IN VITRO EVALUATION OF ANTIOXIDANT-MEDIATED APOPTOSIS ON CANCER CELLS AND NON-TOXICITY OF ETHANOLIC EXTRACT OF TERMINALIA CHEBULA RETZ AND PYROGALLOL

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ABSTRACT

Objective: Antioxidants are very important for human health, and hence, external supplementation is recommended to protect cells from the deleterious effects of excessive reactive oxygen species conditions that are capable of causing direct damage to deoxyribonucleic acid (DNA) which might lead to the cause of cancer. In our research work, we have aimed to evaluate the antioxidant potential of *Terminalia chebula* and pyrogallol to relate to its apoptotic nature as well as prove its non-toxicity.

Methods: Investigations were carried out to determine the antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl and lipid peroxidation inhibition assay of the ethanolic extract of *T. chebula* and pyrogallol. The apoptotic nature of tumor cells was determined by comet assay. To prove the non-toxicity of the extract as well as compound, the hemolysis assay was carried out.

Results: The results of our study established the antioxidant potential of the plant which may serve as additional evidence for the antiproliferative activity of the plant. It is considered safe for human use.

Conclusion: Plant-based traditional medicine system continues to play an essential role in health care. The ethanolic extract of *T. chebula* and pyrogallol possesses antioxidant and anticancer properties.

Keywords: *Terminalia chebula*, Antioxidant, Apoptosis, Hemolysis.

INTRODUCTION

*Terminalia chebula* (Combretaceae), also known as the “king of medicine,” seems to hold the prime place at the beginning of the list in the “Ayurvedic Materia Medica” because of its incredible power of healing. The fruit possesses numerous health benefits that may be attributed to the presence of various phytochemicals such as terpenes, alkaloids, anthocyanins, flavonoids, anthocyanins polyphenols, alkaloids, and glycosides [1]. The use of natural substances to inhibit carcinogenesis is a rapid and currently evolving aspect of research [2].

Several research reports are suggestive of the fact that the anticancer activity of medicinal plants may be due to the presence of antioxidants in them [3]. Antioxidants are very important for human health, and antioxidant supplementation is essential to provide cellular protection from the deleterious effect of excessive reactive oxygen species (ROS) concentration [4].

Production of free radicals owing to environmental pollution, harmful ultraviolet rays, and hazardous toxic chemicals poses a threat to our body causing diseases such as cancer, arthritis, diabetes, skin disorders, cataract, and age-related disease in human beings. To protect the cells from damage due to ROS, we require an antioxidant that functions interactively and synergistically to neutralize the free radicals. Chemicals present in natural products like plants might be an age-old science, but it is keeping pace with enormous potential in the current century. One of the important plants that hold good for such medication in Ayurveda is *T. chebula* which is rich in tannins, gallic acid, ellagic acid, and other essential compounds [5].

METHODS

Plant extract preparation

The dried fruits of *T. chebula* Retz were collected from Pachamalai Hills and authenticated (HDP 001) by Rapinat Herbarium, St. Josephs College, Tiruchirappalli. It was crushed, powdered, and extracted with 95% ethanol, using Soxhlet apparatus. After the completion of extraction, the extract was filtered and the solvent was removed by distillation under reduced pressure [6].

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay was used to analyze the antioxidant property of the given samples. Different concentrations of the samples (100 µl) were added to 0.9 ml of the methanolic solution of DPPH (2.5 mg/100 ml), and the reactants were incubated at room temperature for 30 min in the dark. Different concentrations of butylated hydroxylated hydroxytoluene (BHT) were used as a standard, and the solvent (distilled water) was used instead of extract in control. After 30 min, the absorbance was measured at 515 nm using a spectrophotometer, and the radical scavenging activity of the extract was calculated and expressed on a percentage basis [7].

Lipid peroxidation inhibition potential

Lipid peroxidation inhibition potential of the given samples was investigated in goat brain homogenate model. Goat brain was collected from freshly sacrificed animal in a local slaughterhouse and kept in phosphate-buffered saline (PBS) under cold condition until analysis. Tissue was cut using a sterile blade and weighed 500 mg and then homogenized with 5 ml PBS buffer (pH 7.0). The brain homogenate (0.3 ml) was preincubated with 0.3 ml of plant extract (1 mg/ml) and then 0.1 ml of 3% *H*₂*O*₂ was added and incubated at 37°C for 10 min. BHT (1 mg/ml) was used as a positive control, while 0.3 ml of PBS was used instead of extract in control. The reaction was stopped by adding 1.5 ml of thiobarbituric acid (TBA) reagent (0.375 g of TBA dissolved in 10% trichloroacetic acid [TCA]). The contents were then heated at 80°C for 20 min, cooled down to room temperature, and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 530 nm, and based on the OD value, the malondialdehyde (MDA) level and lipid peroxidation inhibition capacity of the sample were calculated [8].
Comet assay
Briefly, Michigan cancer cell foundation (MCF-7) cells were seeded in a six-well plate at a density of 10,000 cells/well and incubated for 24 at 37°C in a humidified 5% CO₂ incubator. The wells were washed with sterile PBS and treated with 384.5 µg/ml of the ethanolic extract of T. chebula and 83.5 µg/ml of pyrogallol (based on half maximal inhibitory concentration [IC50] dose) in a serum-free dulbecco's minimal essential media (DMEM) medium and incubated for 24 h at 37°C in a humidified 5% CO₂ incubator. The cells were harvested by trypsinization in a 1.5 ml tube, and comet assay was performed based on the protocol of Nandha kumar et al. with slight modifications. The microscopic slides were sequentially coated with 200 µL 0.75% normal melting agarose as the first layer and 100 µL of 0.5% low melting agarose as the second layer. The next step was to add 20 µL cell suspensions to 60 µL of 0.5% low melting agarose, which was distributed on the slides as the third layer. Then, the slides were incubated in celllysis buffer for overnight at 4°C. After that, the slides were immersed in double-distilled water for 3 times followed by incubation in unwinding solution for 20 min. The slides were placed in a horizontal gel electrophoresis tank containing electrophoresis solution. The electrophoresis was conducted at 25 V for 25 min. Then, the slides were incubated in neutralization buffer for 10 min followed by immersion in double-distilled water thrice and air-dried. The cells were stained with ethidium bromide and observed under a fluorescent microscope. All steps were carried out under dim light to minimize extra DNA damage [9].

Determination of cellular toxicity using sheep erythrocytes (hemolysis)
Briefly, 10-fold serial dilutions of the extract and pyrogallol were made in PBS. A total volume of 0.8 ml for each dilution was placed in an Eppendorf tube. A negative control (saline only) and a positive control (standard, 5 mg/ml) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to make up a final volume of 1 ml. Solutions were incubated at 37°C for 30 min, and all tubes were centrifuged for 5 min and then observed for hemolysis. Complete hemolysis was indicated by a clear red solution without any deposit of erythrocytes [10].

RESULTS
Preparation of ethanolic extract
500 g of fruit powder of T. chebula yielded 12.25% w/w of crude ethanolic extract. The greenish-black colored residue was obtained.

Pyrogallol - as the promising bioactive compound
Based on the results of the gas chromatography-mass spectrometry analysis, pyrogallol was reported to be the predominant compound having a peak area of 82.7% at the retention time (RT) 22.41 [11]. Hence, further work on the compound was carried out using commercially available pyrogallol to serve as a promising bioactive compound.

Free radical scavenging activity by DPPH method
The DPPH radical scavenging ability of the crude ethanolic extract of the fruit of T. chebula at different concentrations (100, 200, 300, 400, and 500 µg/ml) was compared with the reference standard ascorbic acid. The activity of the extract was found to be dose dependent. The percentage of antioxidant activity of extract from low to high dose was 18%, 22%, 47%, and 50% with inhibition percentage being maximum at dosage 500 µg/ml which recorded 77% while that of ascorbic acid is 95% at the same concentration. The antioxidant property of DPPH radicals scavenging ability is due to its hydrogen-donating ability. Standard ascorbic acid exhibited 86.6%, 90%, 91.6%, 95%, and 98.3% of antioxidant activity at concentrations of 100, 200, 300, 400, and 500 µg/ml, respectively. The percentage of antioxidant activity of pyrogallol was reported as 38%, 51%, 64%, 75%, and 84%, respectively (Table 1).

Lipid peroxidation inhibition potential
Lipid peroxidation inhibition potential was performed in goat brain homogenate model. Normal brain homogenate served as blank.
for this clinical menace [26-29]. Different antioxidant assays such as 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), DPPH, H$_2$O$_2$, superoxide, reducing power, and in vivo assays of antioxidants enzymes from the liver of experimental mice are done to determine the presence as well as the level of antioxidants available in the biological sample. Phenolic compounds have become a cause of concern in traditional medicine due to their antioxidant nature that is being looked on with hope for the treatment and prevention of cancer [30,31]. It has been reported that the three extracts of T. chebula (aqueous, methanol, and 95% ethanol) are the richest source of phenolic compounds [32].

IC$_{50}$ values of T. chebula extract and pyrogallol were 334.54 µg/ml and 83.59 µg/ml, respectively. Our results were in accordance to previous reports on Dillenia suffruticosa by Yin Sim Tor [33]. The acute oral toxicity study carried out by Bag et al. [34] using the ethanolic extract of T. chebula Retz in mice at concentration 100, 250, 500, 1000, 2000, and 4000 mg/kg showed no observable changes associated with toxicity proving the effectiveness of the extracts to be therapeutically safe. The lethal concentration 50% value was determined as more than 4 g/kg. In our study also, no hemolysis of red blood cells was observed. This indicates the treatment of plant products and its derivatives to be safe and non-toxic at permissible levels. Thus, herbal lead molecules seem to possess antitumor activity by the induction of apoptosis [35].

CONCLUSION
The ethanolic extract and pyrogallol harbor the adequate efficacy to scavenge the free radicals generated as a result of oxidative stress confirming the antioxidant nature. It is capable of inducing DNA damage leading to apoptosis of tumor cells. These are safe and non-toxic based on toxicity studies at the recommended doses.

AUTHORS’ CONTRIBUTION
Dr. R. Jasmine - Provided the idea and carried out the correction of the manuscript. Deena Priscilla - Executed the research work and prepared the manuscript.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

REFERENCES