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Cytotoxic and antigenotoxic properties of phenolic compound isolated from the fruit of *Terminalia chebula* on HeLa cell

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Abstract

Background: DNA in a human cell is subjected to constant assault from both environmental factors and normal metabolic processes. Accumulation of DNA damage drives the progression of many health disorders like aging, cancer, diabetes, and neurodegenerative disorders.

Results: The present study focuses on the isolation of phenolic compound from the fruit of *Terminalia chebula* and its protective role on induced DNA damage. Diethyl ether and ethyl acetate extract of *Terminalia chebula* fruit were subjected to column chromatographic purification, and the fractions obtained were tested for the presence of phenolics. Fraction-12 isolated from diethyl ether extract was identified as gallic acid, which is used for cytotoxic and DNA damage protection activity assays. To select a non-toxic concentration of isolated compound, cytotoxicity was assessed by MTT assay. Gallic acid showed moderate toxicity at the highest concentration tested (i.e., percentage cell viability at 100 µg/ml is 40.51 ± 1.31). Antigenotoxic effect of gallic acid on HeLa cells was carried by alkaline comet assay. The compound showed significant protective abilities against hydrogen peroxide-induced DNA damage in HeLa cells.

Conclusion: These results show the importance of gallic acid isolated from *Terminalia chebula* fruit, as protector of oxidative stress-induced DNA damage.

Keywords: *Terminalia chebula*, Cytotoxicity, Antigenotoxicity, HeLa cell

1 Background

DNA is the essential carrier of genetic information in living organisms, which contains all genetic information needed for the growth, development, reproduction, and ultimately death of the organism. Even though DNA is stable and versatile, it undergoes several types of spontaneous changes and may lead to damage in DNA. It occurs at a rate of 10,000 to 10,00,000 lesions per cell per day. DNA itself has many elaborate mechanisms to solve the damages. DNA damage, if not repaired properly, may lead to genomic instability, mutation, aging, cancer, and several other diseases.

Oxidation is a natural cellular metabolic process which results in the formation of free radicals known as reactive oxygen species (ROS) or reactive nitrogen species (RNS). Common examples are peroxy radical (RO₂·),

hydroperoxyl (HO₂·), superoxide (O₂⁻), hydroxyl (OH·), nitrogen dioxide (NO₂·), free radicals, etc. [9]. Once formed, they initiate a chain reaction by reacting with other molecules in their vicinity to attain stability. Excessive production of free radicals causes an imbalance between the generation of ROS and the antioxidant enzymes, which can lead to oxidative stress and damage to biological molecules such as DNA, protein, and lipids.

Phenolics are claimed to protect against many health disorders like cancer, diabetes, cardiovascular diseases, and aging, mainly by preventing initial DNA damages. Recently, there are many methods available for the estimation of DNA damage and repair like bacterial Ames test, the scoring of chromosome aberration, and micronuclei and sister chromatid exchanges [1, 16]. Among these classical techniques, single cell gel electrophoresis assay or comet assay is a widely accepted and commonly used technique. Comet assay is a rapid, inexpensive, short-term screening technique developed to study the antigenotoxic potential of natural compounds [22]. The

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assay can be carried out at neutral pH or high pH (above pH 13). At neutral pH, DNA migration is due to the presence of double-strand breaks, while at alkaline pH, DNA migration is associated with the presence of single-strand breaks, alkali labile sites, and single-strand breaks associated with incomplete excision repair sites [17]. The alkaline version is more acceptable since it allows the detection of a wide range of damages. Comets are scored, either by computer-based image analysis software or by visual scoring to calculate tail length, % tail DNA, which are the most appropriate parameters to be taken into account for the study.

Comet assay normally requires a supercoiled cellular genomic DNA of any cell line which is not proliferating, which can be made into single-cell suspension [12]. Therefore, HeLa cell line was chosen for this study. Hydrogen peroxide was selected to elicit oxidative stress, since it is freely diffusible and can potentially reach the nucleus to interact with DNA and cause damage to DNA by generating hydroxyl radical, $\cdot\text{OH}$. These highly reactive hydroxyl radical causes strand breaks and base damage in DNA. It also modifies purine and pyrimidine and causes significant production of DNA base damage products, 8-OH-Gua, FapyGua, etc. [4].

In the present study, the antigenotoxic activity of gallic acid, a phenolic compound isolated from *Terminalia chebula*, on hydrogen peroxide-induced DNA damage in human cervical cancer cell line (HeLa) was investigated by comet assay.

2 Methods

2.1 Preparation of extracts

Fresh fruits of *Terminalia chebula* were collected from Kannur district of Kerala, India, and were taxonomically identified by Dr. Sujanalal P, Scientist, Kerala Forest Research Institute (KFRI), Thrissur, Kerala, India. They were washed with running water followed by double-distilled water. It was air-dried in the dark and was powdered coarsely. Shade-dried fruit powder of the plant was weighed and extracted with 80% methanol using the Soxhlet method, for 24 h. The methanol extract was decanted, and the extract was filtered through Whatman filter paper No 1. The solvent extract was concentrated, and the dried extract was stored at 4 °C for further analysis.

2.2 Extraction and isolation of phenolics from *Terminalia chebula*

Eighty percent of methanolic extracts were washed with petroleum ether to remove fatty matter. The filtrate was partitioned with diethyl ether and ethyl acetate. Diethyl ether and ethyl acetate fractions were concentrated by vacuum oven and further subjected to column chromatographic purification. The column was eluted with hexane to ethyl acetate and ethyl acetate to methanol in varying ratios [20]. Fractions of 20 ml with each solvent

system were collected and were analyzed by TLC. The fractions showing similar R_f values were pooled together and concentrated in vacuum to isolate the active principles. All the pooled fractions were tested for the presence of phenolics.

2.3 Tests for phenolics

2.3.1 Ferric chloride test

The sample was treated with few drops of ferric chloride solution; the formation of bluish-black color indicated the presence of phenolic compounds.

2.4 Structural characterization of the compound

Many fractions showing positive results for phenolics were collected and were also subjected to in vitro antioxidant activity studies. The active fraction which showed the highest antioxidant activity was selected for further structural elucidation. The chemical structure of the isolated compound was determined by proton nuclear magnetic resonance spectroscopy (^1H NMR) and mass spectroscopy. NMR spectra were taken on a Bruker Ascend 500 MHz spectrometer for ^1H NMR and 125 MHz for ^{13}C NMR. The spectra were recorded using CD_3COCD_3 (deuterated acetone) as solvent and TMS (tetramethyl silane) as the internal standard. Mass spectrum of the purified compound was recorded on a Thermo Fisher Scientific high-resolution mass spectrometer (HRMS).

2.5 Cytotoxicity of phenolic compound isolated from diethyl ether extract of *Terminalia chebula* towards HeLa cell line

Cytotoxicity of isolated phenolic compound from diethyl ether extract of *Terminalia chebula* fruit towards HeLa cell lines was evaluated by MTT assay. Initially, cervical cancer cells line HeLa was obtained from NCCS, Pune, India, and maintained in DMEM (Sigma Aldrich, USA) supplemented with L-glutamine, 10% FBS, sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and amphotericin B (2.5 $\mu\text{g}/\text{ml}$). Cells were cultured in 25 cm^2 tissue culture flasks and kept at 37 °C in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). Sub-culturing of the cells was performed by trypsinization, and the cells were maintained in DMEM. The experiment was repeated twice in triplicates.

2×10^3 cells were seeded in a 96-well plate and incubated for 24 h. After 24 h treatment, the cells were treated with different concentrations of extracts. Untreated cells served as negative control, and the cells were incubated for 24 h at 37 °C and 5% CO_2 . At the end of the opted time of incubation, 10 μl MTT (5 mg/ml) was added to each well and the plates were incubated for 4 h. Supernatants were removed, and the resultant formazan crystals formed were dissolved in 100 μl DMSO. The extent of MTT reduction was measured at 590 nm with reference wavelength at 620 nm using

a microwell plate reader [2, 21]. All absorbance values were corrected against blank wells which contained growth media alone. For each test concentration, the mean absorbance of the triplicated wells was noted. Mean absorbance of the cells grown in the absence of test compound was taken as 100% cell survival.

Percentage cell viability was calculated by using the following formula:

$$\% \text{Cell viability} = \frac{\text{Absorbance of sample} \times 100}{\text{Absorbance of control}}$$

2.6 In vitro antigenotoxic activity of phenolic compound isolated from *Terminalia chebula* fruit by comet assay

Initially, the cell lines (cervical cancer cell HeLa) were obtained from NCCS, Pune, India, and maintained in DMEM (Sigma Aldrich, USA) supplemented with L-glutamine, 10% FBS, sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (2.5 µg/ml). Cells were cultured in 25 cm² tissue culture flasks and kept at 37 °C in a humidified 5% CO₂ incubator (NBS Eppendorf, Germany). Sub-culturing of the cells was performed by trypsinization, and the cells were maintained in DMEM. The experiment was repeated twice in triplicates.

The cells were treated with the isolated compound for 1 h at 37 °C. The concentration of compounds was chosen from the range of non-cytotoxic concentration as assessed by MTT assay. DNA damage was induced by 50 µM H₂O₂ for 15 min and incubated for 24 h. The adherent cells were then trypsinized, centrifuged, and re-suspended in ice-cold PBS and were mixed well with 50 µl of 0.5% low melting point agarose (LMPA) at pH 7.4 at 40 °C. Microscopic slides were cleaned using alcohol and cleared in flame. Then, the slides were frosted and coated with 1% normal melting point agarose (NMA) and stored at 4 °C. 1.6 ml sample was immediately pipetted onto a frosted glass slide pre-coated with a layer of 1% normal melting point agarose prepared in PBS and covered with a glass coverslip. The agarose was immediately placed in a refrigerator for 5 min to allow complete agarose solidification. The coverslip was removed, and slides were immersed in a lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, NaOH to pH 10 and 1% Triton X-100) at 4 °C for 1 h. DNA was allowed to unwind for 20 min in freshly prepared alkaline electrophoresis buffer (1 mM Na₂EDTA, 0.3 N NaOH, pH 13). The slides were then placed in a horizontal electrophoresis tank, and electrophoresis was performed at 12 V/cm for 20 min at an ambient temperature of 4 °C. The slides were then washed three times with 1× Tris for 5 min with neutralizing buffer (0.4 M Tris-HCl buffer, pH 7.4) before staining with 20 µl ethidium bromide (20 µg/ml) [10, 13].

3 Results

3.1 Structural elucidation of the compound

3.1.1 Appearance: White solid. Melting point: 250 °C

The following characteristic peaks are observed in the ¹H NMR spectrum (Additional file 1: Figure S1). Signals at *H* = 8.280, 8.236, and 7.152 ppm and their related coupling constant represent protons at C-1, C-2, and C-3 respectively. From the high-resolution mass spectrum (Additional file 2: Figure S2) of the isolated compound, it is clear that the base peak was observed at *m/z* value of 170 coincidence with the molecular weight of gallic acid. The other peaks at *m/z* values of 153, 127, and 109 are due to the loss of acid and hydroxyl functional groups. ¹³C NMR: δ C 166.94 (C-1), 145.14 (C-4 and C-6), 137.79 (C-5), 121.20 (C-2), 109.1 (C-3 and C-7) (Additional file 3: Figure S3).

Based on the chemical and spectral analysis, and comparison with the literature [19], the structure of the isolated compound was elucidated as gallic acid, a phenolic acid (Fig. 1).

3.2 Cytotoxic effects of gallic acid on HeLa cell by MTT assay

To select a non-toxic concentration of isolated compound, the study of cytotoxicity was performed preceding the study of antigenotoxicity. The cytotoxicity was evaluated by MTT assay. For the MTT test, the following concentrations of the isolated compound at doses of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0.78 µg/ml were tested. Percentage of cell viability was calculated in each concentration, and the results are shown in Table 1.

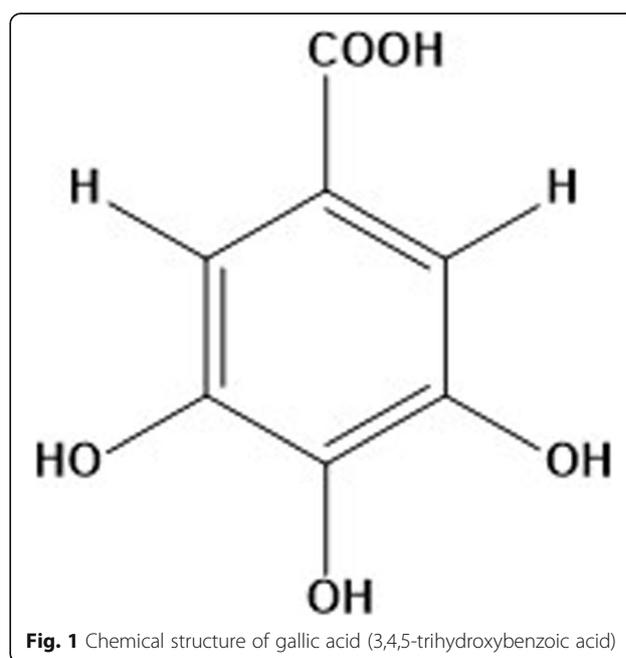


Table 1 Percentage cell viability of gallic acid isolated from the fruit of *Terminalia chebula* by the MTT assay on HeLa cells

Concentration ($\mu\text{g/ml}$)	% cell viability
100	40.51 ± 1.31
50	59.02 ± 1.73
25	66.51 ± 0.35
12.5	70.15 ± 1.25
6.25	82.11 ± 0.75
3.12	$96.45 \pm 1.79^*$
1.56	$96.98 \pm 2.36^*$
0.78	$98.67 \pm 3.03^*$

Results are expressed as mean \pm SEM of six experiments

*Statistical significance ($P < 0.05$) evaluated using Student's t test

Gallic acid showed moderate toxicity at the highest concentration (100 $\mu\text{g/ml}$) tested. IC_{50} value is found to be $72.68 \pm 2.08 \mu\text{g/ml}$. Cell viability greater than 90% is required to analyze the comet assay. Since the aim of the study was to evaluate the antigenotoxic activity of the compound, the lowest non-toxic concentration was chosen, i.e., 3.12 $\mu\text{g/ml}$ for the isolated phenolic compound.

3.3 Antigenotoxic effects of gallic acid isolated from the fruit of *Terminalia chebula* by comet assay on HeLa cells

Alkaline comet assay was implemented to evaluate the antigenotoxic effect of isolated compounds on HeLa cells. Comet length, tail length, and % tail DNA (% DNA in the tail of the comet) were measured using the software CometScore. Figure 2 shows the representative images for DNA damage in HeLa cells treated with 50 μM H_2O_2 and the isolated phenolic compound. Before exposure to mutagen (hydrogen peroxide), the cells were incubated with the non-toxic concentration of isolated compounds for 1 h. The cells were then exposed to different concentration of H_2O_2 and incubated for different time periods to find the suitable concentration of H_2O_2 . Since the cells treated with 50 μM H_2O_2 for 15 min were found to be a suitable concentration to induce a high level of DNA damage, it was chosen for further studies.

Table 2 shows the data for the antigenotoxicity assessment. A total of 100 cells were evaluated per sample for comet length, tail length, and % tail DNA measurement. The percentage of DNA in the tail at 50 μM H_2O_2 was 20.2 ± 2.18 . The percentage of DNA damage was found to decrease as shown by a decrease in % tail DNA, by incubation with the isolated compound. The tail length is the distance of DNA migration from the center of the head, which shows the extent of DNA damage. DNA tail length was found to be higher when the cells were exposed to hydrogen peroxide. DNA tail length for hydrogen peroxide-exposed cells was around 25.5 ± 2.14 , whereas in cells pre-treated with isolated compound tail length was reduced to 9.8 ± 1.4 . However, tail length for positive control ascorbic acid was 4.2 ± 1.02 . % tail DNA for the isolated compound was observed to be 8.7 ± 1.3 . Overall, the result indicated that the compound showed significant protective abilities against hydrogen peroxide-induced DNA damage in HeLa cells at a concentration of 3.12 $\mu\text{g/ml}$.

4 Discussion

The present study is focused on a promising DNA protective activity of isolated phenolic compound, gallic acid, from *Terminalia chebula* on cervical cancer cell. In this study, we examined the antigenotoxic potential of gallic acid against DNA damage induced by hydrogen peroxide. The compound was examined for cytotoxic activity in HeLa cell lines by MTT assay. For antigenotoxic activity study, the isolated phenolic compound was added to cells 1 h before administration of the stress-inducing agent. The antigenotoxic activity of the isolated phenolic compounds was confirmed from the decreased hydrogen peroxide-induced DNA damage in HeLa cells as evaluated by comet assay.

Many methods are available for evaluating antigenotoxic activity, and those methods vary substantially in terms of complexity and chemistry. The comet assay measures the effectiveness of the compounds to protect cells from genotoxic damage [14]. In vitro pre-treatment of HeLa cells with gallic acid is very effective in preventing induced oxidative DNA damage. Even low

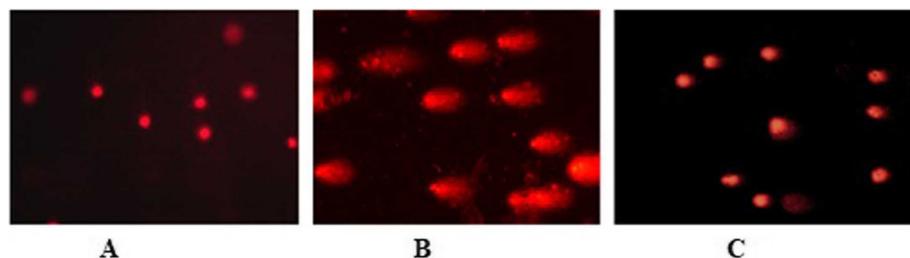


Fig. 2 Representative photographs depict the extent of DNA damage in HeLa cells treated with control and isolated compound from *Terminalia chebula*. **a** Untreated HeLa cells. **b** Control (HeLa cells treated with 50 μM H_2O_2). **c** Control + isolated compound

Table 2 Level of DNA damage in normal, control, and phenolic compound isolated from *Terminalia chebula* on HeLa cell

Sample	Comet length (μm)	Tail length (μm)	% tail DNA
Untreated HeLa	28.85 \pm 2.4	3.5 \pm 0.08	4.1 \pm 0.2
H ₂ O ₂	91.37 \pm 12.2*	25.5 \pm 2.14*	20.2 \pm 2.18*
Isolated compound	77.6 \pm 4.33*	9.8 \pm 1.4*	8.7 \pm 1.3*
Ascorbic acid	21.4 \pm 1.3*	4.2 \pm 0.03*	4.3 \pm 0.08

Results are expressed as mean \pm SEM ($n = 6$). Statistical significance (P) calculated by one-way ANOVA using SPSS 20.0. * $P < 0.05$ relative to untreated control

concentration such as 3.12 $\mu\text{g/ml}$ gallic acid provides a large effect. Untreated cells showed no significant DNA tails.

Many authors have reported that the increased antigenotoxic potential of isolated compounds may be because isolated phenolic compounds increase the cell's antioxidant capacity. Antioxidant capacity of compounds is associated with the following mechanism such as scavenging reactive oxygen species, donating hydrogen atoms or electrons, chelating metal cations, and enhancing the production of antioxidant enzymes and thus acting at a prevention level [5, 18, 24]. The beneficial effects of gallic acid have been attributed to their antioxidant properties. Gallic acid emerged as the strongest antioxidant in well-known assays like DPPH assay, Trolox equivalent antioxidant capacity assay (TEAC I-III), total radical-trapping antioxidant parameter assay (TRAP), photochemiluminescence assay (PCL), and ferric reducing ability of plasma assay (FRAP) [3]. Our results are in agreement with the previous report on the cytotoxicity study of gallic acid on HeLa cell [23]. Also, to the best of our knowledge, this is the first study to describe the antigenotoxicity of gallic acid on HeLa cell. However, Ferik et al. have investigated by the experiments with rats that gallic acid reduces oxidatively damaged DNA in the lymphocytes, liver, colon, and lungs. Gallic acid protects these organs against γ -irradiation-induced strand breaks and formation of oxidatively damaged DNA bases [7]. Gandhi and crew have evaluated in vivo studies that administration of gallic acid to animal prior to whole body radiation exposure reduced the peroxidation of lipids and the damage to the cellular DNA [8].

At this stage, it is not possible to say the exact mechanism(s) behind the antigenotoxic activity. It is possible to hypothesize that observed antigenotoxic potential of isolated phenolic compound could be assigned synergistic action of other mechanism such as (i) restriction of penetration of the toxins into the cells, (ii) direct inactivation of toxins by scavenging, (iii) stimulation of detoxification, (iv) cytotoxicity increase or decrease, (v) stimulation of DNA repair enzymes, and (vi) structure-activity relationship, [6, 11, 15]. The observed protective effect of the compound towards HeLa cell line may

correspond to a synergistic participation of the abovesaid mechanism(s).

Taken together, it has been estimated that the daily intake of gallic acid via food consumption may improve the health status of individuals under oxidative stress. Since gallic acid is contained in a large variety of foods, it is very easy to increase its daily intake by dietary measures.

5 Conclusion

In conclusion, gallic acid, which occurs in many plant-derived foods, reduces the DNA damage induced by hydrogen peroxide in HeLa cell. Further investigations are necessary for a better understanding of mechanisms underlying the antigenotoxic effect of gallic acid on HeLa cell.

6 Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s43088-019-0017-9>.

Additional file 1: Figure S1. Supplementary Figure 1.

Additional file 2: Figure S2. Supplementary Figure 2.

Additional file 3: Figure S3. Supplementary Figure 3.

Abbreviations

DMEM: Dulbecco's modified Eagle medium; DMSO: Dimethyl sulfoxide; DNA: Deoxyribonucleic acid; FBS: Fetal bovine serum; H₂O₂: Hydrogen peroxide; LMPA: Low melting point agarose; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Na₂EDTA: Disodium ethylenediaminetetraacetate dihydrate; NaCl: Sodium chloride; NaOH: Sodium hydroxide; NMA: Normal melting point agarose; ROS: Reactive oxygen species; SEM: Standard error of the mean; TLC: Thin-layer chromatography

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Authors' contributions

SS and SK conceived of the presented idea. JJ performed the extraction procedure. ATM helped the cell culture work. DAT contributed to the interpretation of the results. SK wrote the manuscript with support from SAP. All authors discussed the results and contributed to the final manuscript. SS supervised the project. All authors read and approved the final manuscript.

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Competing interests

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