg)	8.68 ± 0.54**	42.84 ± 3.72**	36.44 ± 2.52**	11.86 ± 2.18
GLEt (10 µg)	14.62 ± 1.82**	36.48 ± 3.46**	39.82 ± 2.02**	11.12 ± 0.92
GLEt (20 µg)	26.84 ± 2.42**	31.68 ± 2.14**	33.56 ± 4.16**	10.86 ± 2.18

Cells were treated with a range of chosen concentrations of GLEt (0–20 µg) for 24 h. After fixation in 70% ice-cold ethanol at 4 °C and staining with PI for 30 min, cell cycle profile was analysed using flow cytometry. Percentages of cells in various phases of the cell cycle and those of cells containing subdiploid DNA content (sub G1) were determined. Data represent mean ± SD of three independent experiments. Significance compared to controls, ** $P < 0.01$ as determined by ANOVA followed by DMRT test (Duncan's multiple range test).

annexin V/FITC and PI staining indicated that percentages of double-positive cells in late apoptosis were increased in a concentration-dependent manner after 24 h treatment with GLEt (Fig. 5).

Discussion

Numerous studies have emphasized relationships between apoptotic cell death and cancer, and increasing evidence suggests that defects in apoptotic signalling pathway are major contributors in many cancer cells and malignant tissues (21,22). Deregulated apoptotic signalling confers high survivability and resistance of tumour

cells to therapeutic agents. As compounds displaying apoptosis-inducing activity are considered potential anti-tumour agents (23), many efforts have been made to discover new drugs through isolation of apoptosis-inducing agents from natural products. According to standards of the National Cancer Institute, crude extracts with IC₅₀ less than 20 µg/ml are considered to be active against tested cancer cells/lines (24).

Gymnema montanum is a rich source of polyphenols, and it has traditionally been used to treat disorders such as diabetes, high cholesterol, wounds, inflammation and gastrointestinal ailments. In earlier studies, we have demonstrated anti-diabetic, anti-hyperlipidemic, antioxidant

Figure 5. Analysis of apoptotic frequencies of GLEt in HL60 cells measured by flow cytometry using annexin-V labelling. The intensity of the annexin-V-Fluos signal is represented on the X-axis and the intensity of the propidium iodide (PI) signal is represented on the Y-axis. The lower left quadrant contains viable cells; the upper left quadrant contains PI-positive cells and the two right quadrants contain annexin-V positive cells. Apoptotic cells are located in the two right quadrants.

and anti-microbial effects of ethanolic extract of *G. montanum* leaves (11–14), however, mechanisms of any anti-proliferative activity of *G. montanum* had not been explored till date. Here and for the first time, we report cytotoxic effects of ethanolic leaf extract of *G. montanum* on human leukaemic HL-60 cells.

HL-60 cells are derived from peripheral blood leucocytes of an adult human female with acute promyelocytic leukaemia (25); it has widely been used for studies of anti-leukaemic agents or mechanisms of apoptosis (26–28) as they tend to undergo apoptosis in response to various stimuli (29,30). Thus, our present results can be discussed with reference to these previous observations. New data obtained suggest that it would be worthwhile to investigate this plant extract further, as a candidate to be a generally non-toxic, yet anti-leukaemic agent, by using further types of myeloid leukaemia cell lines.

Relationships between concentration of extracts and their anti-proliferative effects on HL-60 cells, were investigated by MTT assay; it was found that GLEt has a significant inhibitory effect on HL-60 cell proliferation. Data from the present study indicate that GLEt efficiently blocked proliferation of HL-60 cells *in vitro*, without showing cytotoxicity to normal human peripheral blood mononuclear cells, treated side-by-side. The strong anti-proliferative activity of GLEt may be due to presence of bioactive components, especially polyphenols, which are known to induce anti-proliferative outcomes. Numerous studies have documented that polyphenols are able to influence a variety of cell functions by modulating cell signalling, altering proliferation and reducing proliferation in many cancer cell types (31,32).

The present report suggests involvement of ROS in apoptosis induction in HL-60 cells. This effect was confirmed by MDA estimation, reduction in enzyme antioxidant levels and depletion in intracellular glutathione status. This could be attributed to presence of different classes of cytotoxic compound in the extract. Lipid peroxidation is the most extensively investigated process participating in toxic activity leading to necrosis or apoptosis of these human leukaemic cells. MDA is a breakdown product of lipid peroxidation and its assessment is considered to be a reliable marker of oxidative damage (33,34). Here, GLEt treatment resulted in dose-dependent elevation in lipid peroxidation in HL-60 cells. These results signify that GLEt treatment effectively promotes subsequent lipid peroxidation-related damage dose-dependently, indicating increased levels of MDA, thereby initiating the apoptotic cascade.

Diminished antioxidant levels are a well-known cause of cellular dysfunction and are involved in apop-

totic pathways of malignant cells (35). In this study, exposure of HL-60 cells to GLEt reduced intracellular antioxidant levels, such as those of SOD, CAT, GPx and GSH. These antioxidants are widely reported to play major roles in cell protection, specially, SOD with its superoxide anion scavenging activity (followed by H₂O₂ scavenging activity of CAT), and with formation of glutathione. Reduced levels of these antioxidants, along with increase in lipid peroxidation and/or ROS generation is thus indicative of GLEt's anti-proliferative activity.

Mitochondrial damage is an important early event in apoptosis and is consistent with reduced intracellular antioxidants and changes in mitochondrial membrane potential (36). Our results also show that GLEt treatment reduced intracellular antioxidant production leading to loss of mitochondrial membrane potential. Several studies have suggested that decrease in antioxidant production, preceding loss of mitochondrial membrane potential, is correlated with release of cytochrome c and caspase-3 activation in various cell types, leading to induction of apoptosis. Cytochrome c acts in connection with other cytosolic factors to cause activation of executioner caspase-3, in turn leading to downstream apoptotic events (37). Once in the cytosol, cytochrome c binds to APAF-1 and procaspase-9 in the presence of dATP to form the apoptosome complex. This activates caspase-9, which in turn cleaves and thereby activates caspase-3 (38). In this study, results suggested that GLEt exerted anti-proliferative effects on HL-60 cells and induced cell death through such activation of caspase-3, in a dose-dependent manner.

Apoptosis is a major form of cell death, characterized by a series of stereotypical morphological changes such as chromatin condensation, shrinkage of cells and bleb formation, internucleosomal DNA fragmentation, sub-G1 DNA accumulation (39) and formation of apoptotic bodies. From our study, according to cell cycle distribution pattern analysed by flow cytometry, GLEt-treated HL-60 cells were found to be arrested at G0/G1 (Table 1), which may result in inhibition of cell proliferation; also, this has been implicated as event in cell differentiation.

To confirm apoptosis induced by GLEt, we further employed flow cytometry to determine extent and causes of apoptosis. GLEt significantly induced annexin V binding, and increased the sub-G1 population. To further determine and confirm whether the growth inhibitory effect of GLEt was related to induction of apoptosis, the subdiploid (character of apoptosis) fraction was measured by flow cytometry. After exposure of cells to GLEt (5, 10 and 20 µg/ml) for 24 h, there was continuous increase in the sub-G1 fraction made up of the

apoptotic body population, implying together the extent of cell death (Table 1). Damage was more apparent at higher concentrations of GLEt over the period of study.

Furthermore, flow cytometry studies indicated that GLEt treatment significantly increased apoptotic cell death, as shown by proportion of annexin-V-positive cells. Concentration dependent increase in percentage of apoptotic cells was observed with increasing concentrations of extract; proportion of apoptotic cells increased up to 17.6% at 20 µg/ml. Several previous investigators working with natural products have reported that apoptosis may be associated with cytotoxicity of such compounds, in tumour cells.

Underlying mechanisms of anti-proliferative, cytotoxic action of GLEt could be by activation of the caspase pathway, possibly due to phenolic phytochemicals present in the extract. Numerous laboratory and epidemiological studies have shown that phenolic phytochemicals have antioxidative, anti-microbial, anti-carcinogenic, anti-proliferative and anti-inflammatory activities (40,41). Apart from antioxidant activity, polyphenols are well documented to act as chemopreventive agents exerting a direct, pro-apoptotic effect on tumour cells, which block numerical expansion of several types of human cancer cell lines at different phases of the cell cycle.

Antioxidant or pro-oxidant characteristics of a compound depend on redox potential of individual molecules and inorganic chemistry of the cell (42). From previously reported phytochemical analysis of GLEt by GC-MS, it was found to contain 11.57% carvacrol, 6.77% erythritol, 4.58% gallic acid (GA) and 3.09% quercetin, which demonstrate apoptosis-inducing properties in various cancer cell lines (33,34).

Carvacrol has been reported to induce cell death *via* phospholipase C (PLC)-dependent Ca^{2+} release, involving ROS-mediated apoptosis, in human glioblastoma cells (43) and caspase-3 associated apoptosis in oral cancer cells (44). Furthermore, anti-tumour effects of carvacrol has been reported in HepG2 cells, indicating its profound role in induction of apoptosis by direct activation of both mitochondrial and mitogen-activated protein kinase pathways (45).

Zhao *et al.* (46) have reported mannosylerythritol lipid (MEL)-mediated apoptosis and growth arrest in G1 phase of mouse malignant melanoma cells. Moreover, MEL exposure stimulated expression of differentiation markers in melanoma cells, such as tyrosinase activity, and enhanced production of melanin, an indication that MEL triggered both apoptotic and cell differentiation programmes.

Yeh *et al.* (47) have reported gallic acid-induced apoptosis in HL-60 cells through G0/G1 arrest, inhibition of cyclins D and E and also by upregulating the

mitochondrial dependent pathway involving caspase-8, caspase-9, caspase-3, AIF and endo G. Quercetin has anti-proliferative effects on cell cycle arrest at the G1 phase through induction of p21, and also downregulates cyclin B1 and cyclin-dependent kinase (CDK) 1, both essential in progression to G2/M phase of the cell cycle, in human breast carcinoma cell lines (48) and in HepG2 cells (49). These results suggest that apoptotic effects of GLEt may be rationalized considering possible synergistic effects among polyphenols present in our extract. Hence, this research evidence suggests that GLEt possibly possesses anti-proliferative and anti-tumour properties, which may play a significant role in its cytotoxicity to human leukaemic, HL-60 cells.

In conclusion, here we have reported that *G. montanum* induced apoptosis in HL-60 cells was mediated by altered mitochondrial membrane potential, cell cycle arrest and/or activation of caspases. On the basis of phytochemical composition of the extract, we speculate that synergistic effects of phenolic compounds may be involved in mechanisms of action of the extract against human leukaemic cells. These findings may provide a platform for the use of *G. montanum* as a novel therapeutic agent towards treatment of human leukaemia.

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