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Inhibition of cancer cell growth by crude extract and the phenolics of *Terminalia chebula* retz. fruit

Ammar Saleem^{a,*}, Michael Husheem^b, Pirkko Härkönen^b, Kalevi Pihlaja^a^a Department of Chemistry, University of Turku, Kiinamyllynkatu 10, FIN-20014 Turku, Finland^b Department of Anatomy, Institute of Biomedicine, University of Turku, FIN-20520 Turku, Finland

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Abstract

A 70% methanol extract of *Terminalia chebula* fruit, was studied for its effects on growth in several malignant cell lines including a human (MCF-7) and mouse (S115) breast cancer cell line, a human osteosarcoma cell line (HOS-1), a human prostate cancer cell line (PC-3) and a non-tumorigenic, immortalized human prostate cell line (PNT1A) using assays for proliferation (³H-thymidine incorporation and coulter counting), cell viability (ATP determination) and cell death (flow cytometry and Hoechst DNA staining). In all cell lines studied, the extract decreased cell viability, inhibited cell proliferation, and induced cell death in a dose dependent manner. Flow cytometry and other analyses showed that some apoptosis was induced by the extract at lower concentrations, but at higher concentrations, necrosis was the major mechanism of cell death. ATP assay guided chromatographic fractionation of the extract yielded ellagic acid, 2,4-chebulyl-β-D-glucopyranose (a new natural product), and chebulinic acid which were tested by ATP assay on HOS-1 cell line in comparison to three known antigrowth phenolics of *Terminalia*, gallic acid, ethyl gallate, luteolin, and tannic acid. Chebulinic acid (IC₅₀ = 53.2 μM ± 0.16) > tannic acid (IC₅₀ = 59.0 μg/ml ± 0.19) > and ellagic acid (IC₅₀ = 78.5 μM ± 0.24), were the most growth inhibitory phenolics of *T. chebula* fruit in our study. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Terminalia chebula* Retz.; Cytotoxicity; Apoptosis; Phenolics; Hydrolyzable tannins

1. Introduction

The plants of genus *Terminalia*, comprising of 250 species, are widely distributed in tropical areas of the world (Fabry et al., 1998). Fruits of *Terminalia chebula* Retz. (*T. chebula* Retz.) (Combretaceae), commonly known as black Myroblans in English and Harad in Hindi, indigenous in Pakistan and India among many Asian and African countries, is a popular folk medicine and has been studied for its homeostatic, antitussive, laxative, diuretic and cardiogenic activities (Barthakur and Arnold, 1991; Singh, 1990). At least seven *Terminalia* species were traditionally used for the treatment of cancers (Hartwell, 1982). Lee et al. (1995) studied the cytotoxicity of methanolic extract of *T. chebula* and its isolated compounds, gallic acid, 1,2,3,4,6-penta-*O*-gal-

loyl-β-D-galcopyranose, chebulagic acid, and chebulinic acid. These compounds showed moderate in vitro cytotoxicity against cultured human tumor cell lines including A-549, SK-OV-3, SK-MEL-2, XF-389, and HCT-15. Gallic acid, ethyl gallate and luteolin, isolated from *T. arjuna* showed strong growth inhibition of P388, OVCAR-3, SF-295, A498, NCI-H460, KM20L2 and SK-MEL-5 cells (Pettit et al., 1996). Luteolin, a widely distributed flavone, has shown in vitro growth inhibitory effect against MCF-7 (Han et al., 2001).

We studied the effects of the extract on several malignant cell lines including human prostate cancer cell line (PC-3), human (MCF-7) and mouse (S115) breast cancer cell lines human osteosarcoma (HOS-1) and a non-tumorigenic, an immortalized human prostate cell line (PNT1 A) using assays for cell viability (ATP determination), proliferation (³H-thymidine incorporation, determination of cell numbers by coulter counting) and cell death (flow cytometry and Hoechst DNA staining). The chromatographic work was tar-

* Corresponding author. Tel.: +358-2-333-6830; fax: +358-2-333-6700

E-mail address: amsale@utu.fi (A. Saleem).

geted towards the isolation of antigrowth phenolics and yielded ellagic (3) (an important precursor of anticancer drugs), 2,4-chebulyl- β -D-glucopyranose (4) (a new compound), and chebulinic acid (5) (a gallotannin of *T. chebula* fruit extract). The cytotoxicities of the isolated compounds were determined in HOS-1 cells in comparison to growth inhibitory phenolics from *Terminalia*, gallic acid (1), ethyl gallate (2) luteolin (6) and tannic acid (a mixture of gallotannins).

2. Materials and methods

2.1. Plant collection, identification and documentation

The dried fruit of *T. chebula* was purchased from a local herbal market in Lahore, Pakistan in March 1997 and was identified by Professor Zahoor Ahmed, Department of Taxonomy, and University of The Punjab, Lahore, Pakistan. Specimen was deposited in Turku University Herbarium, (TUR 354114).

2.2. Reagents and chemicals

The RPMI-1640 (culture medium), Dulbecco's Modified Eagles Medium (DMEM), testosterone, gallic acid, ethyl gallate, tannic acid, luteolin (5,7,3',4'-tetrahydroxyflavone) and estrogen were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid and para-formaldehyde were obtained from Merck, (Darmstadt, Germany). 4-(2-hydroxyethyl)-L-piperazineethanesulfonic acid (HEPES), Fetal Bovine Serum (FBS), phosphate buffer saline (PBS), and L-glutamate were from Gibco, (Paisley, Scotland). [3 H]-thymidine solution and Sephadex LH-20 (particle size, 18–111 μ m) were obtained from Amersham Pharmacia Biotech AB, (Uppsala, Sweden). ATP monitoring reagent was from BioThema, (Dalarna, Sweden). Zap-Oglobin reagent and isoton solution (azide free balanced electrolyte) were bought from Beckman Coulter Limited (London, England). Hoechst-33342 dye and Annexin-V-FLUOS Staining Kit were from Roche Diagnostics GmbH, (Mannheim, Germany). Cell culture dishes were from NUNC, (Roskilde, Denmark). All chemicals for chromatography were of HPLC grade except acetone.

2.3. Preparation of *T. chebula* fruit extract

Dried fruits (500 mg) were homogenized for 40 s with Ultra-Turrax T25 (Janke and Kunkel, IKA-Labortechnik, Germany) in 70% methanol at room temperature. The homogenate was continuously stirred for 1 h and centrifuged at 1500 \times g for 10 min. The pellet was extracted twice and the extract was evaporated to dryness under reduced pressure at 35 $^{\circ}$ C. The dried

extract was re-dissolved in deionized water at 40 mg/ml and stored at -18° C until needed.

2.4. Extraction and solvent partitioning

The dried fruits of *T. chebula* (200 g) were extracted (4 \times) with 1 l of 70% methanol by 1 h continuous stirring. Methanol was evaporated at reduced pressure by rotatory evaporator and the extract was freeze-dried yielding 62 g dry extract. Thirty grams of the freeze-dried extract was redissolved in 750 ml of deionized water and partitioned (3 \times) with an equal volume of *n*-hexane, chloroform and 1-butanol (BUT). The *n*-hexane and chloroform extracts were dried with anhydrous sodium sulfate and evaporated under reduced pressure. Aqueous (AQ) and BUT fractions were centrifuged and residues removed. The BUT fraction was concentrated under reduced pressure and water fraction was freeze-dried. The AQ fraction was redissolved in deionized water and the BUT fraction in 9:1 (methanol:water).

2.5. Column chromatography

The AQ and BUT fractions were separated with size exclusion chromatography using Sephadex LH-20 column (40 \times 3.8 cm i.d., Pharmacia, Umeå, Sweden) by consecutive elution with water, 30, 50 and 80% methanol and 30, 50 and 80% acetone. The AQ and BUT sub-fractions were evaporated to dryness and redissolved in water and 3:2 (methanol:water) respectively.

2.6. Preparative HPLC analysis

The fraction BUT6 (80% acetone) was dissolved in 3:2 (methanol:water) and subjected to LiChroprep RP-18 (44 \times 3.7 cm i.d., 40–63 μ m), (Merck, Darmstadt, Germany). Two solvents were used: (A) acetonitrile and (B) 5% formic acid (AQ). The elution profile was linear gradient 0–25% A in B. Flow rate was 4 ml/min. Forty-four fractions were collected and similar fractions were pooled based on HPLC with UV detection at 280 nm.

2.7. Isolation of ellagic acid (3)

The AQ layer was freeze-dried (yield 12.8 g), of which 9.98 g was redissolved in water and chromatographed on Sephadex LH-20. Ellagic acid was separated from AQ8 as a white precipitate (120 mg) (Fig. 1).

2.8. Isolation of 2,4-chebulyl- β -D-glucopyranose (4)

The compound was isolated as glassy crystals (85 mg), after the purification of 30% methanol fraction with semi-preparative HPLC on LiChroprep RP-C18 column.

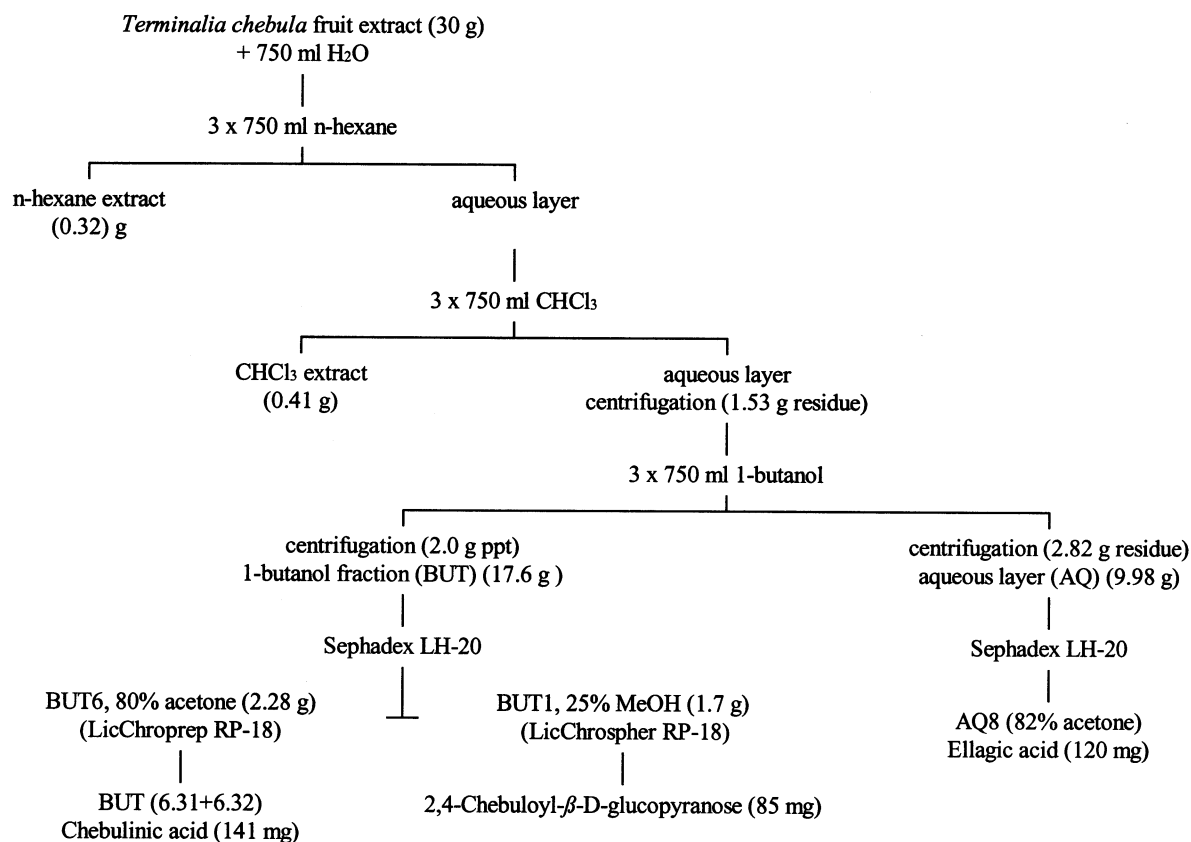


Fig. 1. Scheme showing the isolation of ellagic acid, 2,4-chebulyl- β -D-glucopyranose, and chebulinic acid from *T. chebula* fruit extract.

2.9. Isolation of chebulinic acid (5)

The BUT was chromatographed on Sephadex LH-20 providing eight sub-fractions. The BUT1, BUT2, and BUT3 (30, 50 and 80% methanol) were moderately active, but BUT6 (80% acetone) was strongly active. Further fractionation of BUT6 was proceeded with low pressure liquid chromatography, which yielded 141 mg chebulinic acid as off-white powder.

2.10. Cell culture

MCF-7 cells were grown in RPMI-1640 medium with 5% FBS, 4 μ g/ml insulin and 10^{-8} M estrogen as described previously (Wärri et al., 1993). S115 cells were maintained in DMEM with 4% FBS and 10^{-8} M testosterone (Ruohola et al., 1995). PNT1A cells were maintained in RPMI-1640 medium with 10% FBS and L-glutamate (2.0 mM). PC-3 and HOS cells were maintained in DMEM with 10% FBS. Cells were cultured at 37 °C with 5% CO₂ at 95% humidity. For each assay, the *T. chebula* fruit extract was diluted in serum free medium and added to each cell line after 24 h of cell growth. For morphological analysis, the cells were cultured in 24-well plates and investigated after 24 and 72 h under a phase contrast microscope (Leica DMRB, Wetzl, Germany).

2.11. Cell growth analysis

Cells (1000, 2000 and 3000 per well) were grown in 96 well cell culture plates. The number of parallel wells was 3–5 in each experiment. Cell growth was measured by determinations for [³H]-thymidine incorporation, ATP determination or by counting cell numbers with Coulter counting (Ruohola et al., 1995, 2001). For measuring [³H]-thymidine incorporation into DNA, 1000 cells in 0.1 ml per well were cultured in 96 well plate for defined time periods. [³H]-thymidine solution (50 μ Ci per well) was added to cells. After 3 h of incubation, the cells were trypsinised and harvested on filter paper. [³H]-thymidine incorporated into DNA was quantified by Liquid Scintillation Counter, Microbeta 1450 (Wallac OY, Turku, Finland).

ATP level of the cultures was measured to determine the relative number of living cells. One-thousand cells/0.1 ml per well were cultured in 96 well microplate. A 0.1 ml of 1% trichloroacetic acid was added to cells to cease the cell function and release ATP. A 0.1 ml ATP monitoring reagent solution containing buffer (2 mM EDTA, 0.1 M tris acetate, pH 7.7) was added to 0.5 ml of cell lysate. ATP was determined by luminometric assay with Victor² (Wallac OY, Turku, Finland).

Cell numbers were determined with a Coulter Particle Counter (Wärri et al., 1993; Ruohola et al., 1995).

Three-thousand cells/0.5 ml per well was seeded in 24 well cell culture plates. Culture medium was removed from cells and 0.2 ml of Zap–Oglobin reagent and 1.0 ml buffer (10 mM HEPES+1.5 mM MgCl₂, pH 7.7) were added to cells for 2 min to release the nuclei (Creencia et al., 1996). Ten ml isoton solution was added and the nuclei were counted with a Coulter Counter (Coulter Electronics, Luton, England).

2.12. Hoechst DNA staining

Three-thousand cells per well were plated on glass slides in 24 wells cell culture plate and incubated with the extract for 72 h. Cells were fixed with 3% paraformaldehyde for 15 min, washed twice with PBS and stained for 15 min in dark at 37 °C. Hoechst dye was prepared in PBS (1.25 µg/ml) and cells were stained in it for 15 min. Four hundred cells from three different microscopic fields were observed and the proportion of normal cells and the cells presenting apoptotic morphology (fragmented or shrunken nuclei) were counted under fluorescence microscope (Leica DMRB, Weitzl, Germany) at 418 nm.

2.13. Flow cytometry

Cells were incubated with the extract for 24 h, scraped and washed twice with PBS, centrifuged (2000 × *g*) for 5 min at 0 °C, and incubated with 0.2 µg/ml Annexin-V-FLUOS and 1.4 µg/ml DNA stain propidium iodide for 15 min at room temperature. Measurements were carried out on flow cytometer, FACS Calibur, (Becton Dickinson Immunocytometry Systems; San. Jose, CA) at 488 nm excitation and 530/30 nm band pass filter for fluorescence detection and 670 nm high pass filter for propidium iodide detection.

2.14. Statistical analyses

The data were collected after 24 and 72 h of *T. chebula* extract treatments in all assays. Results from both time points showed similar trends. The 24 h data mostly did not, however, show statistical significance and are not reported. The IC₅₀ values were calculated by non-linear curve fit at four data points and reported as means of ±S.D. *N* = 3, for all assays except for ATP determination and [³H]-thymidine incorporation, where *N* = 5. All experiments were repeated twice.

3. Results

3.1. ATP assay guided partitioning and fractionation

Dried *T. chebula* fruits were extracted with 70% methanol and then separated into *n*-hexane, chloroform

and BUT (Fig. 1). The *n*-hexane and chloroform fractions were inactive while the BUT and AQ fractions were active and their further separations were accomplished using column chromatography on Sephadex LH-20. The AQ8 (82% acetone) yielded ellagic acid. Among BUT fractions, BUT1 and BUT6 were strongly active fractions and they were further purified by preparative HPLC, yielding 2,4-chebulyl-β-D-glucopyranose from BUT1 (20% methanol) and chebulinic acid from pooled sub-fractions BUT (6.31+6.32). The structural elucidation of isolated compounds was carried out by HPLC–DAD, HPLC–ESI-MS, ¹H-NMR and ¹³C-NMR spectrometry.

3.2. Cell growth

A thousand, 2000, and 3000 cells of each cell line were grown for 144 h to determine a suitable number of cells to be used in proliferation assays. The growth of cell lines was measured by ATP assay. The data collected at 48, 72 and 144 h indicated that S115 cells were the fastest and HOS-1 and PC-3 the slowest growing (data not shown). The cell growth was linear for 144 h for all cells at the plating density of 1000 cells per well except for MCF-7. Hence, 1000 cells were chosen as appropriate number of cells to be used.

3.3. Effect of *T. chebula* fruit extract on cell proliferation

The effect of *T. chebula* on different cell lines was studied by measuring cell numbers by Coulter counting and relative numbers of viable cells using ATP determination assay after treatment of the cultures with the extract for various time periods. The treatment of all cell lines with the extract for 144 h markedly decreased cell numbers determined by two methods, showing IC₅₀ at µg/ml level. Within cell lines it showed the highest activity on PNT1A cells (IC₅₀ = 42.0 µg/ml ± 0.5) and weakest on PC-3 cells (IC₅₀ = 79.0 µg/ml ± 0.8) (Table 1). Corresponding results were obtained in both hormone dependent and independent cell lines. The IC₅₀ values obtained for various cell lines were rather similar. They were also in the same range as the IC₅₀ values obtained by counting cell numbers.

In order to determine whether growth inhibitory effect of *T. chebula* extract was due to the inhibition of proliferation or induction of cell death, we analyzed its effect on the rate of cell proliferation by measuring [³H]-thymidine incorporation into DNA in various cell lines. The time course studies of PC-3 cells showed that at a high concentrations (400 µg/ml) the number of viable cells decreased rapidly (in less than 1 h) whereas, at lower concentrations (13.3, 20.0, 40.0 and 66.6 µg/ml), the cell numbers started to decrease markedly after 24 h (Fig. 2) and no cell inhibition was observed at 8.0 µg/ml. These results suggest that at high concentrations the

Table 1
Cytotoxic effect of *T. chebula* fruit extract on selected cell lines

Assays	Cell lines				
	PNT1A	PC-3	S115	MCF-7	HOS-1
ATP determination ^a	44.0 ± 2.3 *	38.0 ± 1.2 *	+ ^{Te} 36.0 ± 0.0 - ^{Te} 36.5 ± 1.0	+ ^E 42.0 ± 0.8 - ^E 47.0 ± 0.3	43.0 ± 0.2 *
Coulter Counter	42.0 ± 0.5	79.0 ± 0.8	56.0 ± 0.3	49.0 ± 1.0	39.0 ± 0.9

IC₅₀ (µg/ml of the extract which inhibits cell growth by 50% vs. control) are means ± S.D., N = 5.

^a Relative number of viable cells was determined by measuring ATP levels of cultures grown for 72 h in the presence of 0–800 µg/ml of *T. chebula* fruit extract; +^E, -^E, in the presence/absence of estrogen; +^{Te}, -^{Te}, in the presence/absence of testosterone; *, hormone independent cell lines.

extract has an immediate toxic effect on PC-3 cells as well as on all the cell lines tested (data not shown) whereas, at lower concentrations it induces a series of cellular events which lead to inhibition of proliferation and/or induction of cell death in a concentration and dose dependent manner.

3.4. Effect of *T. chebula* extract on cell death

The ability of *T. chebula* extract to induce cell death was estimated by analyzing its effect on cell morphology (Fig. 3). The observation of PC-3 cells under a phase contrast microscope showed that after 72 h of treatment, most cells were attached in the cultures containing 40 µg/ml of the extract. They were viable and adherent cells at 66.6 µg/ml although a great proportion of cells were poorly attached and several looked dead, whereas all

cells were shrunken and detached when the concentration of the extract was 400.0 µg/ml. The results are shown for PC-3 cells but similar results were obtained with various cell lines although there were differences in the sensitivity of various cell lines to the extract (data not shown).

The proportion of dead cells and type of cell death after treatment of different cell lines with various concentrations of the extract was estimated by Hoechst DNA staining (Fig. 3) and flow cytometry. Hoechst staining was used to estimate the proportion of apoptotic cells in the cultures exposed to 40.0, 66.6 and 400.0 µg/ml of *T. chebula* extract. The apoptotic cells were recognized on the basis of nuclear condensation and DNA fragmentation of Hoechst-stained cells under a fluorescent microscope. The nuclei showed normal morphology in untreated control cell. In PC-3 cultures

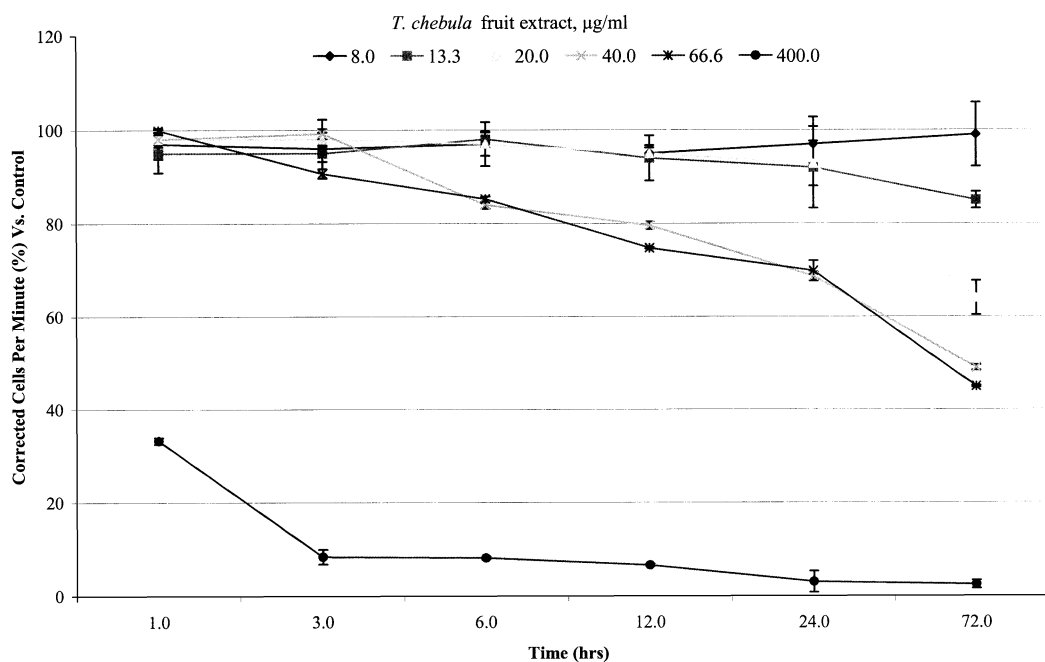


Fig. 2. Time course effect of [³H]-thymidine incorporation in PC-3 cells grown in 8.0, 13.3, 20.0, 40.0, 66.6 or 400.0 µg/ml of *T. chebula* fruit extract. DNA labeling was measured after 0.5, 3, 6, 12, 24 or 72 h treatment of the cultures with the extract; 100% = level of DNA labeling in non-treated control cells (CCPM/well). Results are means ± S.D., N = 5.

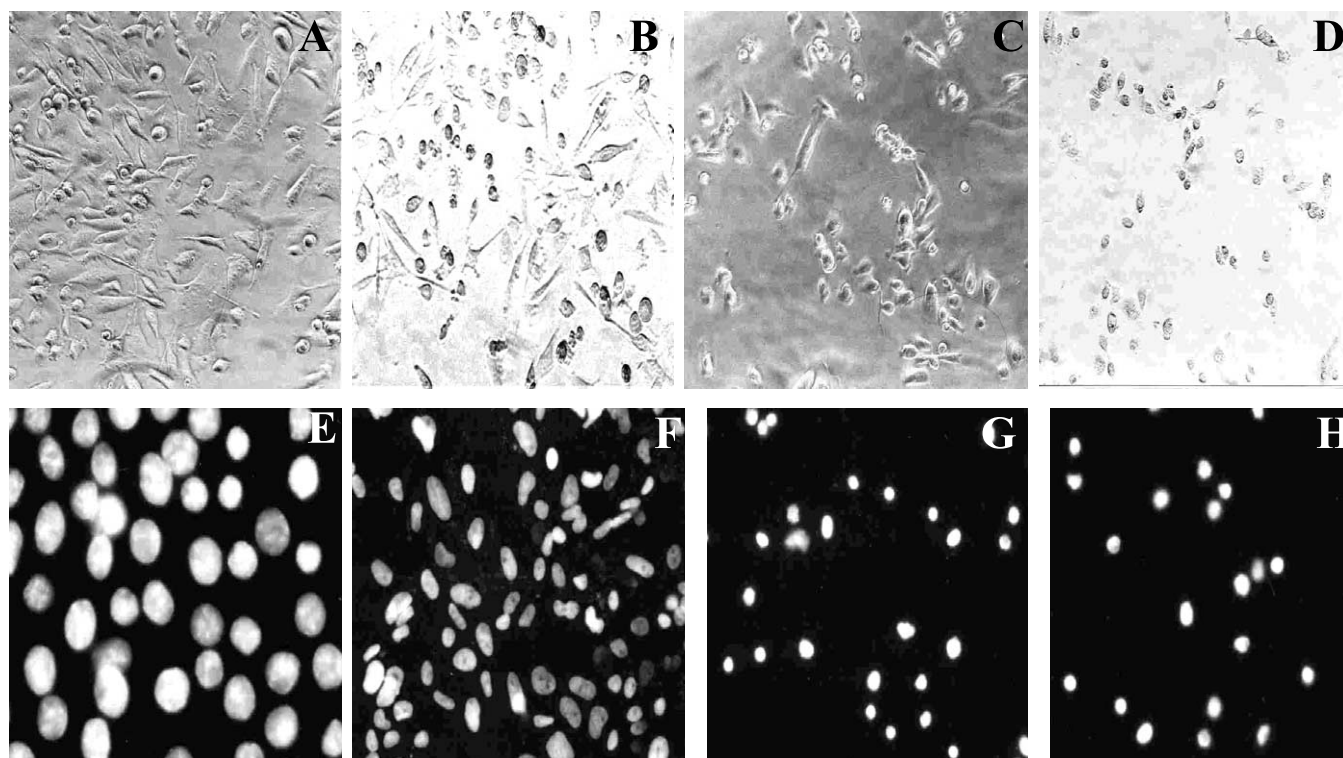


Fig. 3. Phase contrast (A–D) and Hoechst DNA staining (E–H) analysis of PC-3 cells grown for 72 h in the presence of *T. chebula* fruit extract. (A, E) untreated cells, (B, F) 40.0 µg/ml, (C, G) 66.6 µg/ml and (D, H) 400 µg/ml. Microscope magnification (40×280).

treated with 40.0 µg/ml of *T. chebula* extract for 72 h, most cells presented normal nuclear morphology although there were some apoptotic nuclei. Very few or no viable cells were observed in PC-3 cultures treated with 66.6 or 400.0 µg/ml but all the nuclei were shrunken, fragmented and pyknotic compared with non-treated cells.

The effect of *T. chebula* extract on cell death was also analyzed with flow cytometry (Fig. 4). The assay was carried out to further investigate the mechanism of cell death by *T. chebula* extract. By this method the relative proportion of viable cells can be differentiated from those undergoing early stages of apoptosis (annexin-stained, non-disrupted cells) or from those later dying by necrosis (disrupted or lysed cells). The flow cytometric analysis of PC-3 and PNT1A cells showed that after a treatment of the cells with lower concentrations (8.0–40.0 µg/ml) for 72 h, cells undergoing apoptosis (annexin-stained cells) were observed, whereas at higher concentrations (above 40 µg/ml), most dead cells were necrotic or lysed by apoptosis. The cell lines differed in their susceptibility to the cell death inducing capacity of the extract. In PC-3 and PNT1A cells the highest proportion of apoptotic cells was observed at 8.0 µg/ml of the extract, whereas in HOS cells an increase in apoptotic cells was observed only at higher concentrations. In all cell lines, however, the proportion of necrotic cells increased in a dose dependent manner

and the level was by far higher than that of apoptotic cells.

3.5. Effects of *T. chebula* phenolics

Cytotoxicities of six phenolics of *Terminalia* and tannic acid (a mixture of tannins) were assessed by ATP determination using HOS-1 cells as a model (Table 2). Since the cell lines responded in a rather similar way to crude extract, we assumed that one cell treated with phenolic compounds could serve as an example of their activity. However, it is possible that different cell lines differ in their responses to phenolic compounds. Except ethyl gallate in HOS-1 cells all samples showed dose dependence at six data points. The cytotoxic activities (IC_{50} values) were obtained in the range of 53.2–120 µM, which shows an overall moderate effect of these compounds. Chebulinic acid ($IC_{50} = 53.2 \mu\text{M} \pm 0.16$) was the most cytotoxic while ellagic acid ($IC_{50} = 78.5 \mu\text{M} \pm 0.24$) showed slightly weaker activity. The 2,4-chebulyl- β -D-glucopyranose ($IC_{50} = 120.0 \pm 3.25 \mu\text{M}$) was the weakest cytotoxic compound in our test system. This compound is a hydrolyzed form of chebulinic acid, (removal of three galloyl moieties) and chebulanin (removal of a galloyl moiety). The structural elucidation data (HPLC–DAD, HPLC–ESI-MS, $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$) are reported in Saleem et al., 2002.

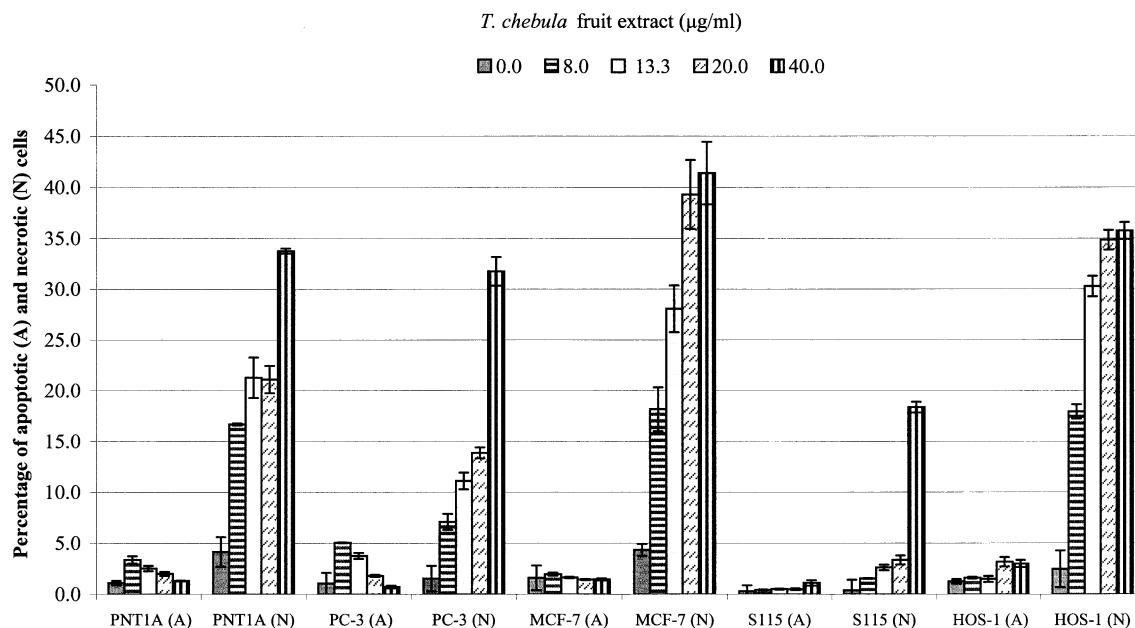


Fig. 4. Proportion (%) of apoptotic (A) and necrotic (N) cells determined by flow cytometry after 72 h culture of PNT1A, PC-3, MCF-7, S115 and HOS-1 cells in 0.0, 8.0, 13.3, 20.0 or 40.0 µg/ml of *T. chebula* fruit extract. The columns are means \pm S.D. of apoptotic or necrotic cells, $N = 3$.

Table 2

Cytotoxicity of *Terminalia* phenolics in HOS-1 cells determined by the level of ATP

Compound	IC ₅₀ (µM)
Gallic acid	114.7 \pm 0.05
Ethyl gallate	No dose dependence
Ellagic acid	78.5 \pm 0.24
2,4-Chebulyl- β -D-glucopyranose	120 \pm 3.25
Chebulinic acid	53.2 \pm 0.16
Luteolin	92.7 \pm 0.18
Tannic acid	59.0 \pm 0.19 (µg/ml)

Cells were treated with the compounds for 72 h.

4. Discussion

Our results demonstrate that *T. chebula* extract has growth inhibitory and cytotoxic effects on several human cancer cell lines. Our results are in agreement with those of Lee et al. (1995), who reported the antigrowth and cytotoxic effects of the components of *T. chebula* extract, gallic acid and chebulinic acid. In contrast, Sato et al. (1997) found no inhibitory effect of ethanol extract of *T. chebula* fruit on MCF-7 cells in MTT assay. This might have been, however, due to the difference in the extraction method used. In addition no details of the assay (such as concentrations used etc.) were given, which makes the comparison of their results to ours difficult. Since it is possible that different cell lines exhibit different sensitivities towards the plant extracts, we included cell lines of different species (human and mouse) and origins (mammary gland/breast

cancer (MCF-7, S115), prostate/prostate cancer (PNT1A and PC-3), bone/osteosarcoma (HOS-1)) with different properties (hormone sensitivity, growth rate, tumorigenicity) in our study. Although various cell lines did differ in their sensitivity to *T. chebula* extract they all responded to it basically by inhibition of proliferation and induction of cell death: apoptosis and necrosis. The effect was not dependent on the presence or absence of hormone in hormone responsive cell lines (MCF-7 and S115).

At high concentrations (400 µg/ml) *T. chebula* extract had an immediate toxic effect possibly caused by an interference of cell membrane integrity or mitochondrial function in the cells. At lower concentrations (100 µg/ml or less) *T. chebula* extract inhibited cell growth time and dose dependency, which was obviously caused by both inhibition of proliferation and induction of cell death. Both effects were evident only after a treatment of some hours (up to 24 h). The delay suggests that *T. chebula* extract initiates cellular processes, which lead to the inhibition of proliferation and induction of cell death.

The mechanisms of inhibition of proliferation or induction of cell death remain to be studied. Flow cytometric analysis of various cell lines revealed different proportions of apoptotic and necrotic cells at different concentrations of the extract. It should be noted that the cells dying by apoptosis eventually are degraded as necrotic cells. The annexin-staining used allows detection of the cells at an early stage of apoptosis (Van Engeland et al., 1996) and in a rather narrow time-window, which is then followed by a longer period of DNA fragmentation and lysis detected as

'necrotic' cells. It is thus understandable that in the flow cytometric analysis the percentage of apoptotic (= annexin-stained cells) at a particular time point is rather low in case of each cell line and that of 'necrotic' cells accumulated much higher. However, the increase of apoptotic cells at very low concentrations demonstrates a specific induction of cell death at least partly by this mechanism.

Polyphenolic compounds of plants, especially gallic acid and caffeic acid derivatives, and flavonoids are well known as dietary antioxidants (Cai et al., 1997). They also exhibit contrasting pharmacological actions like prooxidant toxicity and apoptosis (Sergediene et al., 1999). Gallic acid (3,4,5-trihydroxybenzoic acid), a naturally occurring polyphenol, is obtained by the hydrolysis of tannins in plants. It is well known to show antioxidant, antimutagenic, cytotoxic and anticarcinogenic activities in a variety of in vivo and in vitro studies and its three adjacent hydroxyl groups are believed to be responsible for its cytotoxic potential (Inoue et al., 1995). Several derivatives of gallic acid including ethyl gallate, 2,3,4-trihydroxybenzoic acid and ellagic acid, induce apoptotic cell death in various cancer cell lines including human stomach cancer (KATO III) and human colon adenocarcinoma (COLO 205) (Yoshioka et al., 2000).

Although a wealth of information is available about the antitumor activities of ellagitannins and related compounds (Yang et al., 1999; Miyamoto et al., 1993), few reports present such data about gallotannins. Ellagic acid, generated by the hydrolysis of ellagitannins in plants, is well known as a natural dietary antioxidant and chemopreventive agent (Perchellet et al., 1992). It is a potent antagonist of the mutagenicity of various aromatic hydrocarbons and a possible prototype of a new class of anti-cancer drug molecules (Das et al., 1985). According to a study by Smith, et al. (2001), ellagic acid showed moderate dibenzof[a,]pyrene induced DNA adduct inhibition in MCF-7 cells. Among 57 tannins and related compounds studied for their cytotoxicities against human tumor cells, chebulinic acid showed selective cytotoxicity against PRMI-7951 melanoma cells, ($ED_{50} = 0.8 \mu\text{g/ml}$) (Kashiwada et al., 1992). The 2,4-chebulyl- β -D-glucopyranose (without galloyl moiety) showed about 44% less inhibition as compared with chebulinic acid (with three galloyl moieties). This proposes that the presence of galloyl moieties is crucial for the cytotoxic potential of gallotannin-like compounds, (Fig. 5).

Our HPLC and LC-MS studies of tannin rich fraction (BUT) indicated chebulinic acid as the major tannin of *Terminalia* fruit extract but chebulagic acid, a closely related ellagitannin, was present in minute quantity. Tannic acid was included in our study besides six phenolics of *Terminalia* due to its ability to show chemoprotective, skin antitumor (Nepka et al., 1999;

Perchellet et al., 1992) and growth suppressor activities against HTC-15 and HepG2 cell lines (Kamei et al., 1999). Tannic acid (Sigma Chemical Co.) was analyzed by HPLC–DAD and HPLC–ESI-MS, and was found to contain gallic acid and di-, tri-, tetra- and penta-galloyl glucoses, besides many minor unidentified compounds.

Our recent study (Saleem et al. 2001) has reported that among 37 medicinal plant extracts, *T. chebula* fruit extract has higher phenolic contents and stronger in vitro lipid peroxidation inhibition capability. The antioxidants are known to play a key role in reducing cancer cell proliferation, (Chinery et al., 1998) and tannins are known as strong lipid peroxidation inhibitors (Okuda et al., 1983). Hence, due to the presence of tannins and related compounds as the major phenolics of *T. chebula* extract, they could be the molecules responsible for the strong antioxidant and antigrowth activities found in our studies. However, further chemical work and pharmacological evidences, at molecular level, are required to establish the possible correlation among the mentioned activities of the extract.

5. Conclusions

The present study was undertaken to evaluate the cytotoxic activity of *T. chebula* fruit extract that is used in the treatment of cancer in Asia and Africa. *T. chebula* fruit extract decreases the number of cells in immortalized and cancer cell lines by inhibiting the rate of cell proliferation and inducing cell death. At low concentrations *T. chebula* extract is able to initiate cellular pathways that lead to apoptosis whereas at high concentrations the extract has direct toxic effects, which lead to rapid necrotic cell death. Among the phenolics of *T. chebula* fruit extract, chebulinic acid and ellagic acid showed moderate inhibition and they may be responsible for the inhibiting cell proliferation observed with *T. chebula* fruit extract.

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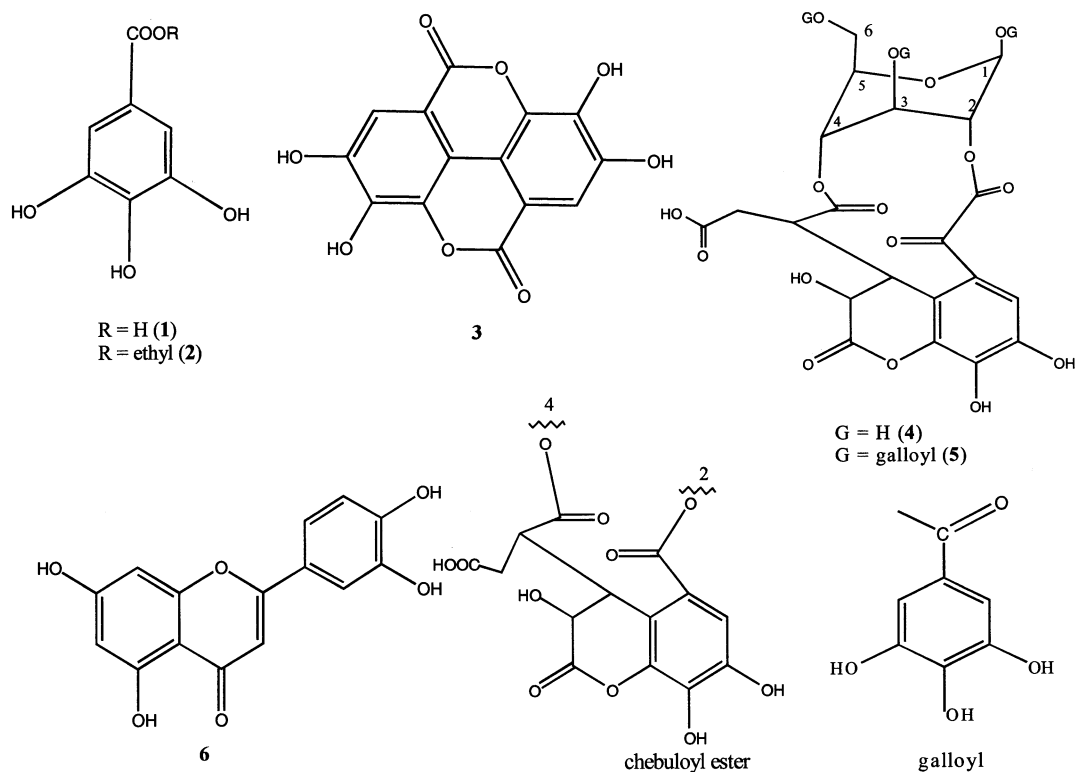


Fig. 5. Cytotoxic phenolics of *Terminalia*. 1, Gallic acid; 2, ethyl gallate; 3, ellagic acid; 4, 2,4-chebulyl- β -D-glucopyranose; 5, chebulinic acid; 6, luteolin.

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