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# *In vitro* evaluation of *Elaeagnus conferta* Roxb.- As a potent antioxidant and anti-inflammatory agent

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The key objective of the study was to identify bioactive compounds present in *Elaeagnus conferta* Roxb. and to determine their antioxidant and anti-inflammatory efficacy. Qualitative and quantitative preliminary screening of phyto-constituents was carried out using different solvent extracts, namely petroleum ether, ethyl acetate, acetone, ethanol, hydro-ethanol, and water, followed by *in vitro* antioxidant and anti-inflammatory assay. Among the six extracts, hydro-ethanolic extract revealed the presence of most of the secondary metabolites, which was used for further analysis. Antioxidant activity was determined by DPPH, Hydrogen peroxide scavenging assay and Nitric oxide scavenging assay. The results showed significant antioxidant potential in a strongly dose-dependent manner. Based on the antioxidant results, the *in vitro* anti-inflammatory assays were carried out. The anti-inflammatory assay inhibition of protein denaturation using egg albumin, bovine serum albumin and HRBC membrane stabilisation was done, which revealed that this plant species may serve as an excellent source of natural antioxidant and anti-inflammatory compounds. This study suggests that *E. conferta* Roxb. has potent efficacy to act as an anti-inflammatory agent and can possibly be used as a plant source in the pharmaceutical industry.

**Keywords:** Anti-inflammatory, Anti-oxidant assay, DPPH, *Elaeagnus conferta*, HRBC membrane stabilization, Protein denaturation assay

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## Introduction

In recent years, many researchers focus on exploring natural compounds for their anti-inflammatory and anti-oxidant properties. Plants play an important role in the development of new medicines, and the discovery of natural substances as replacements for synthetic compounds has gained more interest in many countries. Plants are rich sources of bioactive compounds which are crucial for uncovering novel therapeutic compounds. Anti-inflammatory and antioxidant properties aid in preventing and coping with several chronic diseases by decreasing cellular damage and inflammation.

*Elaeagnus conferta* Roxb. (=*Elaeagnus kologa* Schltdl.) also referred to as *E. dendroidea* (Schltdl.) Schltdl, *E. grandifolia* Bojer ex Schltdl, *E. kologa* Schltdl and *E. latifolia* L.) thorny shrub with elliptic, obovate leaves belongs to the Elaegnaceae family. It is widely distributed in the Western Ghats, particularly in the Shola forest of Nilgiris district of Tamil Nadu, India. The leaves are silvery and contain rusty scales below. The flowers are present in axillary clusters with a slight silvery colour. The fruits are oblong, woolly, and thin; they turn red when ripe[1]. "Huligehanninagida" is the local name of the plant, and the fruits are called "Kolangaannu" by the local communities in the Nilgiris district of Tamil Nadu, India[2]. The clans of Nilgiris, including the Todas and Kotasutilise the plant's decoction against dropsy, cutaneous infection, venereal sores, deafness, anasarca, and dysentery[1].

The biological activities of the extracts of plant leaf that have been revealed so far are anti-bacterial[3] and insecticidal activity, while antioxidant capacity and phenolic contents are validated only in the fruits[2]. Comprehensive studies on *E. conferta* are limited, in particular concerning its antioxidant and anti-inflammatory properties, despite the body of literature already available on plant-derived chemicals. This research gap emphasises the significance of the need for the current study to thoroughly assess *E. conferta's* medicinal potential and provide valuable insights. We intended to shed light on the medicinal use of *E. conferta* leaves for the amelioration of

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inflammatory diseases utilising existing ethnomedicinal knowledge.

## Materials and Methods

### Chemicals

All the chemicals used in the present investigation were purchased from HI MEDIA Pvt. Ltd. Only analytical-grade chemicals were used.

### Collection and identification of plant materials

The plant *E. conferta* was collected from The Niligiris in and around Kotagiri, Tamil Nadu, India (October 2020). The plant material was identified and authenticated by the Botanical Survey of India, Coimbatore, and the voucher number of the specimen is BSI/SRC/5/23/2019/Tech/162. The fresh leaves were collected, washed, shade-dried at room temperature, powdered, and stored in an air-tight container.

### Extract preparation

The dried powdered leaf weighing about 10 g was extracted by maceration with 100 mL petroleum ether, ethyl acetate, acetone, ethanol, hydro ethanol, and water for three days at room temperature with occasional shaking. The concentrate was shifted, and the marc was re-removed by a similar procedure until plant materials were depleted. The gathered filtrates were pooled and evaporated to dryness to yield the dry extracts.

### Determination of plant extract yield

The yields of extracts were expressed in percentage using the formula:

$$\text{Yield percentage (\%)} = \frac{W_1}{W_2} \text{ X } 100$$

Where $W_1$ is the dry weight of extract after evaporating the solvent, and $W_2$ is the weight of the plant powder initially soaked in the solvent.

### Preliminary phytochemical screening of plant extracts

The dried samples extracted with solvents of increasing order of polarity, namely, petroleum ether, ethyl acetate, acetone, ethanol, hydro ethanol, and water, were subjected to preliminary screening for the presence of active phytoconstituents using standard protocols[4-7].

### Quantitative estimation of chemical constituents

#### *Total phenolic content*

Folin-Ciocalteu colourimetric method was used to analyse the sample with slight modifications[8]. About 0.5 mL of the plant extracts (petroleum ether, ethyl acetate, acetone, ethanol, hydro ethanol, and water) was taken and added with 2.25 mL of Folin-Ciocalteu phenol reagent and left undisturbed for 5 min. After which, 2.25 mL of 6% sodium carbonate was added and mixed. This mixture could stand at room temperature for about 90 min. A spectrophotometer was used to measure the absorbance of the contents at 725 nm. The calibration curve was plotted using gallic acid (20-100 µg/mL) as standard, which was processed in the same manner as the sample. The total phenolic content was expressed in gallic acid equivalents (GAE) per gram.

#### *Total flavonoid content*

The total flavonoid content of the sample was determined using the aluminium chloride colourimetric method with few adaptations[9]. Quercetin (20-100 µg/mL) was used as standard. Exactly 1 mL of 2% aluminium chloride was added to 0.5 mL of the extracts/ quercetin. The solution was mixed and incubated for 60 min at room temperature. The absorbance of the reaction mixture was measured at 420 nm using a UV-Vis Spectrophotometer and expressed as quercetin equivalents (QE) pergram.

#### *Tannin content*

The tannin content was estimated by the method described by Senguttuvan *et al.*[10]. Tannic acid with a concentration ranging from 20-100 µg/mL was used as standard. Exactly 0.5 mL of the sample/standard was added to a clean, dry test tube, followed by 0.5 mL Folin-Ciocalteu reagent and 1 mL of 35% Sodium carbonate. The total volume was made up to 5 mL using distilled water. The solution was centrifuged, and the absorbance of the supernatant was recorded at 725 nm using a UV-Vis Spectrophotometer. The concentration of tannin was calculated as tannic acid equivalent (TAE).

#### *Alkaloid content*

Exactly 5 g of the powder was taken in a 250 mL beaker, to which 200 mL of 20% acetic acid was added. The contents were mixed, covered, and left undisturbed for 4 hours. After 4 hours, the mixture was filtered. This solution was heated in a water bath until the volume came to one-quarter of the original volume. Once the volume has reduced to one-quarter, ammonium hydroxide is added dropwise until a precipitate is formed. The solution was left intact, and the precipitate was filtered, dried, and weighed[11]. The alkaloid content was estimated using the formula:

$$\text{\% Alkaloid} = \frac{\text{Weight of residue}}{\text{Weight of sample taken}} \text{ X } 100$$

***In vitro* antioxidant assay**

The hydro-ethanolic leaf extract of *E. conferta* was subjected to assays based on electron transfer, namely DPPH method, Hydrogen peroxide radical scavenging assay and Nitric oxide radical scavenging assay.

***DPPH radical scavenging activity***

This assay was carried out in accordance with the procedure described by Aslam Khan *et al.*[11]. The sample, as well as the standard (ascorbic acid), were prepared at concentrations ranging from 100-500 µg. Exactly 1 mL of the sample/ standard was added to 1 mL of 2,2'- diphenyl-1picrylhydrazyl DPPH solution (0.3 mM) and 1 mL of ethanol. The contents were vortexed and maintained in the dark at room temperature for 30 min. The addition of DPPH marks a colour change (purple colour fades), and a decrease in absorbance is seen at 518 nm using a UV-Vis spectrophotometer. The control is devoid of the plant sample. The percentage of inhibition is calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of the control} - \text{absorbance of sample}}{\text{Absorbance of the control}} \text{ X } 100$$

***Hydrogen peroxide radical scavenging assay***

The method described by Rabia Naz *et al.*[12], was used to examine the potential of plant extract to scavenge hydrogen peroxide. Exactly 2 mM hydrogen peroxide solution was prepared in 50 mM (pH 7.4) phosphate buffer solution. The plant powder was prepared in varying concentrations (100-500 µg). 0.5 mL of extract was transferred into vials, followed by adding 0.4 mL phosphate buffer solution and mixed. Hydrogen peroxide 0.6 mL was added to the same and vortexed. The absorbance at 230 nm using a UV-Vis spectrophotometer after 10 min was read against a blank solution (phosphate buffer without hydrogen peroxide). The standard ascorbic acid was treated in the same manner.

$$\text{Percentage scavenged} = \frac{\text{Absorbance of the control} - \text{absorbance of sample}}{\text{Absorbance of the control}} \text{ X } 100$$

***Nitric oxide radical scavenging assay***

The sodium nitroprusside generates nitric oxide in an aqueous solution at physiological $p^H$, which reacts with oxygen to produce nitrite ions, which are measured by adding a Griess reagent and read at 540 nm[13]. About 3 mL of 10 mM sodium nitroprusside was added to 1 mL of varying concentration (100-500 µg) of plant extract mixed and maintained at 25°C for 150 min. After which 500 µL of Griess reagent was added, and the absorbance of chromophore was recorded. The Phosphate buffer saline and sodium nitroprusside equal volume were taken as blank and treated the same as that of the sample.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of the control} - \text{absorbance of sample}}{\text{Absorbance of the control}} \text{ X } 100$$

**Evaluation of *in vitro* anti-inflammatory activity**

***Membrane stabilisation***

The anti-inflammatory efficacy can be evaluated using the HRBC membrane stabilisation method[14]. The samples were collected in heparin tubes from healthy individuals. Equal volumes of blood and Alsever medium were mixed and centrifuged at 3000 rpm for 10 min. The supernatant was removed carefully, and the packed cells were washed with 0.85% iso-saline ($p^H$ 7.2) two to three times. Lastly, 10% HRBC suspension was prepared using iso-saline. About 0.5 mL of the plant extract (100- 500 µg), 1 mL phosphate buffer (0.15 M, pH 7.4), 2 mL hyposaline (0.36%), and 0.5 mL HRBC suspension comprise the assay mixture. The reference drug used is Diclofenac. The control contains 2 mL distilled water in the place of hyposaline. The mixture was maintained at 37°C for about 30 min and centrifuged at 3000 rpm for 10 min. The supernatant (haemoglobin content) was measured at 560 nm using a UV-Vis spectrophotometer.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of the control} - \text{absorbance of sample}}{\text{Absorbance of the control}} \text{ X } 100$$

***Inhibition of protein denaturation using egg albumin***

Diclofenac/ sample (100-500 µg) 0.5 mL, egg albumin 0.2 mL and 2.8 mL phosphate buffered saline ($p^H$ 6.4) constituted the reaction mixture made up to 5 mL, respectively. The mixture was maintained at 37°C for 15 min and then heated at 70°C for 5 min. The absorbance was read at 660 nm using a UV-Vis spectrophotometer[15].

$$\text{Inhibition (\%)} = \frac{\text{Absorbance of the control} - \text{absorbance of sample}}{\text{Absorbance of the control}} \text{ X } 100$$

***Inhibition of protein denaturation using Bovine serum albumin***

Aspirin was used as a standard drug (100-500 µg). 0.5 mL of the sample/standard, 0.45 mL of BSA was attuned to $p^H$ 6.3 by adding 1N HCl. The samples could stand for 20 min at room temperature, followed by heating for 30 min at 57°C. The mixture was

cooled and added with 2.5 mL of phosphate buffer, and the absorbance was read at 660 nm using a UV-Vis spectrophotometer[16]. The percentage inhibition was deliberated using the following formula:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \text{ X } 100$$

**Statistical analysis**

All the values are expressed as mean±SD for six observations. The $IC_{50}$ values were calculated from regression analysis in MS- Excel.

## Results and Discussion

**Extraction yield**

The percentage yield of the crude extracts (petroleum ether, ethyl acetate, acetone, ethanol, hydro ethanol, and water) is tabulated in Table 1.

The hydro-ethanolic extract of the leaf exhibited a higher yield of 7%, followed by water and ethanol. Petroleum ether yielded only 5.8%, the lowest among all the six extracts.

Table 1 — % Yield of extracts

| Solvent | % Yield |
|---|---|
| Petroleum ether | 5.8 |
| Ethyl acetate | 6.0 |
| Acetone | 5.9 |
| Ethanol | 6.1 |
| Hydro-ethanol | 7.0 |
| Water | 6.5 |

**Preliminary phytochemical screening of plant extracts**

The phytochemical screening of all six extracts showed the presence and absence of different phytoconstituents according to the polarity of the solvents used for extraction. Table 2 depicts the phytoconstituents in *E. conferta*. Inulin, tannin, steroids, and triterpenoids were present in all six solvent extracts, whereas alkaloids, lignin, and glycoside were present only in ethanol, hydro-ethanol, and water extracts. Flavonoids were absent in the acetone extract. The presence of most of the secondary metabolites was shown in polar solvents. A higher number of secondary metabolites with a higher degree of precipitation was found in the hydro-ethanolic leaf extract. Existing evidence reports that plants of the Elaegnaceae family contain flavonoids, alkaloids, and biologically active lipids[17], which support our findings in this study.

**Quantitative estimation of chemical constituents**

***Total phenolic content***

Total phenolic content ranged between 2.7 mg GAE/g to 5.5 mg GAE/g of dry weight (Table 3). Hydro ethanolic extract demonstrated the highest phenolic content, and ethyl acetate was the lowest. Phenolic compounds, the significant plant constituents known for powerful chain-breaking antioxidant properties, react with active oxygen radicals and form water due to the presence of hydroxyl groups in them[18]. Numerous biological

Table 2 — Phytoconstituents present in *Elaeagnus conferta* Roxb.

| Test | Petroleum ether | Ethyl acetate | Acetone | Ethanol | Hydro ethanol | Water |
|---|---|---|---|---|---|---|
| Flavonoids | + | + | ++ | ++ | +++ | ++ |
| Alkaloids | - | - | - | + | +++ | + |
| Lignin | - | - | ++ | +++ | +++ | ++ |
| Glycoside | - | - | - | + | +++ | + |
| Inulin | ++ | + | + | + | +++ | + |
| Tannin | + | ++ | ++ | +++ | +++ | +++ |
| Steroids | +++ | ++ | ++ | +++ | +++ | +++ |
| Triterpenoids | +++ | ++ | ++ | +++ | +++ | +++ |

+++, ++, + and – indicate highly present, moderately present, low and absent, respectively

Table 3 — Levels of phytoconstituents in *Elaeagnus conferta* Roxb.

| Solvent | Phenols mg/g | Tannins mg/g | Flavonoid mg/g | Alkaloid mg/g |
|---|---|---|---|---|
| Petroleum ether | 4.46±0.15 | 5.63±0.15 | 1.66±0.2 | Nil |
| Ethyl acetate | 2.7±0.1 | 5.46±0.15 | 1.73±0.15 | Nil |
| Acetone | 5.36±0.12 | 4.8±0.2 | 2.77±0.25 | Nil |
| Ethanol | 3.46±0.11 | 5.46±0.2 | 2.4±0.2 | 3.43±0.20 |
| **Hydro-ethanol** | **5.66±0.12** | **6.00±0.13** | **2.6±0.26** | **4.23±0.25** |
| Water | 5.03±0.15 | 6.06±0.23 | 2.43±0.21 | 2.56±0.15 |

activities, such as antioxidant, anti-inflammatory, and anti-carcinogenic effects, have been linked to these compounds.

***Total flavonoid content***

The total flavonoid content of the leaf extract was calculated as mg quercetin equivalent/g dry weight. The total flavonoid content in petroleum ether 1.5 mg quercetin/g was the lowest, while 2.9 mg quercetin/g was found in hydro-ethanolic extract. Ethanol and aqueous extract had the same 2.29 mg quercetin/g (Table 3). Flavonoids are polyphenolic compounds that exhibit a wide range of biological effects. Because of their phenolic hydroxyl bond, they can successfully scavenge the reactive oxygen molecules and are, therefore, strong antioxidants[19].

***Tannin content***

The tannin content of the leaf extract was expressed in terms of mg tannic acid equivalent per g dry weight. The hydro-ethanolic extract had the maximum tannin content, followed by aqueous and ethanolic extract. The tannin content of hydro-ethanolic (highest) and acetone (lowest) extract was found to be 6 mg tannic acid/g and 4.6 mg tannic acid/g, respectively (Table 3). Plant-based medicines predominantly contain tannins as an active ingredient[20].

***Alkaloid content***

The gravimetric analysis for total alkaloid content in leaf extract exhibited its presence only in ethanol, aqueous and hydro-ethanolic extracts. Hydro-ethanolic extract contained 4 mg, followed by 3.2 mg and 2.4 mg in ethanol and aqueous, respectively (Table 3).

***In vitro* antioxidant activity of *E. conferta***

From the above preliminary analysis, it was evident that the hydro-ethanolic extract showed a predominant presence of phytoconstituents. Hence, it was used for further *in vitro* analysis. The plant extract was prepared in varying concentrations ranging from 100-500 µg/mL.

***DPPH radical scavenging activity***

The DPPH radical scavenging activity of leaf extract and standard ascorbic acid are depicted in Fig. 1a. The radical scavenging activity was highest at 500 µg for both the standard and the plant extract. The $IC_{50}$ of the standard and plant extract was assessed to be 25.56 µg/mL and 70.08 µg/mL, respectively. The mechanism involved is the loss of colour of the DPPH solution. When a solution of

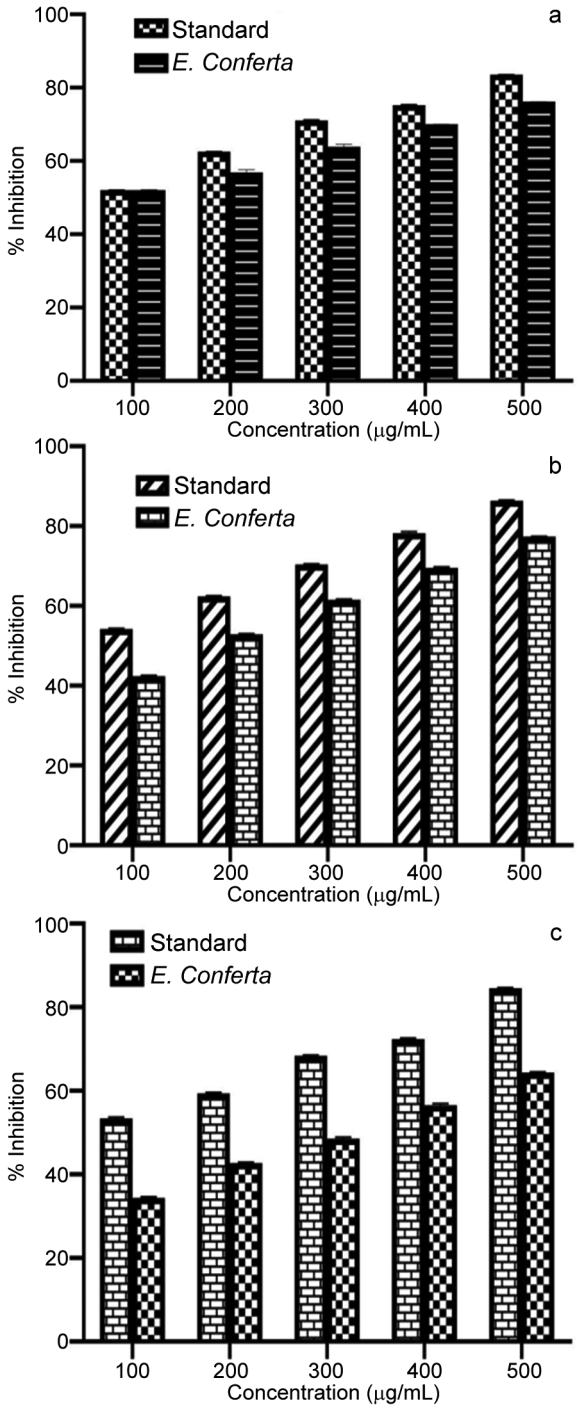


Fig. 1 — a) DPPH radical scavenging activity of *Elaeagnus conferta*; b) Hydrogen peroxide radical scavenging assay of *Elaeagnus conferta*; and c) Nitric oxide radical scavenging assay.

DPPH is mixed with the plant sample capable of donating hydrogen atoms, the reduced form of the radical is generated, indicated by the loss of violet colour. There was a significant decrease in absorbance with increased concentration of the

standard/plant extract. The decrease in absorbance marks the increase in the percentage inhibition. Previous research on *Elaeagnus* species, *E. umbellata,* would support the outcome of the current investigation[21].

***Hydrogen peroxide radical scavenging assay***

Fig. 1b represents the hydrogen peroxide scavenging activity of hydro-ethanolic extract and standard. The leaf extract expressed a strong dose-dependent inhibition with increasing concentration of plant extract. The $IC_{50}$ of the plant extract was 46.95 µg/mL, and that of the standard was 178.26 µg/mL. Hydrogen peroxide inactivates a few enzymes by oxidation of thiol (SH) groups, which acts as a weak oxidising agent. It can cross the cell membrane and potentially react with $Fe^{2+}$ and $Cu^{2+}$. This leads to the formation of hydroxyl radicals, which marks the beginning of many toxic reactions[22]. A compound's reducing ability can be a key indicator of its possible antioxidant activity[23]. In *Hippophae rhamnoides* L., the same form of assay was carried out. Similar efficacy was demonstrated by those belonging to the same family as *E. conferta*[24].

***Nitric oxide radical scavenging assay***

The nitric oxide radical scavenging potential increased with an increase in the concentration of the plant extract and standard. The maximum inhibition of 64.28% for the plant and 84.5% for the standard was observed (Fig. 1c). The $IC_{50}$ 69.88 and 312.93 µg/mL were expressed in the plant and standard, respectively. Nitric oxide is a chemical mediator involved in controlling numerous physiological processes. During reduction with oxygen or superoxides, $NO_2$, $N_2O_4$, and $N_3O_4$ formed are highly reactive. However, over-accumulation can cause deleterious effects on cellular components. The nitrite ions bind to the Griess reagent, forming purple azo dye. The decline in the intensity of colour represents the presence of scavengers in the plant extract. *E. indica*, which belongs to the same family, has comparable activities[25].

***In vitro* anti-inflammatory activity**

***Membrane stabilisation***

The hydro-ethanolic extract is used for this analysis. Fig. 2a depicts the HRBC membrane stabilisation assay results. The percentage protection increased with the increase in concentration of the selected plant extract. The plant extract showed nearly the same percentage of protection as the standard

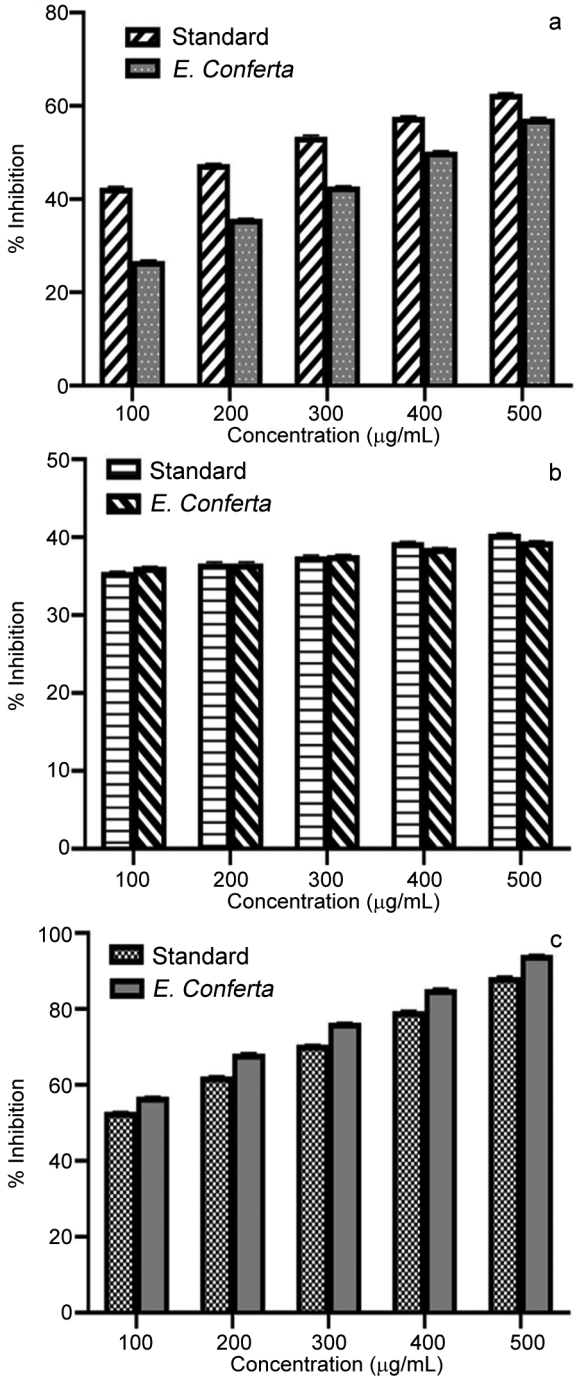


Fig. 2 — a) HRBC membrane stabilization assay of *Elaeagnus conferta*; b) Inhibition of protein denaturation using egg albumin of *Elaeagnus conferta*; and c) Inhibition of protein denaturation using Bovine serum albumin.

aspirin. The percentage protection ranged from 42.34–62.75% for the standard and 26.57–57.14% for the sample. The $IC_{50}$ of the standard and plant extract was measured to be 248 µg/mL and 400 µg/mL, respectively. The rupture of the RBC membrane is

marked by excessive accumulation of intracellular fluid associated with hypotonic hemolysis. RBC membrane injury enhances the vulnerability of the cell to secondary damage, which is predominantly caused by free radicals, lipid peroxidation and release of PLA2. During improved permeability caused by inflammatory mediators, membrane stabilization prevents the leakage of serum proteins and fluids into the RBC[26]. The extract inhibited hypotonicity-induced lysis of the erythrocyte membrane, which showed the ability of the extract to induce or promote the efflux of intracellular components.

***Inhibition of protein denaturation using egg albumin***

Anti-denaturation of egg albumin method was used to determine the anti-inflammatory activity. The present outcome shows a concentration-dependent inhibition. The $IC_{50}$ values of the standard 1275.85 µg/mL and plant extract 1776.86 µg/mL were determined from this assay. A maximum of 40.51 and 39.35% inhibition was found in the standard and plant extract, respectively. The plant extract showed inhibition equal to that of the standard (Fig. 2b). Protein stabilisation is measured by the per cent inhibition of protein denatured with respect to that of the control. *E. conferta* showed a significant inhibitory effect on protein denaturation. This was comparable to that of the standard, which may be due to their phenolic content.

***Inhibition of protein denaturation using Bovine serum albumin***

The effect of the plant extract/standard against the heat denaturation of BSA is depicted in Fig. 2c. The anti-denaturation was a completely dose-dependent inhibition. The inhibition of denaturation exhibited by plant extract was higher compared to that of the standard. The percentage inhibition ranged between 52.94–88.25% for the standard and 56.88–94.2% for the plant extract. The $IC_{50}$ of the plant extract was 15 and 65.5 µg/mL for the standard. The inhibitory effect of the plant extract was markedly higher than that of the standard.

## Conclusion

The quest for plant sources in developing pharmaceuticals, cosmetics, and food brings new products into the market. *In vitro* antioxidant and *in vitro* anti-inflammatory studies were carried out to validate the efficacy of *E. conferta* scientifically. Based on the results of the present study, it was concluded that the hydro-ethanolic extract of *E. conferta* possesses significant antioxidant and anti-inflammatory activity.

The efficacy of *E. conferta* may be due to the presence of phenol, flavonoid and alkaloid compounds. Further experimentation to isolate bioactive compounds and *in vivo* studies are to be performed to understand the mechanism of action of *E. conferta* as an effective herbal antioxidant.

## Conflict of interest

The authors declare that there is no conflict of interest.

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