Chapter 7

In vitro Antioxidant Analysis and the DNA Damage Protective Activity of Leaf Extract of the *Excoecaria agallocha* Linn Mangrove Plant

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Additional information is available at the end of the chapter

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1. Introduction

Reactive oxygen species (ROS) are various forms of activated oxygen, which include free-radicals, e.g., superoxide anions (O_2^-), hydroxyl radicals (·OH), non-free-radical compounds (H_2O_2) and singlet oxygen ($^1O_2$), which can be formed by different mechanisms in living organisms. Oxidative damage of DNA molecules associated with electron-transfer reactions is an important phenomenon in living cells, which can lead to mutations and contribute to carcinogenesis and the aging processes. ROS species are considered as important causative factors in the development of certain diseases such as diabetes, stroke, arteriosclerosis, cancer and cardiovascular diseases, in addition to the aging process. Prior administration of antioxidant provides a close relationship between FRSA and the involvement of endocrinological responses, which help to reverse the effect [1, 2]. Plants are rich sources of phytochemicals such as saponin, tannin, flavanoids, phenolic and alkaloids, which possess a variety of biological activities including antioxidant potential. Antioxidants provide protection to living organisms from damage caused by uncontrolled production of ROS and concomitant lipid peroxidation, protein damage and DNA stand breaking. Natural antioxidants are in high demand for application as bio-pharmaceuticals, nutraceuticals and food additives.

Terrestrial plants are considered potent sources of bioactive compounds and pharmacologically active compounds, however, little is known about the therapeutical potential of mangrove plants. Exploration of the chemical constituents of mangrove plants is necessary to find new therapeutic agents and this information is very important to the local community. Important reasons for studying the chemical constituents of mangrove plants are first, mangroves are a type of tropical forest that grows easily and has not as yet been widely utilized. Secondly, the chemical aspects of mangrove plants are very important because of the potential to develop compounds of agrochemical and medical value.

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The plants of the genus *Excoecaria* (family: Euphorbiaceae) comprise nearly 40 species which are distributed throughout the mangrove regions of tropical Africa, Asia and northwest Australia. The most widely reported mangrove species is *Excoecaria agallocha* Linn. The latex of this plant has been used as a purgative and abortifacient, as well as in the treatment of ulcers, rheumatism, leprosy and paralysis. The leaves and latex of this tree have been used as fish poison in India, New Caledonia and Malaysia. The bark and wood is used in Thailand as a remedy for flatulence. Recently, much attention has been paid to *Excoecaria* species due to their cytotoxic and anti-HIV activities [3].

In this study we investigated the antimicrobial and antioxidant potential of methanol extract of *Excoecaria agallocha* Linn leaf. The DPPH and the oxidative DNA damage preventive activity and antioxidant potential of the crude methanol extract and sequential hexane, water and methanol extract of *E. agallocha* Linn leaf were also investigated. We found that water extract of *E. agallocha* Linn was more effective and could scavenge reactive oxygen species (ROS) thus preventing DNA strand scission by •OH generated in the Fenton reaction on pCAMBIA1301 DNA.

2. Material and methods

2.1. Plant materials and extraction procedure

The plants of *E. agallocha* were collected during the month of November 2009 from Ayiramthengu located near Alleppy in Kerala, India, at an average temperature of 28 - 34 °C. Collected plant material was dried in the shade and the leaves were then separated from the stem and pulverized to a fine powder in a grinder. The powdered leaf (10 g) was extracted sequentially with 100 ml of hexane and methanol by Soxhlet at a temperature not exceeding the boiling point of the solvent. To hexane extract water was added and separated in a separating funnel. The extracts were filtered using Whatman No. 1 filter paper and then concentrated in a vacuum at 40 °C using a rotary evaporator. The residues obtained were stored in a deep freezer at $^{-}$80 °C until use.

3. Determination of antimicrobial activities

3.1. Microorganisms

Microbial cultures *Bacillus subtilis* (MTCC- 441), *S. pyogenes* (MTCC- 442), *E. coli* (MTCC - 443), *Salmonella typhi* (MTCC - 733), *K. pneumoniae* (MTCC - 109), *S. marcescens* (MTCC -*97), *Vibrio cholerae* (01) and *Vibrio cholerae* (08) belonging to bacterial species and *Candida albicans* (MTCC - 3017) yeast were used in this study. Microorganisms were provided by IMTech, Chandigarh, India.

3.2. Antimicrobial activity by Disc-diffusion assay

The dried plant extracts were dissolved in the same solvent (methanol and distilled water) to a final concentration of 100 mg/ml and sterilized by filtration by 0.45 μm Millipore filters.

Antimicrobial tests were then carried out by disc-diffusion method [4] using 100 μl of suspension containing 10^8 CFU/ml of bacteria spread on nutrient agar (NA). The discs (6 mm in diameter) were impregnated with 5 μl of the extracts (500 μg/disc) at the concentration of 100 mg/ml and placed on the inoculated agar. Negative controls were prepared using the same solvents employed to dissolve the plant extracts. Ampicillin (10 μg/disc) was used as a positive reference standard to determine the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 ^oC for 24 h for clinical bacterial strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay in this experiment was repeated twice.

4. Evaluation of antioxidant activity

4.1. Reducing power assay

The reducing power of *E. agallocha* was determined as per the reported method [5]. Different concentrations of plant extract (100 -2000 μg/μl) in 1 ml of methanol were mixed with a phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1 %). The mixture was incubated at 50 ^oC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1 %), and the absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

4.2. Metal chelating effect

The chelation of ferrous ions by the extract was estimated as per the method of Dinis [6]. Different concentrations of the extract (100-2000 μg/μl) were added to a solution of 1 mM $FeCl_2$ (50 μl). The reaction was initiated by the addition of 1 mM ferrozine (0.1 ml) and the mixture was finally quantified to 1 ml with methanol, shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. All analyses were done in triplicate and average values were taken. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given below: % Inhibition $[(A_0 - A_1)/A_0 \times 100]$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the sample of *Excocaria* extract. $FeCl_2$ and ferrozine complex formation molecules are present in the control.

4.3. Nitric oxide radical inhibition activity

Nitric oxide, generated from sodium nitroprusside in an aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction [7]. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in a phosphate buffer saline and the extract (100 – 2000 μg/μl) were incubated at 25°C for 150 min. After incubation, 0.5 ml

of the reaction mixture was removed and 0.5 ml of Griess reagent (1 % (w/v) sulfanilamide, 2 % (v/v) H_3PO_4 and 0.1 % (w/v) naphthylethylene diamine hydrochloride were added. The absorbance of the chromophore formed was measured at 546 nm.

4.4. Lipid peroxidation and thiobarbituric acid reaction

A modified TBARS assay [8] was used to measure the lipid peroxide formed using egg yolk homogenate as lipid rich media [9]. Egg homogenate (0.5 ml of 10 %, v/v) and 0.1 ml of extract were added to a test tube and made up to 1 ml with distilled water, 0.05 ml of $FeSO_4$ (0.07 M) was added to induce lipid peroxidation and the mixture was incubated for 30 min. Then, 1.5 ml of 20 % acetic acid (pH 3.5) and 1.5 ml of 0.8 % (w/v) thiobarbituric acid in 1.1 % sodium dodecyl sulphate were added, the resulting mixture was vortexed and then heated at 95 °C for 1 h. After cooling, 5.0 ml of butanol was added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm. Inhibition of lipid peroxidation percent by the extract was calculated as 100- $[(A_1/A_2) \times 100]$; where A_1 is the absorbance value in the presence of extract and A_2 of the fully oxidized control.

4.5. Determination of DPPH radical scavenging capacity

Quantitative estimation of the free-radical scavenging activity was measured by DPPH assay [10]. The reaction mixture contained a different concentration (100-2000μg/μl) of test extract and 2.9 ml of DPPH (60 μM) in methanol. These reaction mixtures were taken in test tubes and incubated at 37 ^oC for 30 min, the absorbance was measured at 517 nm. The percentage of radical scavenging activity by the sample treatment was determined by comparison with the methanol treated control group. BHT and ascorbic acid was used as a positive control. The DPPH radical concentration was calculated using the following equation: scavenging effect (%) = $(DPPH^{\cdot})_T$ / $(DPPH^{\cdot})_{T=0}$ x 100, where $(DPPH^{\cdot})_T$ is the concentration of $DPPH^{\cdot}$ at 30 min time and $(DPPH^{\cdot})_{T=0}$, the concentration at zero time (initial concentration).

4.6. Total antioxidant activity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate / Mo (V) complex at the acid pH [11]. The tubes containing 0.1 ml of the extract and the 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM Ammonium molybedate) were incubated at 95 ^oC for 90 min. After the mixture was cooled to room temperature, absorbances were taken at 695 nm against the blank. The antioxidant capacity was expressed as AAE.

4.7. DNA nicking induced by hydroxyl radical

The DNA damage protective activity of *E. agallocha* L. extract was performed using super coiled pCAMBIA1301 DNA. Plasmid DNA isolation was done using GenElute™ Plasmid

Miniprep Kit (Sigma - Aldrich USA). A mixture of 10 μl of hexane, water and methanol extract (100 μg/μl), and plasmid DNA (0.5μg) was incubated for 10 min at room temperature followed by the addition of 10 μl of Fenton's reagent (30 mM H_2O_2, 50 μM ascorbic acid and 80 μM of $FeCl_3$). The final volume of the mixture was made up to 20 μl with double distilled water and incubated for 30 min at 37 ^{o}C. The DNA was analysed on 1 % agarose gel using ethidium bromide staining and photographed in Gel Doc. Quercetin (50 μM) was used as a positive control [12].

4.8. Phytochemical analysis

Chemical tests were carried out on the aqueous extract of the powdered specimens using standard procedures to identify the constituents as described by Harborne [13].

5. Results and discussion

5.1. Antimicrobial activity

This paper illustrates the antimicrobial, antioxidant and DNA protective effect of *Excoecaria agallocha* Linn leaf extract. The phytochemical constituent of *E. agallocha* Linn can be summarized as follows: methanolic leaf extract shows the presence of saponin, tannin and a high content of terpinoids, whereas cardiac glycosides are absent. The antimicrobial activities of methanol extract of *Excoecaria agallocha* Linn leaf is summarized in Table 1. The extract of *E. agallocha* reported to have significant *in vitro* antibacterial activity against *Staphylococcus aureus, Shigella dysenteriae, Shigella sonnei* and *Enterococci* with the zones of inhibition ranging from 11 to 15 mm, and no significant activity was reported against *Shigella flexneri* and *Staphylococcus epidermis* at test concentrations [14].

Microbes / Sample	+ve	1	2	3	4
B.subtilis	17	NI	NI	NI	NI
S.pyogenes	NI	NI	NI	NI	NI
E.coli	13	12	NI	NI	10
S.typhi	15	12	NI	NI	11
K.pneumoniae	>10	12	NI	NI	10
S.marcescens	36	11	10	NI	10
V. Cholerae (01)	10	11	10	NI	10
V. Cholerae (08)	15	10	NI	NI	10

Ampicillin used as positive control and the solvent used as negative control, activity expressed in millimole – mm concentration. (NI- no inhibition).

Table 1. Antimicrobial activity of different fractions (500mg/6mm disc) of *Excoecaria agallocha* Linn.

5.2. Reducing power assay

The reducing power of *E. agallocha* and reference compound ascorbic acid increases steadily with the increase in concentration. The absorbances at 700 nm of *E. agallocha* 4.00 (2000μg/μl) and ascorbic acid 4.5 (1000 μg/μl) shows that *E. agallocha* can act as electron donor and can react with free-radicals to convert them to more stable products and thereby terminate radical chain reactions (Figure 1A). The IC_{50} value of *E. agallocha* is observed to be at 62.96 μg. The reducing power of plant compounds might be due to the di- and mono-hydroxyl substitution in the aromatic ring which possesses potential hydrogen donating abilities [15]. The reducing properties are generally associated with the presence of reductones [16], which have been shown to exert antioxidant activity by breaking the free-radical chain by donating a hydrogen atom.

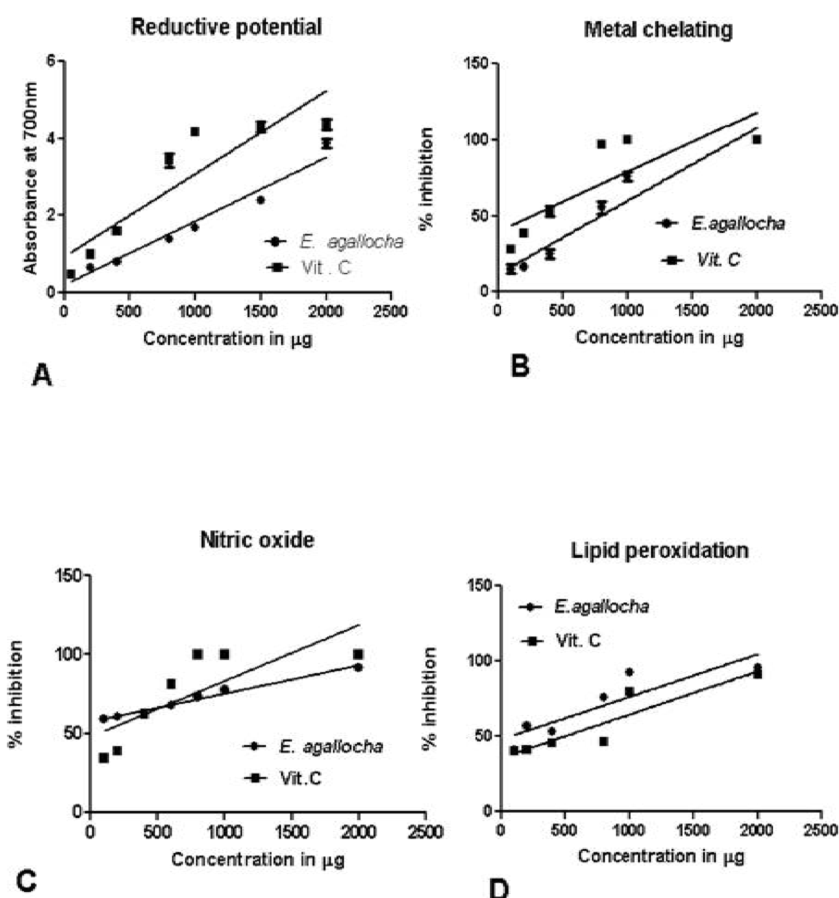

Figure 1. The concentration dependent (100 -2000 μg/μl) antioxidant activity: A) Reducing power, B) Metal chelating, C) Nitric oxide and D) Lipid peroxidation of methanol extract of leaf *E. agallocha* Linn (mean ± SD, $n = 3$).

5.3. Metal chelating effect

It has been proposed that transition metals catalyse the formation of the first few radicals to start the propagation of radical chain reaction in lipid peroxidation. Chelating agents may inhibit lipid oxidation by stabilizing transition metals. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. As shown in Figure 1B, the ferrozine - Fe^{2+} complex is not complete in presence of the plant extract, indicating its ability to chelate the iron. The absorbance of ferrozine-Fe^{2+} complex decreased linearly in a dose dependent manner (100– 2000μg/μl) and the IC_{50} value is estimated as 2.47 μg. The metal chelating activity of *E. agallocha* was evaluated against Fe^{2+}. The standard compounds ascorbic acid and BHT did not exhibit any metal chelating activity at the tested concentrations (100–2000 μg/μl). Reaction of ascorbic acid and gallic acid with $FeCl_2$ might enhance the degradation of ascorbic acid and gallic acid, and increase the ascorbyl and gallic acid radical concentrations [17].

5.4. Nitric oxide radical inhibition activity

The antioxidant system protects the pathogens against the ROS-induced oxidative damage. Nitric oxide radical generated from the sodium nitropruside is measured by the Greiss reduction. Sodium nitropruside at physiological pH spontaneously generates nitric oxide, which thereby interacts with oxygen to produce nitrate ions that can be estimated using Greiss reagents. Thus, the scavengers of nitric oxide compete with the oxygen, leading to reduced production of nitric oxide. The chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm. The methanolic leaf extract of *E. agallocha* has shown a more significant effect than that of ascorbic acid and the results are explained in Figure 1C. The IC_{50} of *E. agallocha* is estimated as 4.8 μg/μl.

5.5. Lipid peroxidation and thiobarbituric acid reaction

Egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of ferrous sulphate with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen with TBA absorbing at 532 nm [18]. Peroxidation of lipids has been shown to be the cumulative effect of reactive oxygen species, which disturb the assembly of the membrane causing changes in fluidity and permeability, alterations of ion transport and inhibition of metabolic processes [19]. The extract of *E. agallocha* exhibited strong lipid peroxidation inhibition in a concentration dependent manner (Figure 1D). The IC_{50} value for the inhibition of lipid peroxidation is observed to be 100 μg/μl. This activity was higher than that of ethanolic and hexane extract of *Ziziphus mauratiana* and *Z. spina-christi* reported using egg yolk as media of peroxidation [20]. The studies made on *E. agallocha* leaf extract suggest that it could play a role in protecting the physicochemical properties of membrane bilayers from free-radical induced severe cellular dysfunction.

5.6. Determination of DPPH radical scavenging capacity

Methanolic leaf extract of *E. agallocha* has shown good free-radical scavenging activity at all tested concentrations. DPPH· is a stable free-radical and can accept an electron or hydrogen radical becoming a stable diamagnetic molecule [21]. DPPH is purple in colour which turns yellow; the intensity of the yellow colour depends upon the amount and nature of radical scavenger present in the sample and standard compounds. The scavenging activity increases with an increase in concentration of the extract, as well as ascorbic acid, and levels off with further increases in concentration - IC_{50} value is at 67.50 µg/µl (Figure 2A). The residual concentration of DPPH depends exclusively on the structure of the phenolic compound, since there are two theoretical termination reactions: one between DPPH radicals and the other between DPPH· and phenol radical (Phe O·). However, the former reaction cannot occur due to steric hindrance and the latter reaction competes with the Phe O· coupling termination reaction [22, 23]. The accessibility of the radical centre of DPPH· to each polyphenol could also influence the order of the antioxidant power. Recently, the free-radical scavenging potential possessed by *Desmodium gangeticum* chloroform root extract was reported [24].

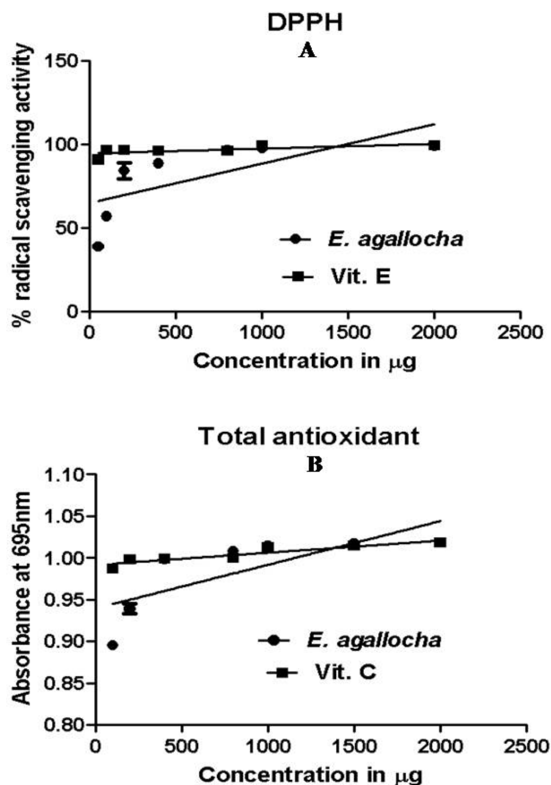

Figure 2. A) DPPH scavenging activity in concentration dependent (100 -2000 µg/µl) manner of methanol extract of leaf of *E. agallocha* Linn and B) Total antioxidant activity checked by phosphomolybdenum assay (mean ± SD, *n* = 3).

5.7. Total antioxidant activity

The total antioxidant potential of *E. agallocha* was investigated and compared against ascorbic acid, the results are explained in Figure 2B. The methanolic extract of *E. agallocha* is observed to be more effective - the IC_{50} value is calculated as 3.36 μg. The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, α-tocopherol and carotenoids [25]. Ascorbic acid, glutathione, cysteine, tocopherols, polyphenols and aromatic amines have the ability to donate hydrogen and electrons, and can thus be detected by this assay. Oxidative stress is the condition in which an imbalance between oxidant stimuli and physiological antioxidants exist leading to the damage of a cell. The body's physiological response to oxidative stress is through several antioxidant systems which include enzymes like superoxide dismutase, catalase, glutathione peroxidase and a variety of large molecules such as albumin and ferrtin, and small molecules such as ascorbic acid, tocopherol, etc. These antioxidants can be found as water-soluble or lipid-soluble molecules and localized transiently throughout tissues, cells and cell types.

5.8. DNA nicking induced by hydroxyl radical

Hydroxyl radical is the most reactive among reactive oxygen species, it has the shortest half life compared with others and is considered to be responsible for much of the biological damage in free-radical pathology. The radical has the capacity to cause strand breakage in DNA, which contributes to carcinogenesis, mutagenesis and cytotoxicity [11]. The DNA protective effect of hexane water and methanol extract of *E. agallocha* Linn leaf was checked against Fenton's induced DNA damage of pCAMBIA 1301 DNA. The protection offered against DNA damage by *E. agallocha* (10- 200 μg/μl) (results not shown) was concentration dependent. At concentration 100 μg/μl protection was more effective and slightly close to that of 5U of Catalase and 50 μM of quercetin tried (Figure 3). Native DNA has shown three forms, form I open circular form, form II single supercoiled band and below it form III, whereas DNA + Fenton's reagent and hexane fraction has exhibited complete degradation of DNA. Water fraction of *E. agallocha* Linn has shown very good protective activity and has retained all three forms. These results indicate that the water extract of *E. agallocha* Linn effectively mitigates the oxidative stresses on susceptible biomolecules, such as DNA.

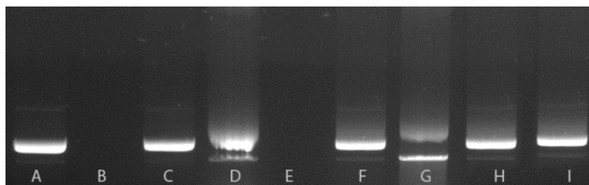

Figure 3. DNA protective effect of hexane, water and methanol extract of leaf of *E. agallocha* Linn, was checked against Fenton's induced DNA damage of pCAMBIA 1301 DNA. Effect of fraction of *E. agallocha* Linn. on oxidative DNA nicking caused by hydroxyl radicals. Lane A: native pCAMBIA 1301 DNA; Lane B: DNA + Fenton's reagent; Lane C: DNA + Fenton's reagent + *E. agallocha.* Linn water extract (50 μg/ml), Lane D: DNA + Fenton's reagent + *E. agallocha.* Linn methanol extract (100 μg/ml),

Lane E: DNA + Fenton's reagent + *E. agallocha.* Linn hexane extract, Lane F: DNA + Fenton's reagent + *E. agallocha.* Linn water extract (100μg/ml), Lane G: DNA + Fenton's reagent + *E. agallocha* Linn. crude methanol extract (100μg/ml) and Lane H: DNA + Fenton's reagent + quercetin (50 μM), Lane I: DNA + Fenton's reagent + Catalase (5 units).

6. Conclusion

In conclusion, the result obtained in the present study shows that the methanolic extract of *E. agallocha* contains a number of antioxidant compounds that can effectively scavenge ROS. Antioxidant properties of botanical extracts should be assessed in an array of model systems using several different indices because the effectiveness of such antioxidant material is largely dependent upon the chemical and physical properties of the system to which they are added and a single analytical protocol adopted to monitor lipid oxidation may not be sufficient to make a valid judgement. Hence, it may be concluded that the strong radical scavenging activity and oxidative DNA damage preventive activity of *E. agallocha* Linn may be correlated with its rich content of flavonoids.

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