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Cytotoxic investigation of stem bark extracts of *Terminalia chebula* Retz.

KEYWORDS: *Terminalia chebula* Stem Bark, Trypan Blue Assay, MTT Assay, DNA Fragmentation Assay, Acute Oral Toxicity Assay.

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ABSTRACT *Terminalia chebula a native plant in India and Southeast Asia commonly known as Haritaki has been reported to exhibit a variety of biological activities. The present study aimed to investigate the cytotoxicity of the stem bark extracts of Terminalia chebula Retz. Methanol, chloroform, petroleum ether and aqueous extracts were used for the study. Percentage cell viability of cell lines was carried out by using trypan blue dye exclusion technique. The viability percentage of cell lines analysed after exposing the cells to various solvent extracts showed difference in cell tolerating ability. These extract was screened for its cytotoxicity against HeLa and cell lines at different concentrations to determine the growth inhibition by MTT assay. It was found that the % growth inhibition increased with increasing concentration steadily upto 100mg/ml on HeLa cell line. Methanolic extracts shows higher cytotoxicity at a concentration of 100mg/ml. The DNA fragmenting ability of bark methanolic extract is effective when compared to other solvent extracts. This was mainly due to the presence of quinones and phenolic compounds in the methanol extract. Chloroform and petroleum extracts showed less DNA fragmentation due to the absence of these metabolites. It was followed by acute oral toxicity studies. The bark extract of T. chebula when administered for analysing the acute oral toxicity test showed no deleterious effects were observed in the experimental animals.*

INTRODUCTION

Plants contain wide variety of chemical constituent with different potential biological activites. Traditional health care systems using medicinal plants can be recognized and used as a starting point for the development of novelties in drugs. The use of plant substances for medication is believed to be less toxic compared to that of synthetic chemical compounds. Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordon, 2001).The biological activites of the extracts can be influenced by the extraction solvents, conditions and storage (Kim and Choe, 2004).

The plants of genus *Terminalia*, comprising of 250 species which are widely distributed in tropical areas of the world (Fabry *et al*., 1998). *Terminalia chebula* Retz., an important medicinal plant distributed in the Sub-Himalayan tracks and the eastern, western and southern parts of India. It has been extensively used in ayurvedic, unani and homoeopathic medicine. The fruits are astringent, gastroprotective, purgative and laxative and are used to alleviate asthma, piles and cough (Chatterjee and Pakrasi, 2000). It has been reported to exhibit a wide variety of biological properties like antidiabetic (Sabu *et al*., 2002), anticancer (Saleem et., 2002), antioxidant (Cheng *et al*., 2003), antimicrobial (Sato *et al*., 1997), antimutagenic (Kaur *et al*., 2002) and antiviral (Ahn *et al*., 2002) activity. Lee *et al* (1995) studied the cytotoxicity of methanolic extract of *Terminalia chebula* and its isolated compounds. Gallic acid and chebulic acid were isolated from extract of *Terminalia chebula*, as an active principle that blocked the cytotoxic T-Lymphocytes (CTC) mediated cytotoxicity (Hamada *et al*., 1997). The isolated compounds like 1,2,3,4,6-penta-O-galloyl-β-D-gulcopyranose, chebulagic acid, gallic acid and chebulinic acid showed moderate *in vitro* cytotoxicity aganist human tumor cell lines. Therefore, the present study has been undertaken to investigate the cytotoxicity of stem bark extracts of *Terminalia chebula* Retz. The acute oral toxicity were also carried out and showed no signs of toxicity against stem bark extracts.

MATERIALS AND METHODS

Collection of the plant

The stem barks of *Terminalia chebula* were collected from Nilgiri district, Tamil Nadu, India.

Solvents Extract preparation

The plant materials were washed and dried in an oven at 45°C. Powdered samples were successively extracted in a soxhlet apparatus using low, medium and high polar solvents such as petroleum ether (40-60°C), chloroform (59.5-61.5°C) and methanol (64-65.5°C) for 16-18 hours. The extract was concentrated and stored in refrigerator (4°C).

Viable cell count using trypan blue (Freshney, 2006)

Trypan blue is a vital dye. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless membrane is damaged. Therefore all the cells which exclude the dye are viable.

Trypan Blue Staining of Cells

About 0.5ml of a suitable cell suspension (dilute cells in complete medium without serum to an approximate concentration of $1x10^5$ to $2x10^5$ cells per ml) was placed in a screw cap test tube. Followed by 0.1ml of 0.4% trypan blue stain was added and mixed thoroughly. The mixture was allowed to stand for 5min at 15 to 30°C (room temperature). Cell was counted by filling the cell suspension in a haemocytometer. Viable and non-viable cells were observed under a microscope.

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MTT assay for cell proliferation (Freshney, 2006)
The cells were harvested (4.5-5.0x10^4 cells/well) and inoculated in 24 well microtitre plates.The cultured cells were washed with warm RPMI-1640 without phenol red and then inoculated with and without the extract at different concentrations. After 72h incubation the medium was aspirated. MTT working solution was added into the wells being assayed, for example 1.0ml for each well of 12-well plate. Plates were incubated at 37°C for 30min to 3hrs (this time depends on cell density and cell type). At the end of the incubation period, the medium can be moved if working with attached cells. The converted dye is solubilized with 1ml acidic isopropanol (0.04M HCl in absolute isopropanol). The mixture was pipetted up and down several times to make sure that the converted dye dissolved completely. The dye solution was transferred with the cells into a 1.5ml eppendorff tube and centrifuged at 13,000rpm for 2min. The supernatant was transferred into a new eppendorff tube. Absorbance of the converted dye was measured at a wavelength of 570nm with background subtraction at 650nm. ELTEK–400 reader was used to calculate the inhibition of cell viability by means of the formula,

% inhibition = (1-absorbancy of treated cells/absorbancy of untreated cells)x100

DNA fragmentation assay (Freshney, 2006)
In 96 flat-wells plate, 1x10^5 target cells (36 wells of 96 per well) with desired concentration of effectors (10^5 target cells per well) were incubated. After incubation, the cell sample was collected in 1.5ml eppendorff tube, spinned down and resuspended with 0.5ml PBS in 1.5ml eppendorff tubes. About 55µl of lysis buffer was added and left for 20min on ice (4°C). Eppendorff tubes were centrifuged in cold at 12,000g for 30minutes. The samples were transferred to new 1.5ml eppendorff tubes and the supernatant was extracted with 1:1 mixture of phenol:chloroform (gentle agitation for 5min followed by centrifugation). The mixture was precipitated in two equivalence of cold ethanol and one-tenth equivalence of sodium acetate. The content was centrifuged and the precipitates were transferred in 30µl of deionized water-RNase solution (0.4ml water+5µl of RNase). About 5µl of loading buffer was added and incubated for 30 minutes at 37°C. About 2µl of Hind III marker was added on the outer lanes. 1.2% gel was run at 50V for 5min before increasing to 100V.

Acute oral toxicity testing (Walum, 1998)
Healthy young female adult Swiss Albino mice, nulliparous and non-pregnant were selected. The animals weighing 25-30g were acclimatized to the housing conditions of 22°C±3°C room temperature, 40 to 60% humidity and 12hours light/12hours dark cycle. Water and standard food pellets were provided.

Acute oral toxicity study was performed as per organization for economic cooperation and development (OECD) guideline 423 methods. Two groups of animals each consisting of three animals were administered, a single of 0.5ml (350mg/kg body weight) using specially designed mice oral needle. 0.2% carboxy methylcellulose (CMC) was used as vehicle. Animals were fasted 3hours prior to dosaging (water was provided) and after dosaging food was withheld for 2hours. Animals were observed individually after dosaging periodically for a total of 7 days.

RESULTS
Trypan Blue Assay
Percentage cell viability of cell lines was carried out by using trypan blue dye exclusion technique. In Table-1, the % viability of *HeLa* cell line was presented. The viability percentage of cell lines analysed after exposing the cells to various solvent extracts showed difference in cell tolerating ability. The toxic effect of the extracts was due to the extracted metabolites of the bark, which had injured the cell membrane and leading to the uptake of trypan blue dye showing variations in their viability. The effectiveness of methanol in extracting the secondary metabolites of the bark imparts high toxic nature to the cells decreasing its viability.

Table-1: Trypan Blue Assay of different extracts of *T. chebula bark*

	Total	Live	Viability (%)	Toxicity	% of Toxicity
Control	23	23			
Methanol	20	18	0.9	0.1	10
Chloroform	20	19	0.95	0.05	5
Petroleum ether	21	20	0.952381	0.047619	4.761905
Aqueous	22	21	0.954545	0.045455	4.545455

MTT Assay
The cytotoxicity study was carried out for plant extract of *Terminalia chebula*. These extract was screened for its cytotoxicity against *HeLa* and cell lines at different concentrations to determine the growth inhibition by MTT assay**.** It was found that the % growth inhibition increased with increasing concentration steadily upto 100mg/ml on *HeLa* cell line (Table-2). Different extracts shows different cytotoxicity at different concentrations (Figure-1). Methanolic extracts shows higher cytotoxicity at a concentration of 100mg/ml. Reports have shown that crude plant extracts are more active pharmacologically than their isolated active principles. This may be due to the synergistic effects of the various components present in the extracts.

Table-2: MTT assay to find the level of cytotoxicity of various extracts

Replication parameters	1	2	3	t/c	1-(t/c)	1-(t/c)*100
Control (HELA only)	1.444	1.432	1.438	0.910991	0.089009	8.90
DMSO	1.399	1.391	1.395	0.88375	0.11625	11.62
Methanol (solvent only)	1.354	1.357	1.3555	0.858727	0.141273	14.13
100	1.345	1.323	1.334	0.845106	0.154894	15.49
50	1.36	1.36	1.36	0.861577	0.138423	13.84
25	1.356	1.354	1.355	0.85841	0.14159	14.16
Chloroform (solvent only)	1.4	1.398	1.399	0.886284	0.113716	11.37

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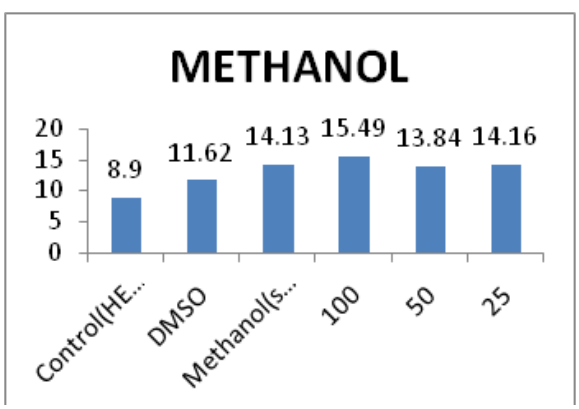
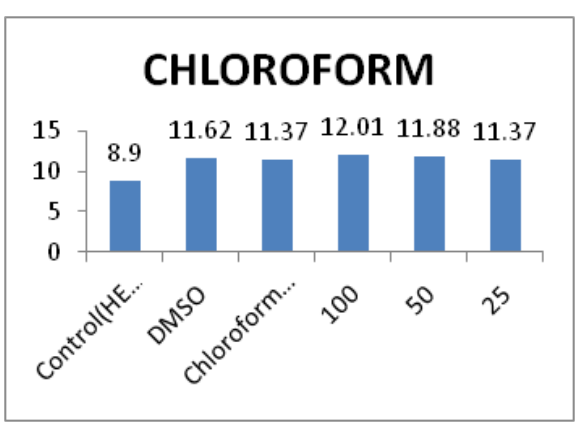
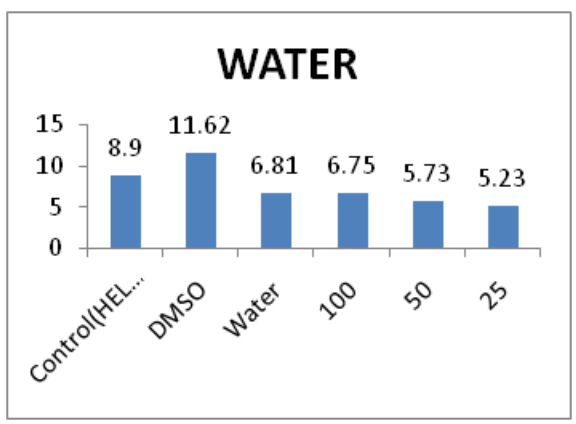
100	1.39	1.388	1.389	0.879949	0.120051	12.01
50	1.391	1.391	1.391	0.881216	0.118784	11.88
25	1.399	1.399	1.399	0.886284	0.113716	11.37
Water	1.494	1.448	1.471	0.931897	0.068103	6.81
100	1.396	1.548	1.472	0.932531	0.067469	6.75
50	1.431	1.545	1.488	0.942667	0.057333	5.73
25	1.448	1.544	1.496	0.947735	0.052265	5.23
Petroleum Ether(solvent only)	1.375	1.361	1.368	0.866646	0.133354	13.34
100	1.345	1.396	1.3705	0.868229	0.131771	13.18
50	1.372	1.353	1.3625	0.863161	0.136839	13.68
25	1.361	1.399	1.38	0.874248	0.125752	12.58



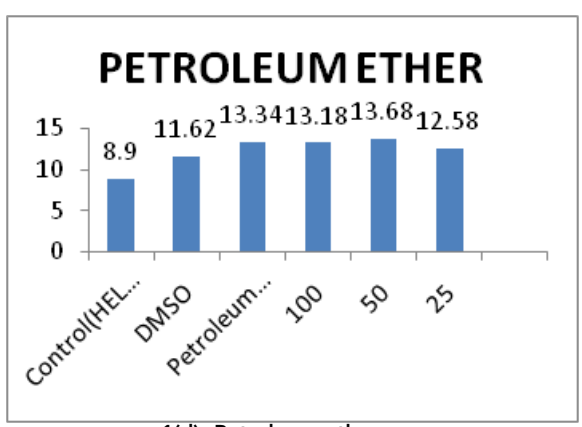
1(a): Methanol



1(b): Chloroform



1(c): Water



1(d): Petroleum ether

Figure-1: Cytotoxicity of different extracts- Methanol, chloroform, water, petroleum ether

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Note: Values were presented in Percentage

HeLa cells the breast cancer cells was preferred for the work due to its high proliferating and cancerous nature under *in vitro* conditions. The cancer cell proliferating ability can be analysed when exposed with different solvent extracts of the bark of *T. chebula*. The MTT assay will determine the toxic and non-toxic concentration of the extracts over the used cancerous cells. When compared with untreated cells, the cell proliferation inhibition percentage of the bark solvent extracts was more. To understand the cytotoxic effect of solvent extract, the aqueous extract was also used simultaneously. As the methanolic extract possess plant metabolites like alkaloids, sterol/terpenoids, quinines, anthraquinones, tannins and phenols, it showed more effective cytotoxicity than other solvent extracts. The bark extract of petroleum ether contain metabolites like coumarins, alkaloids, sterols/terpenoids, quinines and resins which are more similar to methanolic extract also showed good cytotoxic effect. When compared, the bark extract of chloroform which had not extracted any of these metabolites showed less cytotoxic effect on the used *HeLa* cells. The bark solvent extract present metabolites like anthraquinones, tannins and phenols play a major role in preventing oxygen utilization of the cells and negatively interact with their genetic material to stop its replication.

DNA Fragmentation Assay

The presence of plant metabolites like quinones, phenols and quinines had a direct denaturing ability on nucleic acids. These metabolites can alter the bonding ability of the nitrogenous bases in the genetic material. The hydrogen and phosphate bonds in the DNA lose their specificity and strength and get altered leading to random fragmentation of the DNA. The random fragmentation is irreversible process, which affects the proliferating and the replicating ability of the cells. The DNA fragmenting ability of bark methanolic extract was effective when compared to other solvent extracts. This was mainly due to the presence of quinones and phenolic compounds in the methanol extract. Chloroform and petroleum extracts showed less DNA fragmentation due to the absence of these metabolites (Figure-2). When exposed to aqueous extract, very less DNA denaturing effect was observed as no or less inhibitory substances are present. This was mainly due to poor extracting ability of the water on *T. chebula* bark samples.

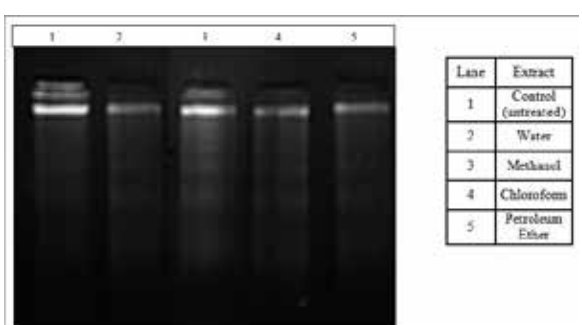

Figure-2: DNA Fragmentation assay of bark extracts of *T. chebula*

Acute Oral Toxicity Assay

The most often disease outbreak due to toxic effect of plant metabolite in new born animal or human babies lung is the hyaline membrane disease or in adult, the respiratory illness. The bark extract of *T. chebula* when administered for analysing the acute oral toxicity test, made no discomfort in the breathing process of administered animals. The motor activity in the administered animals was normal by comparison with control animals. The muscular movement, the mouth action and the throat part were normal indicating there was no motor neurone disease. Usually during the outbreak of motor neurone disease, progressive muscular atrophy (PMA), lateral sclerosis and bulbar palsy were the effects noticed in patients. The reflex action of the extract administered animals is normal. Automatic reaction to a stimulus was noticed during the incubation time indicating the normal functioning of reflexes. The salivation process is normal showing the functions of glands in the body of animals. There was no ptyalism noticed during the time of analysis in animals. Skin has no change in their pigmenting ability and was no dehydration. The hairs are normal and there was no erection of hairs. The muscle tone is normal, which was indicated by the normal state of the muscles without getting fully relaxed. The physical examination of the administered animals after incubation showed no changes in the physical appearance and activity showing that the solvent extracts have no deleterious effects on the body cells or organs of animals (Table-3).

Table-3: Acute Oral Toxicity Assay-Observation of toxic signs in mice

S. No	Observation in	Observed sign
1	Respiratory	Nil
2	Motor activity	Nil
3	Reflexes	Nil
4	Ocular signs	Nil
5	Cardiovascular signs	Nil
6	Salaivation	Nil
7	Skin	Nil
8	Piloerection	Nil
9	Muscle tone	Nil
10	Gastrointestinal signs	Nil
11	Physical examination	Nil

DISCUSSION

The present research report was compared to the test results of Lawal *et al.*, (2013). The *in vitro* cytotoxic activity and possible pro-apoptotic effect of *Securidaca longepedunculata* aqueous root bark extract on Ehrlich ascites carcinoma cells were determined. *In vitro* cytotoxic activity was determined using the trypan blue assay by incubating Ehrlich ascites carcinoma cells with various concentrations of *S. longepedunculata* aqueous extract. *S. longepedunculata* was found cytotoxic to Ehrlich ascites under *in vitro* conditions.

The present test result when compared to the research work conducted by Krishna and Nair, (2010) showed supportive information that all solvent extract samples would reveal more cytotoxic activity when compared to the control and aqueous samples. The researchers investigated the cytotoxic potential of *Tectona grandis* extracts by MTT assay. In their study the chloroform extract of bark exhibited very high activity against chick embryo fibroblast (CEF) and human embryonic kidney (HEK 293) cells with 87% and 95.3% inhibition respectively.

Similarly, investigation by Gaire *et al.,* (2013) revealed that the *Terminalia chebula* fruit extract to protect neuronal cells against ischemia and inflammation in rat pheochromocytoma cells (PC12) using *in vitro* oxygen-glucose deprivation followed by reoxygenation (OGD-R) ischemia and

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hydrogen peroxide (H2O2) induced cell death. Cell survival was evaluated by a 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. They found that *T. chebula* extract, increases the survival of cells subjected to OGD-R by 68% and H2O2 by 91.4%; reduces NO production and death rate of microglia cells stimulated by lipopolysaccharide (LPS).

By comparing the obtained results of MTT assay with these literature survey reports, it could be concluded that *T. chebula* extract has the potential as a natural herbal medicine to protect the cells from damage and the possible mechanism might be the inhibition of oxidative and inflammatory processes.

Lawal *et al*., (2013) determined the *in vivo* cytotoxic activity and possible pro-apoptotic effect of *Securidaca longepedunculata* aqueous root bark extract on Ehrlich ascites carcinoma cells. *In vivo* study was carried out by intraperitoneal administration of varied doses of *Securidaca longepedunculata* to tumour-bearing mice. Isolated DNA from Ehrlich ascites carcinoma cells in treated and untreated animals was used for DNA fragmentation assay on agarose gel. *S. longepedunculata* was found cytotoxic to Ehrlich ascites under *in vivo* conditions. The IC50 of *S. longepedunculatawas67µg/ml.Securidaca longepedunculata* caused a decrease in angiogenesis as observed in the reduction in weight of treated animals and a reduction in volume of ascitic fluid in treated mice. DNA fragmentation assay of Ehrlich ascites carcinoma cells from treated animals depicted a possible pro-apoptotic effect of the *S. longepedunculata* extract due to the ladder forming pattern which was comparable to that of the standard drug (fluorouracil). *Securidaca longepedunculata* aqueous extract had a cytotoxic and pro-apoptotic effect on Ehrlich ascites carcinoma cells.

Suleiman *et al*., (2013) studied the acute oral toxicity of butanol extract of *Terminalia avicennioides* in rats. Serial doses (2-13g/kg) were used. The median lethal dose (LD50) was estimated to be 6.5g/kg. At dosages of 2-8g/kg, the plant extract apparently produced no gross or histopathologic lesions in the brain, heart, intestine, kidney and spleen. Grossly, at 13g/kg the spleen, liver and the kidneys of treated rats were enlarged and congested; the coronary blood vessels were also congested. The researchers concluded that the butanol extract of *T. avicennioides* was relatively non-toxic.

In another experiment, Morshed *et al*., (2011) carried out *in vitro* cytotoxicity test by brine shrimp lethality bioassay. Their results illustrated significant ($p<0.05$) cytotoxicity against *A. salina,* that were expressed as LC50. *Terminalia arjuna* ethanol extract showed brine shrimp cytotoxicity with lethal concentration 50 (LC50) value of 50.11µg/ml.

Bulus *et al*., (2011) used Lorke's method to study the acute toxicity effect of aqueous extracts from the stem bark of *Terminalia avicennioides* (ATA) on white albino rats. The study involved intraperitoneal administration of different doses of the extract to groups of male rats. Signs accompanying toxicity and possible death of animals were monitored for two weeks to ascertain the median lethal dose (LD50) of the extract. At the end of the two week study, all the animals in all the dose groups were sacrificed and the mean internal organ-body weight ratios (OBR) were determined and compared with values from those of the control group. The LD50 was found to be >5000mg/kg body weight. There was no significant weight decrease ($P>0.05$) among dose groups upto 1000mg/kg body weight. Liver congestion was observed with 100mg/kg body weight dose group. This study when compared to the present research showed almost similar results of no deleterious signs and symptoms for dose group upto 1000mg/kg body weight. But when the dose was increased LD50 was found significant along with lower congestions.

In another acute toxicity analysis by Panunto *et al.,* (2011) showed no signs of toxicity such as general behaviour changes, mortality, changes on gross appearance or histopathological changes of the internal organs of rats after orally administering dried fruit water extracts of *T. chebula*. Hematological and blood chemical values in treated groups were normal in comparison with the control group. Non-toxicity effect of *T. chebula* was present as no changes in body weight, internal organ weight and general behaviours. Macroscopic or microscopic of internal organs or tissues in treated rats showed no changes.

From these literature reports, it was interesting to conclude that no deleterious effects were observed in the experimental animals for the bark extracts. When compared to the literature report, the similar deleterious effects could be observed if the dosage in experimental animals was increased. This could be optimized and studied in the near future using the similar bark extracts of *T. chebula*.

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