Antigenotoxic and apoptotic activities of essential oil of Atalantia monophylla Correa

Ramaraj Thirugnanasampandan, Gunasekar Ramya, Madusudhanan Gogulramnath

Abstract:
Objective: To study antigenotoxic and apoptotic activities of hydrodistilled essential oil from the leaves of Atalantia monophylla Correa.

Materials and Methods: Antigenotoxic activity of essential oil was tested against hydrogen peroxide (100 μM)-induced deoxyribonucleic acid (DNA) damage in 3T3-L1 cells. Cervical cancer cell (HeLa) growth inhibitory effect of essential oil was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide assay. Annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI), Hoechst 33258, and acridine orange/ethidium bromide (AO/EtBr) staining techniques were used to identify apoptosis.

Results: DNA protecting the activity of A. monophylla essential oil was high at 125 μg/mL. HeLa cell growth was inhibited dose-dependently and inhibitory concentration 50% was calculated as 43.08 ± 0.02 μg/mL. Annexin V-FITC/PI double staining showed membrane breakage and nuclei staining. Further, Hoechst 33258 and AO/EtBr stain also confirmed the apoptosis in essential oil-treated HeLa cells.

Conclusion: The results obtained suggest that A. monophylla essential oil is a promising natural agent which may be used in preparation of herbal medicine to treat cancer and other diseases.

Key words: Apoptosis staining, comet assay, cytotoxicity, volatile oil

Reactive oxygen species (ROSs) and free radicals-induced oxidative deoxyribonucleic acid (DNA) damage are important in the pathogenesis of many human diseases, including cancer, muscle degeneration, coronary heart disease and aging, etc.[1] Hydrogen peroxide (H₂O₂) is one of the main ROSs which induces oxidative damage in cells, causing a spectrum of DNA lesions, including single and double strand breaks. DNA damage due to H₂O₂ produced hydroxyl radicals (●OH) in the presence of transition metal ions such as iron through Fenton reaction.[2]

Cervical cancer is the second most common cancer among women worldwide and caused with infection of human papillomavirus (HPV) such as 16 and 18. HPV oncoproteins E6 and E7 are considered in initializing and maintaining the malignant growth of HeLa cells. HPV E6 binds to p53 and stimulates its degradation by an ubiquitin-dependent protease system, while E7 causes destabilization and disruption of Rb/E2F repressor complexes. Since growth regulatory machinery is masked by the expression of HPV E6 and E7 proteins, so repression of HPV oncogenes has been a suitable target to arrest malignant growth.[3,4]

Hence, there is a need to develop a plant-based DNA protecting and apoptosis-inducing agent showing more specificity and efficacy with minimal side effects. In this study, we aimed to establish antigenotoxicity against H₂O₂-induced DNA damage in 3T3-L1 cells and apoptosis inducing activities in HeLa cells by essential oil of Atalantia monophylla.

A. monophylla Correa. belong to Rutaceae is an evergreen small thorny tree with yellowish very hard, close-grained wood. Leaves are green, reticulate, ovate, and aromatic in smell. Earlier we have reported the chemical composition of essential of A. monophylla and its antioxidant and antibacterial activities.[5]

How to cite this article: Thirugnanasampandan R, Ramya G, Gogulramnath M. Antigenotoxic and apoptotic activities of essential oil of Atalantia monophylla Correa. Indian J Pharmacol 2016;48:720-4.
Materials and Methods

Antigenotoxicity-cell Culture and Treatment
The 3T3-L1 cells procured from the National Centre for Cell Science (NCCS), Pune, India, and cells (4–5 × 10^4) were cultured in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in 25 cm² tissue culture flasks. The cells were treated with various concentrations of oil (25, 50, 75, 100, and 125 μg/mL) for 60 min in a carbon dioxide (CO₂) incubator. After pretreatment, the cells were exposed to 100 μM of H₂O₂ for 30 min on ice. The cells were harvested, centrifuged for 5 min at 1500 rpm, and resuspended in phosphate-buffered saline (PBS).

Single Cell Gel Electrophoresis (Comet Assay)
Twenty-five microliter of cell suspension was mixed with 75 μL of 0.6% low melting agarose. The suspension was spread on a frosted microscopic slide precoated with 0.8% of normal melting agarose. The cell suspension was covered with a cover slip and kept on ice for 10 min. The coverslips were removed and the slides were incubated overnight in a lysis solution containing 1% sodium dodecyl sulfate, 2.5 M sodium chloride, 100 mM ethylene diamine tetraacetic acid disodium (Na2EDTA), 1% Triton X-100, and 10% dimethyl sulfoxide (DMSO) at 4°C. The slides were arranged in an electrophoresis tank filled with prechilled electrophoretic buffer (1 mM Na2EDTA and 300 mM NaOH) and incubated for 20 min. Electrophoresis was carried out at 25 V (300 mA) for 20 min using a power supply (CBS Scientific Company, USA). After electrophoresis, the slides were washed with 0.4 M Tris (pH 7.5) and stained with ethidium bromide (EtBr) (20 μg/mL). The slides were viewed using an Olympus-CKX42 fluorescence microscope and photographed.

Cell Line and Culture
HeLa cell line procured from NCCS, Pune, India, was maintained in a humidified incubator at 37°C and in a 5% CO₂ atmosphere. DMEM containing 10% FBS was supplemented with antibiotics penicillin (100 units/mL) and streptomycin (100 μg/mL).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide Assay
The assay detected the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) by mitochondrial dehydrogenase to blue formazan product which reflects the normal functioning of mitochondria and cell viability. Briefly, after being harvested from culture flasks, the cells were counted using a hemocytometer and cell viability was determined by trypan blue exclusion. 1 × 10⁵ cells were incubated in a 96-well plate containing 100 μL of the medium per well. Cells permitted to adhere for 24 h and then treated with different concentration of essential oil dissolved in the medium for 48 h; 20 μL of 5 mg/mL MTT in PBS was added to each well and the plate was incubated at 37°C for 4 h. The medium was removed, and 100 μL of DMSO was added to each well. After incubation at 37°C for 10 min, absorbance at 570 nm of the dissolved solution was measured by a microplate reader (CyberLab).

Annexin V-fluorescein Isothiocyanate/Propidium Iodide Double Staining
Apoptotic cells were identified with an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA). Briefly, 1 × 10⁵ cells/mL was treated with different concentrations of essential oil for 12 h at 37°C. Cells were then harvested and resuspended in the binding buffer. Cells were stained with 10 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of propidium iodide (PI) for 15 min at room temperature in the dark and viewed under Olympus CKX42 fluorescence microscope and photographed.

Hoechst 33258 Staining
Hoechst 33258 stain (Sigma-Aldrich, USA) was used to observe the apoptotic morphology of cells. Briefly, 1 × 10⁵ cells/mL were seeded in six-well plates and incubated for 24 h. Afterward, the cells were treated with different concentrations of essential oil for 48 h. Then, the cells were collected and fixed with 4% formaldehyde in PBS for 15 min and stained with Hoechst 33258 (10 mg/mL) at room temperature for 10 min. After washed with PBS, morphological changes including a reduction in volume and nuclear chromatin condensation were observed under Olympus CKX42 fluorescence microscope and photographed.

Acridine Orange/Ethidium Bromide Dual Staining
Morphological analysis of apoptosis by acridine orange/Ethidium Bromide (AO/EtBr; Hi-Media, India) dual staining was performed. Briefly, 2 × 10⁴ cells per well were seeded in 96-well plate, and treated with different concentrations of essential oil for 48 h. After incubation, the plates were centrifuged. Ten microliter of 1 mg/mL AO and EtBr mixture was added to each well. Nuclei were visualized and photographed under Olympus CKX42 fluorescence microscope and photographed.

Statistical Analysis
The date obtained from the cytotoxicity study was analyzed using the SPSS (16.00, SPSS Inc. Chicago, IL) for IC₅₀ calculation.

Figure 1: H₂O₂-induced genotoxicity in 3T3-L1 cells shows different classes of comets (a) Class 0 and 1, (b) Class 2, (c) Class 3, (d) Class 4 Comet tail length expressed in μm, (e and f) Normal cells
Results

DNA-protecting Activity of Oil

H₂O₂-induced DNA damage in 3T3-L1 cells, and different classes of comets were formed, the longer tail length indicates more DNA damage [Figure 1]. Further A. monophylla essential oil treatment showed a reduction in comet tail length. The length of the comet was dependent on the concentration of the oil used. Of the five different concentrations tested, 125 µg/mL oil showed considerable DNA protection with reduced comet tail formation [Figure 2]. Untreated control cells showed no DNA damage or tail formation.

Cytotoxic and Apoptotic Activity of Oil

We investigated the effect of A. monophylla essential oil on HeLa cell proliferation. HeLa cells were exposed to different doses of oil for 24 h, and cell viability was checked by MTT assay. Viability was markedly decreased after treatment with oil. Cell growth was inhibited in a concentration and time-dependent manner. The concentration required to inhibit 50% of cell growth was calculated as 43.08 ± 0.02 µg/mL. To confirm the apoptosis induction of oil in HeLa cells, Annexin V-FITC/PI double staining method was used. After treatment with oil at different concentrations for 12 h, apoptosis was induced in a concentration-dependent manner. The double positive staining of the majority of cells revealed that those cells were at the late apoptotic stage. At the same time, cells were negative to PI indicate that they were at an early stage of apoptosis [Figure 3]. The ability of oil to induce apoptosis in HeLa cells, Hoechst 33258 staining was used. After 24 h of treatment, bright fluorescence was detected at 25–75 µg/mL concentration which indicates apoptotic morphology followed by nuclear condensation and apoptotic body formation was observed at 100–150 µg/mL concentration at 48 h [Figure 4]. Further apoptosis-inducing activity of oil was tested using AO/EtBr-staining technique. Fluorescence microscopic image clearly showed majority of the oil-treated cells were at early apoptosis with morphological changes such as cell breakage, shrinkage, and membrane blebbing [Figure 5].

Discussion

Plant products are well known for the treatment of cancer and other disease since long ago. Many plant species have been reported to have significant antimicrobial, antioxidant, and anticancer properties.[8,9] Phytochemicals have emerged as drugs upon modification, and many are available in the form of crude, and more are yet to reach the market. Essential oil is one among the known secondary metabolites with immense commercial and medicinal values. Essential oils are complex mixtures of volatile substances mainly contains mono-, sesqui-, diterpenes, and phenylpropanoids. Numerous reports are available on chemical composition, biological activities, and possible applications of essential oils in food, pharmaceutical, and cosmetic industries.[10]

Excess ROS formation results in oxidative stress and tissue injury. Using water channels, H₂O₂ cross cell membrane, reach nucleus, attack DNA, and single strand breaks occur.[11] In the present study, we treated 3T3-L1 cells with 100 µM of H₂O₂ for 30 min showed more DNA damage. Subsequent treatment
with different concentration of oil showed a decrease in tail length. DNA protecting the activity of oil may be associated with scavenging of $\text{H}_2\text{O}_2$-induced hydroxyl radical formation through Fenton reaction.\[12\] We assumed that observed antigenotoxic activity of *A. monophylla* essential oil might be due to the presence of active constituents such as sabinene, trans-asarone, and myrcene.\[5\]

Induction of apoptosis is a highly desirable goal for cancer control. Apoptosis regulators have been suitable targets for cancer therapy over several decades.\[13\] However, chemotherapeutic agents have many undesirable effects on patients. Last one-decade research in apoptosis induction has been performed with plants, and their products are gaining much importance than chemopreventive agents with little side effects.\[14\] A significant cytotoxic effect and apoptosis were noted in HeLa cells following treatment with different concentrations of *A. monophylla* essential oil. Anticancer activity of each compound present in the oil was not evaluated against HeLa cell line; therefore, at this stage, it is not possible to say which compound is responsible for the observed activity. It is possible to hypothesize that observed cytotoxic and apoptotic activity of oil could be related to combined action of compounds present. Although synergism is a reason, it is unavoidable to discuss about the cytotoxic and apoptotic activities of major components of the oil.

**Acknowledgments**

We thank management of Kongunadu Arts and Science College for necessary lab facilities to carry out this work.

**Financial Support and Sponsorship**

This work was financially supported by the Department of Science and Technology-Science and Engineering Research Board, New Delhi, India, under grant No. SB/FT/LS-230/2012; dt 02.05.2013.

**Conflicts of Interest**

There are no conflicts of interest.

**References**

1. Lee DH, Timothy R, Gred P. Oxidative DNA damage induced by copper and hydrogen peroxide promotes CGTT tandem mutations at methylated CpG dinucleotides in nucleotide excision repair-deficient cells. Nucleic Acid Res 2002;30:3566-73.
2. Collins AR. Oxidative DNA damage, antioxidants, and cancer. Bioessays 1999;21:238-46.
3. Goodwin EC, DiMaio D. Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. Proc Natl Acad Sci USA 2000;97:12513-8.
4. Godefroy N, Lemaire C, Mignotte B, Vayssiére JL. p53 and
retinoblastoma protein (pRb): A complex network of interactions. Apoptosis 2006;11:659-61.
5. Thirugnanasampandan R, Gunasekar R, Gogulramnath M. Chemical composition analysis, antioxidant and antibacterial activity evaluation of essential oil of *Atalantia monophylla* Correa. Pharmacognosy Res 2015;7 Suppl 1:S52-6.
6. Grover P, Danadevi K, Mahboob M, Rozati R, Banu BS, Rahman MF. Evaluation of genetic damage in workers employed in pesticide production utilizing the comet assay. Mutagenesis 2003;18:201-5.
7. Lau CB, Ho CY, Kim CF, Leung KN, Fung KP, Tse TF, et al. Cytotoxic activities of *Coriolus versicolor* (Yunzhi) extract on human leukemia and lymphoma cells by induction of apoptosis. Life Sci 2004;75:797-808.
8. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 2000;36:1235-47.
9. Ozkan G, Kamiloglu S, Ozdal T, Boyacioglu D, Capanoglu E. Potential use of Turkish medicinal plants in the treatment of various diseases. Molecules 2016;21:257.
10. Sadgrove N, Jones G. A contemporary introduction to essential oils: Chemistry, bioactivity and prospects for Australian agriculture. Agriculture 2015;5:48-2.
11. Wang YK, Hong YJ, Huang ZQ. Protective effects of silybin on human umbilical vein endothelial cell injury induced by H2O2 in vitro. Vascul Pharmacol 2005;43:198-206.
12. Zheng LP, Zou T, Ma YJ, Wang JW, Zhang YQ. Antioxidant and DNA damage protecting activity of exopolysaccharides from the endophytic bacterium *Bacillus cereus* SZ1. Molecules 2016;21. pii: E174.
13. Okun I, Balakin KV, Tkachenko SE, Ivachtchenko AV. Caspase activity modulators as anticancer agents. Anticancer Agents Med Chem 2008;8:322-41.
14. Lu J, Kim SH, Jiang C, Lee H, Guo J. Oriental herbs as a source of novel anti-androgen and prostate cancer chemopreventive agents. Acta Pharmacol Sin 2007;28:1365-72.