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Original Article

IN VITRO BIOMOLECULAR PROTECTIVE EFFECT OF *CINNAMOMUM ZEYLANICUM* BARK EXTRACTS AGAINST OXIDATIVE DAMAGE

VARALAKSHMI B.[1#], VIJAYA ANAND[2], SAMPATH KUMAR[3]

[1]Department of Biochemistry, Shrimati Indira Gandhi College, Trichy, Tamil Nadu, India, [2]Department of Human Genetics and Molecular Biology, Bharathiar University, Coimbatore, Tamil Nadu, India, [3]Department of Biochemistry, Sastra University, Kumbakonan, Tamil Nadu, India
Email: varalakshmisupraja@gmail.com

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ABSTRACT

Objective: Free radicals play an important role in the pathogenesis of many disorders by damaging important biomolecules such as lipids, proteins and DNA. Natural antioxidants are safer, cheaper and potential therapeutics to scavenge free radicals. Hence, this study was aimed to assess the biomolecular protective effect of *Cinnamomum zeylanicum* bark, a culinary spice, on the oxidant-treated membrane lipids and DNA in cell-free systems.

Methods: The total phenolics and flavonoid content of the bark were estimated. The extent of inhibition of lipid peroxidation (LPO) by aqueous, methanolic and chloroform extracts of bark was studied *in vitro* in three different membrane models such as goat RBC ghosts, goat liver homogenate, and goat liver slices which differ in architecture and lipid composition. The extent of inhibition of oxidant-induced DNA damage by the bark extracts was assessed in commercial DNA preparations such as pBR322, herring sperm, and calf thymus DNA.

Results: The total phenolic content was 153.33±23.09 mg of pyrocatechol equivalents/g, and flavonoid was 33.66±1.15 mg of catechin equivalents/g of powdered bark. All the three extracts exhibited a considerable inhibition of LPO in all the membrane systems, and most significant inhibition was exerted by methanolic extract on the RBC ghosts. All the three extracts were able to revert the oxidant-induced DNA damage, and more significant DNA protection was rendered by methanolic extract on calf thymus DNA.

Conclusion: The present study showed that *C. zeylanicum* bark is a nutraceutical rich in phenolic antioxidants that can protect biomolecules against oxidative stress.

Keywords: Lipid and DNA models, Lipid peroxidation (LPO), DNA damage, Antioxidant activity, *C. zeylanicum* bark

INTRODUCTION

An antioxidant is a molecule that is capable of slowdown or even prevents the oxidation of other molecules. The continuing development of the market of antioxidants reflects the hope to cure the wide range of diseases that are believed to be caused or promoted by 'oxidative stresses' [1]. Under normal conditions, the generated reactive oxygen species (ROS) are detoxified by the antioxidants present in the body, and there is a balance between the ROS formed and the antioxidants present. However, due to the overproduction of ROS and/or inadequate antioxidant defense, this equilibrium is hindered favoring the ROS upsurge that culminates in oxidative stress [2]. The ROS is capable of damaging biomolecules (e. g. lipids, proteins, DNA), provoking an immune response, activating oncogenes and enhancing the aging process [3] and eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans [4]. Antioxidants retard the progression of various chronic diseases as well as lipid peroxidation [5]. Antioxidants from natural substances like spices, oilseeds and other plant materials that can be replaced synthetic antioxidants have been of interest. In developing countries, many people still utilized the herbal medicines to meet their health needs [6]. Therefore, there is a growing interest in natural and safer antioxidants [7]. Plants (fruits, vegetables, medicinal herbs, etc.) have a wide range of free radical scavenging molecules, like phenolic compounds (flavonoids, phenolic acids, tannins, coumarins, quinones, lignans, stilbenes), nitrogen compounds (alkaloids, betalains, amines), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity [8].

Cinnamomum zeylanicum (family: Lauraceae) is a small, tropical, evergreen tree most noted for its bark. It is reported that essential oils and other constituents from cinnamon also have important activities, including antimicrobial [9], anti-inflammatory [10], antidiabetic [11] and anticancer agent [12]. This study aimed to evaluate the protective effect of *C. zeylanicum* bark extracts against oxidative stress induced damage to biomolecules such as membrane lipids and DNA.

MATERIALS AND METHODS

Preparation of extracts

C. zeylanicum (family: Lauraceae) is a small, tropical, evergreen tree most noted for its bark.

The bark was collected from Anjarakandy Cinnamon Estate, Kannur district, Kerala, dried and powdered. The species was identified, and a voucher specimen was deposited at the Rapinet herbarium (BV001) St. Joseph's College, Tiruchirappalli. All the chemicals used were of analytical grade. 10g of the bark powder was homogenized in 100 ml of the solvents of varying polarity such as water, methanol, and chloroform. The organic extracts were dried at 60 °C protected from light. The residue was weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain the desired concentration. Aqueous extracts were prepared fresh.

Estimation of total phenolics and flavonoids

The amount of total phenolics in the plant bark was estimated by the method proposed by Mallick and Singh [13] using pyrocatechol as the reference standard. The method proposed by Cameron *et al.* [14] was used to extract and estimate flavonoids and catechin was used as reference standard.

Study of effects of *C. zeylanicum* bark extracts on oxidant-induced damage to lipids

RBC ghosts (plasma membrane lipids), goat liver homogenate (plasma membrane and intracellular lipids) and liver slices (intact cells) were used as lipid source and prepared as follows: The RBC ghost was prepared by the method described by Dodge *et al.* [15].

Briefly, defibrinated goat blood was diluted 1:1 with sterile isotonic KCl (1.15%). The RBCs were pelleted by centrifuging, and the pellet was kept in hypotonic (0.5%) KCl lyses the cells, centrifuged to remove the hemoglobin then the resulting pellet was suspended in tris-buffered saline (TBS). Goat liver was procured fresh from the slaughterhouse, and a 20% liver homogenate was prepared in Tris-HCl buffer. The goat liver slices were prepared by plunging the goat liver into cold, sterile PBS and maintained at 4 °C and thin slices of 1 mm thickness were made.

For the estimation of LPO in RBC ghosts and in goat liver homogenate, different reaction mixtures viz., (i) RBC ghosts/goat liver homogenate as lipid source, *C. zeylanicum* bark extracts (aqueous/methanol/chloroform), $FeSO_4$ and ascorbate as oxidant and buffer (ii) blank was set up as the above mixture without the bark extract and lipid source (iii) a mixture corresponds to 100% oxidation, contained all the constituents except bark extract and (iv) mixture corresponds to auto-oxidation (contain only lipid source) were prepared. The extent of formation of LPO from the oxidant-treated RBC ghosts and goat liver homogenate was measured by absorbance at 535 nm. [16, 17].

For the estimation of LPO in goat liver slices, different reaction mixtures as above were prepared except that H_2O_2 was used as oxidant instead of $FeSO_4$ and ascorbate. The extent of formation of LPO from the damaged lipids by oxidizing agents was measured by absorbance at 535 nm [18].

Study of effects of *C. zeylanicum* bark extracts on oxidant-induced damage to DNA

The effect of the aqueous, methanolic and chloroform extracts of *C. zeylanicum* bark on oxidant-induced DNA damage was assessed in cell-free systems. Commercially available DNA preparations such as pBR322 (circular plasmid DNA), herring sperm DNA (haploid genomic DNA) and calf thymus DNA (diploid eukaryotic DNA) were used for the study. H_2O_2 was used as an oxidant to induce DNA damage.

The electrophoresis method described by Guha *et al.* [19] was used to assess the oxidant-induced DNA damage to pBR322. The pBR322 was treated with/without bark extracts and with/without H_2O_2, then loaded on gel as follows:-Lane 1: DNA (control), Lane 2: DNA+H_2O_2, Lane 3: Aqueous extract+DNA, Lane 4: Aqueous extract+DNA+H_2O_2, Lane 5: Methanolic extract+DNA, Lane 6: Methanolic extract+DNA+H_2O_2, Lane 7: Chloroform extract+DNA, Lane 8: Chloroform extract+DNA+H_2O_2. After electrophoresis the ethidium bromide, stained DNA bands on the gel were viewed under transilluminating UV light and photographed. The density of the DNA bands was analyzed.

The extent of H_2O_2 induced damage to herring sperm DNA and calf thymus DNA was measured spectrophotometrically according to the procedure of Aeschlach *et al.* 1994 [20]. Briefly, the assay mixture contained herring sperm DNA/calf thymus DNA in Tris buffer at pH 7.4, 30% H_2O_2, $MgCl_2$, FeCl3 and bark extracts. The contents were incubated at 37 °C for one hour. The reaction was arrested by the addition of EDTA. The color was developed by the addition of 1%TBA and 25%, HCl and incubating at 37 °C for 15 min. The percentage of thiobarbituric acid reactive substances (TBARS) production was measured by absorbance at 532 nm.

Statistical analysis

All the estimations were done in triplicates, and the experimental results were expressed as means±SD.

RESULTS

Total phenolics and flavoniods in the bark

The total phenolic content was 153.33±23.09 mg of pyrocatechol equivalents/g, and flavonoids were 33.66±1.15 mg of catechin equivalents/g of powdered bark. In the present study, the quantity of total phenolics and flavonoids present in *C. zeylanicum* bark was found to be considerably higher.

Effect of *C. zeylanicum* bark extracts on oxidant-induced damage to membrane lipids

The aqueous, methanolic and chloroform extracts caused a considerable decrease in the extent of LPO in all the membrane systems. The percentage inhibition of LPO was 90.3±3.9, 95.17±0.4, and 73.7±3.5 for RBC ghosts, 84.8±3.5, 86.8±0.1 and 77±0.82 for goat liver homogenate and 77.6±3.32, 91.6±0.77 and 65±4.8 for goat liver slices respectively for aqueous, methanolic and chloroform extracts **(**fig. **1**). The inhibition of LPO was found to be higher in the RBC ghosts than other two membrane models. The methanolic extract of the bark showed a greater extent of protection to the membrane models compared to other two extracts.

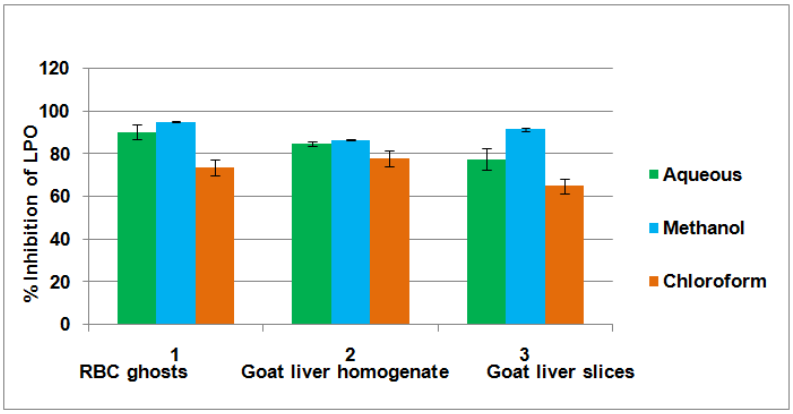

Fig. 1: Effect of *C. zeylanicum* bark extracts on lipid peroxidation of different membrane preparations

Effect of *C. zeylanicum* bark extracts on oxidant-induced damage to DNA

The protective effect of *C. zeylanicum* bark extracts (aqueous, methanolic and chloroform) against oxidant-induced DNA damage was studied on DNA of three different hierarchies viz., pBR322, herring sperm DNA and calf thymus DNA. fig. 2 shows the electrophoretic pattern of pBR322 DNA following exposure to H_2O_2. By visual comparison, it is evident that normal pBR322 (lane 1) showed two bands on agarose gel electrophoresis. The faster-moving band represented the native form of supercoiled circular DNA (scDNA), the slower moving band corresponded to the open circular form (ocDNA).

H_2O_2 exposure caused absolute damage to pBR322 DNA (multiple bands visible) in lane 2. The band pattern in lane 3, 4, 5, 6, 7 and 8 were comparative to that of lane 1. Thus, all the three extracts showed some magnitude of DNA protection in comparison to lane 2. This suggested that all the three extracts showed varying degrees of potential in inhibiting DNA damage due to H_2O_2 exposure. By visual observation, more prominent and sharper bands were noticed in lane 5 and 6 compared to lane 3, 4, 7 and 8. This supports that the

methanolic extract being a better DNA-protector than the aqueous and chloroform extracts.

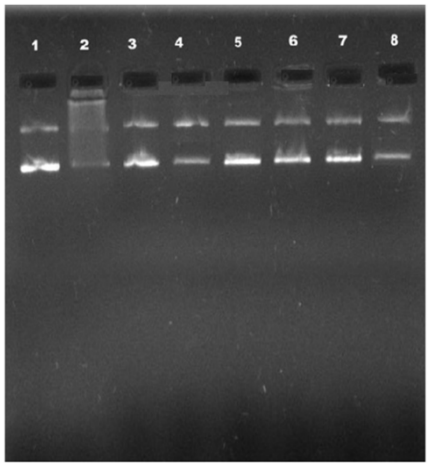

Fig. 2: Electrophoretic pattern of pBR322 DNA treated with oxidant and bark extracts

The extent of DNA protection by the bark extracts against damage induced by H_2O_2 to herring sperm and calf thymus DNA is depicted in fig. 3 and fig. 4 respectively. The values of the oxidant-treated groups were fixed at 100 per cent of damage, and the relative values in percentage were calculated for the other groups. The percentage production of TBARS of H_2O_2 treated herring sperm DNA in the presence of aqueous, methanolic and chloroform bark extracts was found to be 52.4±1.65, 33.29±1.6 and 67.45±1.7 whereas in the absence of H_2O_2 was found to be 22.8±1.4, 20.6±0.59, 22.5±1.2 respectively. The percentage production of TBARS of H_2O_2 treated calf thymus DNA in the presence of aqueous, methanolic and chloroform bark extracts was found to be 45.63±2.7, 33.85±1.36 and 64.53±2.45 whereas in the absence of H_2O_2 was found to be 25.88±1.55, 20.61±1.26 and 28.82±1.49 respectively.

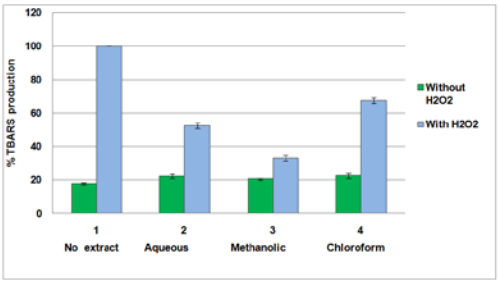

Fig. 3: Effect of *C. zeylanicum* bark extracts on oxidant-treated herring sperm DNA

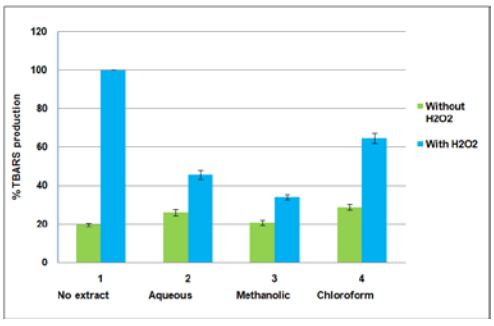

Fig. 4: Effect of *C. zeylanicum* Linn bark extracts on oxidant-treated calf thymus DNA

In the current investigation, all the three extracts of *C. zeylanicum* bark were found to have a significant protection against oxidative damage in purified DNA samples. The maximum inhibition of TBARS was rendered by the methanolic extract followed by the aqueous and chloroform extracts in both the samples of DNA tested.

DISCUSSION

There are many studies supporting the antioxidant capacity of phenolic compounds**.** Flavonoids can quench and or/scavenge ROS and protect the body against the deleterious effects [21]. Natural phenolic compounds are effective and safer antioxidants [22]. It is thought that the capability of phenolic compounds to reduce the free radicals arises because of both their acidity (ability to provide protons) and the delocalized π-electrons (ability to transfer electrons) [23]. Jayaprakasha *et al.* [24] reported that higher the phenolic content of fruit extracts of *C. zeylanicum,* higher the antioxidant activity in the *in vitro* model systems such as β-carotene-linoleate, and 1,1-diphenyl-2-picryl hydrazyl (DPPH). Seeram *et al.*, [25] and Liu *et al.*, [26] respectively reported that intake of polyphenol-rich beverages and Mulberry anthocyanin extracts rich in polyphenols could delay low-density lipoprotein (LDL) oxidation. Grape powder polyphenols attenuate the progression of atherosclerosis in apolipoprotein E-deficient mice and also reduces the macrophage atherogenicity by decreasing macrophage-mediated oxidation of LDL and cellular uptake of oxidized LDL [27]. In the present study, it was observed that the content of total phenolics and flavonoids in the bark was quite high which could play a vital role to protect the body against the deleterious effects of ROS.

Many reports supported the biomolecular protective effect of phytol compounds specifically by phenolic compounds. Sumathi *et al.* [28] observed that extracts of *Withania somnifera* leaves were very effective in preventing the lipid peroxidation in oxidant-challenged different membrane model systems under *in vitro* conditions. Sreelatha and Padma [29] examined the antioxidant properties of the leaf extracts of *Moringa oleifera* and reported that the leaf extracts could inhibit the amount of MDA generated (and thus LPO) in goat liver homogenate. The results of the current investigation are parallel with the results of others. Hence, these findings suggested that the extracts render better protection to intracellular membrane lipids. Oxidants must cross the membrane barriers to exert their effects. Similarly, antioxidants must also traverse the different membranes.

There are many literature supports for DNA protective effects of herbal extracts or phytol compounds. Guha *et al.,* [30] reported that aqueous and methanolic extract of *Lawsonia inermis* Linn, commonly known as Henna inhibited the DNA damage caused by exposure of pBR322 to Cr(VI)-UV (strong oxidant). Guha *et al.* [19] evaluated the free-radical scavenging properties and potential to prevent DNA damage (from oxidative stress) of 56 extracts (polar and non-polar) from 14 medicinal plants. The results showed that 27% of the extracts exhibited considerable plasmid DNA protection, and 37% of the extracts showed partial DNA protection against a high level of H_2O_2-driven oxidative damage. From the results of this study, it could be inferred that aqueous, methanolic and chloroform extracts of the bark of have high antioxidant potential by virtue of high phenolic content. These extracts could inhibit H_2O_2 induced oxidative toxicity to pBR322, herring sperm and calf thymus DNA under *in vitro* conditions.

CONCLUSION

In conclusion, the tested extracts may inhibit the oxidative damage pathways induced by oxidants in living cells also. Thus, *C. zeylanicum* bark can serve as a prospective source of natural phenolics and other metabolites to prevent oxidative stress-induced cell death.

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CONFLICT OF INTERESTS

Declare none

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