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Antioxidative potential and anticancer activity of *Elaeagnus caudata* (Schltdl) against Type-II human lung adenocarcinoma, A549 cells *via* caspase-mediated apoptotic cell death

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This study investigates the phytochemical composition, anti-oxidative potential and anti-cancer activity of silverberry *Elaeagnus caudata*. The aqueous extract of *E. Caudata* has higher phenolic content (259.32 ± 5.18 mg GAE/g) and flavonoid content (1869.25 ± 25.9 mg quercetin equivalent/g) than the methanolic extract. The aqueous extract possessed higher scavenging activities than the methanolic extract for 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions ($O_2^{\bullet -}$) and 2, 2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) with IC_{50} of 61.48 ± 1.06 μg/mL, 335.7 ± 0.61 μg/mL, and 372.2 ± 16.17 μg/mL, respectively. Consistently, the aqueous extract was found to exhibit higher total reducing power than the methanolic extract as well as standard ascorbic acid. The extracts were also analysed for their anti-haemolytic activity and inhibitory effect on lipid peroxidation in an *ex-vivo* condition using mice erythrocyte and liver, respectively. The aqueous extract of *E. caudata* showed higher inhibitory activities against hemolysis and lipid peroxidation with an inhibition rate of 80.77% and 81.58%, respectively. *E. caudata* also induced cell death in A549 cells in a dose and time dependent manner and increased the level and activities of antioxidants, while decreasing the lipid peroxidation (LPO) level in the A549 cells. Induction of DNA damage and elevation of caspase-6 activity in A549 cells following *E. caudata* treatment provide an insight into apoptosis based anti-cancer activities of *E. caudata*.

Keywords: Anti-cancer, Antioxidant, Apoptosis, DNA damage, *Elaeagnus caudata*

Reactive oxygen species (ROS) such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) are fundamental in modulating various physiological functions of the body when present in low to moderate concentrations[1]. However, due to their highly reactive property, excessive generation of ROS hampers the antioxidant defence systems of the body leading to a condition called 'oxidative stress' ensuing tissue damage[2]. Oxidative stress is found to be closely associated with various lifestyle-related disorders such as neurodegenerative disorders, coronary heart disease, diabetes, inflammation, arthritis, lung damage and cancer[2,3]. Although cells are naturally equipped with an impressive repertoire of antioxidants, both enzymatic (such as SOD, CAT, GST and GPx) as well as non-enzymatic antioxidants (such as GSH, ascorbic acid and lipoic acid)to counterbalance ROS thereby preventing oxidative stress, over-production of ROS overwhelms the endogenous antioxidants leading to oxidative cellular damage with likely adverse health outcomes in the long-run[4]. Therefore, maintenance of optimal body function and redox homeostasis in cells may require exogenous antioxidants supplementation.

Cancer is a group of diseases characterized by uncontrolled proliferation of cells with the ability to invade or spread to other cells of the body. Despite the availability of certain cancer treatment modalities, the mortality due to cancer does not seem to decline[5]. Plants and natural products remain a prominent source of anti-cancer agents due to their safety, efficacy and lesser side effects[6]. There is an increasing evidence for the potential of plant-derived compounds as inhibitors of various stages of tumorigenesis and associated inflammatory processes, indicating the importance of plant products in cancer prevention and therapy. A549 cells are human alveolar basal squamous epithelial cells adenocarcinoma obtained from a 58-year-old Caucasian male[7] that grows adherently as a monolayer *in-vitro*. A549 cells have been commonly used for the screening of anticancer properties of various plant extract *in-vitro*[8-10].

The genus *Elaeagnus*, commonly known as Silverberries, consists of deciduous evergreen shrubs

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which are mainly distributed in subtropical regions of Asia, Europe and North America[11]. *Elaeagnus caudata*, locally known as Sarzukpui, has been traditionally used for the treatment of various health problems such as stopping of menses, induction of labor, expulsion of retained placenta and rheumatoid arthritis[12]. Furthermore, it has been used as folk medicine by local herbal practitioners for the treatment of various malignancies. Among the genus *Elaeagnus*, *E. angustifolia*[13], *E. umbellata*[14], *E. pungens*[15], *E. rhamnoides*[16] and *E. glabra*[17]have been extensively studied and reported to have multi-pharmaceuticals importance such as anti-nociceptive, muscle relaxant, anti-angiogenic, cytotoxicity, anti-tumorigenic and anti-microbial. Bioactive groups including flavonoids (such as rutin, epigallocatechin gallate), phenolic compounds (such as ferulic acid, triperpenoids), saponins (such as terpengustifol) and alkaloids (such as harmane and tetrehydroharmol) have also been isolated from various species of the *Elaeagnus*[18,19]. Despite the extensive use of *E. caudata* as traditional medicine, scientific validation of their pharmaceutical property is still limited. Therefore, the present study aimed to investigate the phytochemical constituents, anti-oxidative potentials and anti-cancer activity of *E. caudata.*

Materials and Methods

Chemicals

Gallic acid, quercetin dihydrate, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazinemethosulfate (PMS), 2-deoxyribose, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydrogen peroxide (H_2O_2), bovine serum albumin (BSA), glutathione (GSH) reduced, trypsin EDTA, Eagle`s Minimal Essential Medium (MEM), 3-4, 5-dimethylthiazole-2-yl-2, 5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS),acridine orange, ethidium bromide and Folin-ciocalteu's reagent were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) and thiobarbituric acid (TBA), 1-chloro-2,4 dinitrobenzene (CDNB), 5, 5′ dithio 2-nitrobenzoic acid (DTNB), agarose (low gelling temperature), ethylenediamine tetra-acetic acid (EDTA), Trizma base, Trizma hydrochloride and trichloroacetic acid (TCA) were obtained from Sigma Aldrich Inc (Louis, Germany). Doxorubicin (Getwell Oncology Pvt., Ltd., Haryana, India) was purchased from local pharmacy. The remaining chemicals were purchased from Merck Specialities Pvt., Ltd. (Mumbai, India).

Preparation of extracts

Leaves of *E. caudata* was collected from Serkawn, Lunglei District, Mizoram (22.9081° N, 92.7587° E) and dried in a well ventilated dark room. Identification and authentication of the plant was done by the Department of Horticulture, Aromatic and Medicinal Plants (HAMP), Mizoram University, Aizawl. The dried leaves were grounded into fine powder using electrical grinder. Fat contents and chlorophyll washing was done using petroleum ether and chloroform, respectively, for 72 h followed by sequential extraction using methanol and distilled water as extractant for a minimum of 40 cycles using Soxhlet apparatus. The liquid extracts were filtered and dried in rotary evaporator (Buchi, Germany) under reduced pressure at 40°C for 5 h. The extracts obtained were then collected and stored at 4°C until use. Henceforth, the methanolic extract and aqueous extract of *E. caudata* will be called as EC-ME and EC-AE, respectively.

Phytochemical analysis

Preliminary phytochemical screening was performed using standard methods[20]. Samples of different fractions of *E. caudata* were analysed for the presence of cardiac glycosides, alkaloids, saponins, steroids, tannins, terpenoids, resins and phlobatannins. Results are expressed as '+' for the presence and '−' for the absence of phytochemical.

Estimation of total phenolic content

The total phenolic content of *E. caudata* was estimated using standard method[21]. Briefly, 5 mL of Folin-Ciocalteu's reagent (diluted ten-fold) was mixed with 1 mL of *E. caudata* extracts (0.25-4.0 mg/mL). After 5 min of incubation, 4 mL of sodium carbonate (0.115 mg/mL) was added. The mixture was then incubated in the dark at room temperature for 2 h and the absorbance was recorded at 765 nm. Calibration curve was also prepared by mixing methanol solution of gallic acid (1 mL, 0.25-4.0 mg/mL) with the reagents above and absorbance was recorded at 765 nm using UV-Visible spectrophotometer. The experiment was repeated three times and the total phenolic content was expressed as gallic acid equivalents (GAE) mg/g of the dry extract.

Estimation of total flavonoid content

Total flavonoid content of various extract of *E. caudata* was estimated according to the method previously described with minor modifications[21]. Briefly, 0.25 mL of *E. caudata*

extracts (0.25-4.0 mg/mL) and standard quercetin solution was mixed with 75 µL of 5% (*w/v*) sodium nitrite solution and 1.25 mL of distilled water. After the addition of 150 µL of 10% (*w/v*) aluminium chloride, the solution was allowed to stand for 5 min followed by addition of 0.5 mL of 1 M NaOH. The volume of the solution was made up to 2.5 mL using distilled water. The absorbance was recorded immediately at 510 nm. The total flavonoid content of *E. caudata* was expressed in term of quercetin equivalent (mg/g extract).

Determination of free radical scavenging activity *in vitro*

The ability of different extracts of *E. caudata* to inhibit the generation of various free radicals was carried out as described below.

DPPH radical scavenging activity

The scavenging activity of *E. caudata* extracts for DPPH radical was determined according to the method previously described with minor modifications[22]. Briefly, 0.5 mL of various extracts of *E. caudata* (0.25–1000 µg/mL) was mixed with 1 mL of methanol solution of 0.1 M DPPH followed by 30 min incubation in the dark. The absorbance of the solution at 523 nm was compared with the control. The scavenging activity of the plant extract against DPPH was expressed as IC_{50} which is the concentration (µg/mL) of extract at which 50% of the DPPH radicals was inhibited. Ascorbic acid (ASA) was used as the standard. The test was repeated at all concentration of each sample in triplicate. The scavenging activity was then calculated based on the percentage of DPPH radicals scavenged using the formula:

$$\text{Scavenging}\ (\%) = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

Superoxide radical scavenging activity

Superoxide scavenging activity was determined by the nitroblue tetrazolium (NBT) reduction method with minor modifications[23]. In brief, the reaction mixture was prepared using 0.2 mL of NBT (1 mg/mL in DMSO) and 0.6 mL of plant extract (0.25-1000 µg/mL). Then, the volume of the mixture was made up to 2.8 mL using 2 mL of alkaline DMSO (1 mL DMSO in 5 mM NaOH). The absorbance of the mixture was recorded at 560 nm and pure DMSO was used as blank. Ascorbic acid (ASA) served as the standard and the ability of *E. caudata* extracts to scavenge the superoxide radical was calculated.

$$\%\ \text{scavenging} = (A_e - A_o / A_e) \times 100$$

where, A_o is absorbance without plant extract and A_e is absorbance with the plant extract.

ABTS radical scavenging activity

The scavenging activity of *E. caudata* against ABTS was determined using the method previously described[21]. Briefly, 5 mL each of 7 mM ABTS and 2.45 mM potassium persulfate were mixed for a stock solution. A stock solution was then incubated at room temperature in the dark for 12 h so as to yield a dark-colored solution that contains $ABTS^{\bullet+}$ radicals. A freshly prepared working solution consists of a stock solution diluted with 50% methanol having an initial absorbance of 0.70 (±0.02) at 745 nm. $ABTS^{\bullet+}$ radicals scavenging activity was then assessed by mixing 150 µL of different fractions of various extracts of *E. caudata* (0.25-1000 µg/mL) with 1.5 mL of ABTS working solution. The decrease in absorbance was measured immediately at 745 nm. The test was repeated at all concentration of each sample in triplicate. Ascorbic acid (ASA) served as the standard. The scavenging activity of the plant extract was then calculated using the formula:

$$\text{Scavenging}\ (\%) = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control (solution containing all the reagents except the plant extracts) and A_{sample} is the absorbance of the solution containing the plant extract.

Ferric Reducing Assay

The reducing power of *E. caudata* extracts was estimated using the standard method with minor modifications[24]. Briefly, 2.5 mL each of 0.2 M phosphate buffer (pH- 6.6) and 1% potassium ferricyanide solution were mixed with *E. caudata* extracts (25-1000 µg/mL). After incubation of the mixture at 50°C for 20 min, 2.5 mL of 10% TCA was added. The mixture was then centrifuged at 3000 rpm for 10 min. Equal volume of distilled water was added to the supernatant followed by 0.5 mL of 1% ferric chloride solution. Absorbance of the mixture was measured at 700 nm. The increase in absorbance indicated increasing reducing power of the extract.

***Ex vivo* antioxidant assay**

Anti-haemolytic activity

The inhibition of mice erythrocyte hemolysis by various extracts of *E. caudata* was measured to

determine their antioxidative potential[25]. Blood was collected from Swiss albino mice of same age group (10-12 w) and body weights (25-30 g) by heart puncture in a heparinized tube. The mice erythrocyte hemolysis was induced with H_2O_2 that serve as free radical initiator. A mixture was prepared by adding 0.5 mL of 5% (*v/v*) suspension of RBC in PBS, 0.4 mL (0.5 mg/mL) of different extracts of *E. caudata* and 100 µL of 1 mol/L H_2O_2. The reaction mixture was gently mixed while being incubated at 37°C for 3 h. It was then diluted with 4 mL of PBS and centrifuged at 2000 rpm for 10 min. The supernatant was collected and absorbance was recorded at 540 nm. The rate of inhibition of erythrocyte hemolysis was then calculated.

$$\text{Inhibition rate (\%)} = [1 - (A1 - A2)/ A_0] \times 100$$

where, A_0 is the absorbance of control, A1 is the absorbance of the solution containing the plant extract and A2 is the absorbance without RBC.

Inhibition of lipid peroxidation

Lipid peroxidation inhibitory potential of *E. caudata* extracts was measured according to the method described earlier using mice liver[26]. Liver was excised from Swiss albino mice and 1% liver homogenate was prepared and centrifuged at 3000 rpm at 4°C for 10 min. 0.5 mL of supernatant was mixed with 0.5 mL (0.5 mg/mL) of *E. caudata* extracts, 0.25 mL each of 0.5 mol/L $FeCl_2$ and H_2O_2 and incubated at 37°C for 1 h. Absorbance was measured at 535 nm and the rate of inhibition of lipid peroxidation was calculated using the formula:

$$\text{Inhibition rate (\%)} = [1 - (A1 - A2)/ A_0] \times 100$$

where, A_0 is the absorbance of control, A1 is the absorbance of the solution containing the plant extract and A2 is the absorbance without liver homogenate.

Animals

Colony of inbred Swiss albino mice is being maintained under standard environmental conditions of temperature (22°C ± 5°C) and light (12 h of light and dark, respectively) (Frontier Euro Digital Timer, Taiwan) at the Animal House, Department of Zoology, Mizoram University, India. The animals were having free access to food and water. Caring and handling of animals were carried out based on the guidelines given by WHO, Geneva, Switzerland. The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZU-IAEC/2018/09) and CPCSEA, New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

Cell culture

Type II human lung adenocarcinoma (A549) was obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were then cultured in MEM supplemented with 10% FBS, 1% L-glutamine and 50 µg/mL gentamicin sulfate with loosened caps at 37°C in an incubator containing 5% CO_2 (Eppendorf AG, Hamburg, Germany).

MTT assay

The cytotoxic effect of *E. caudata* was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) reduction assay[27]. Briefly, 1×10^4 cancer cells were seeded in 96-well plate containing 100 µL MEM per well. The cells were allowed to adhere for 24 h and treated with different concentrations of aqueous extracts of *E. caudata* (25-200 µg/mL) for 24 h and 48 h. After treatment with *E. caudata* extracts, 20 µL of MTT (5 mg/mL) was added to each wells and incubated for 3 h. The drug-containing media were removed and cells were washed with FBS free media. Then the formazan crystals were dissolved in 200 µL of DMSO followed by incubation for 15 min after which the absorbance was measured at 560 nm using microplate reader (Spectramax m2e, Molecular Devices). Three independent experiments consisting of three replicates were carried out for each treatment. Cytotoxicity was expressed as inhibition (%) which was calculated by the formula given below:

$$\text{\% inhibition} = \text{Control-Treatment/Control} \times 100$$

Cell morphology analysis by fluorescent staining (Apoptotic assay)

The mode of cell death induced by EC-AE was determined by dual florescent staining using acridine orange/ethidium bromide (AO/EtBr). Briefly, 1×10^5 A549 cells were seeded in six-well plate containing 5 mL of media. Cells were allowed to adhere overnight and treatment was given for 48 h with 50 µg/mL EC-AE. After treatments, cells were washed with sterile 1X PBS and detached with 1X trypsin EDTA. The cells were pelleted and resuspended in 100 µL of FBS free media. Subsequently, 25 µL cell suspension was stained with 2.5 µL each of acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL) in a ratio of 1:1 for

2 min followed by gentle mixing. The morphology of apoptotic cells was then examined on a slide under fluorescent microscope (Thermo Fisher Scientific, EVOSR Fluorescence Imaging, AMEP-4615). A minimum of 300 cells were scored and the apoptotic index was calculated as follows:

Apoptotic index (%) = Number of apoptotic cells scored × 100/Total number of cells counted

Antioxidants/Oxidant assays

For the estimation of antioxidant enzymes activities and lipid peroxidation level, 0.5×10^6 A549 cells were seeded in T-25 flasks containing 5 mL media. The cells were treated with 50 µg/mL of *E. caudata* aqueous extract for 48 h. After treatment, the drug-containing media were discarded and the cells were washed with sterile 1X PBS and harvested with 1X trypsin EDTA. The cancer cells were pelleted, sonicated (PCI Analytics Pvt. Ltd., Mumbai, India) and 5% homogenate was prepared using cold sterile PBS (pH 7.4) and used for the biochemical estimations. Total protein contents were determined by the standard method[28] using bovine serum albumin as standard.

Glutathione (GSH)

The concentration of GSH was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm[29]. Briefly, 1.8 mL of 0.02 M Na_2HPO_4 (pH 8.0) and 40 µL of 10 mM DTNB were mixed with 160 µL of cell homogenateand incubated for 2 min at room temperature. The absorbance of the sample was read against blank, which consisted of distilled water instead of cell homogenate, at 412 nm and the concentration of GSH was calculated from the standard curve and expressed in µmol/mg of total protein.

Glutathione-s-transferase (GST)

The activity of GST was measured following the standard method[30]. Briefly, 850 µL of phosphate buffer (pH 6.5) was mixed with 50 µL of 20 mM CDNB, incubated for 10 min at 37˚C followed by the addition of 50 µL each of cell homogenate and 20 mM GSH. The absorbance of blank and sample was measured at 360nm and the enzyme activity was expressed in unit/mg of total protein.

Superoxide dismutase (SOD)

The SOD activity was estimated using the standard method with minor modifications[31]. Briefly, 100 µL each of cell homogenate and 186 µM PMS were mixed with 300 µL of 3.0 mM NBT and 200 µL of 780 µM NADH. The mixture was incubated for 90 sec at 30°C and 1 mL of acetic acid and 4 mL of n-butanol were added to stop the reaction. The absorbance of sample and blank was measured at 560 nm, and the enzyme activity was expressed in units (1U = 50% inhibition of NBT reduction)/mg protein.

% inhibition = (OD of blank – OD of test/OD of blank) × 100

SOD unit = 1/50 × % inhibition

Lipid peroxidation (LPO)

Malondialdehyde (MDA) formed by the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction of lipids. MDA has been identified as the product of lipid peroxidation (LPO) that reacts with TBA to give a red product. LPO was estimated by the standard method[32]. Briefly, cell homogenate was added to a mixture containing 10% TCA, 0.8% TBA and 0.025N HCl in 1:2 ratio. The mixture was boiled for 10 min in a boiling water bath. After centrifugation, the supernatant was collected and its absorbance was read at 535 nm against the blank. The malondialdehyde (MDA) concentration of the sample was calculated using the extinction coefficient of 1.56×10^{-6} M^{-1} cm^{-1}.

Assessment of genotoxicity of EC-AE using Comet assay

The genotoxic effect of EC-AE in A549 cell was determined using the alkaline single cell gel electrophoresis (Comet Assay) as described earlier[33] with minor modifications. Briefly, 2×10^4 A549 cells treated with 50 µg/mL of EC-AE for 48 h along with the untreated control were suspended in 75 µL of 0.5% low-melting point agarose (LMPA) prepared in 1X PBS and spread onto a frosted slide precoated with 1% normal-melting point agarose (NMPA) and covered with a coverslip. Once the gel got solidified, the coverslip was gently removed and the third layer of 90 µL 0.5% LMPA was added. The slides were then incubated for 2 h in a freshly prepared lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, 1% Triton X-100 and 10% DMSO, pH 10). After lysis, slides were placed on a horizontal electrophoresis tank filled with freshly prepared alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH13) for 20 min to allow unwinding of DNA. Electrophoresis was then carried out for 30 min at 24 V and 300 mA. The slides

were then neutralized by washing with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min. After neutralization, slides were washed with d.H_2O and then stained with ethidium bromide (EtBr) solution (2 μg/mL) for 5 min. Each slide was prepared in triplicate and 100 randomly selected cells from each slide were examined using florescence microscope with a magnification of 200x. Image capture and analysis were performed with Image J software.

Caspase-6 activity assay

To further assess the apoptotic inducing effect of EC-AE, the activity of caspase 6 was determined using commercially available kit (BioVision Incorporated, USA). Briefly, 5×10^5 A549 cells were treated with 50 µg/mL of EC-AE for 48 h in a 6-well plate along with the untreated control. After treatment, cells were washed and lysed in 50 µL of chilled lysis buffer followed by 10 min incubation on ice. The cell lysates were centrifuged at 15,000 × g for 1 min at 4°C, and the supernatant was collected. The assay was performed in a total volume of 100 µL in 96-well plates. 150 µg of protein from each sample was assayed for caspase-6 activity against its specific colorimetric substrate VEID-*p*NA. The mixture was incubated for another 2 h at 37°C and absorbance of free *p*-nitroanilide (*p*NA) produced *via* cleavage from specific substrates by activated caspase- 6 was measured at 405 nm using microplate reader.

Statistical analysis

Results are expressed as mean ± standard error of mean. Student t-test was performed for comparison of cytotoxicity, DNA damage, caspase activity, antioxidant/oxidant status, phytochemical contents and anti-oxidative potential. One-way analysis of variance (ANOVA) followed by Tukey multiple comparisons of means was also performed to test the significant variations on the free radical scavenging activities using SPSS ver.20.0 software (SPSS Inc, Chicago, Illinois, USA). The IC_{50} values were calculated by plotting the% inhibition against the log doses using GraphPad Prism software version 6.0. A P-value of less than 0.5 was considered statistically significant.

Results

Phytochemical analysis

Qualitative phytochemical screening revealed the presence of various naturally occurring compounds such as alkaloids, cardiac glycosides, saponins, tannins, terpenoids, phlobatannins and resins in the aqueous extract of *E. caudata* (EC-AE). Alkaloids, tannins and resins were also found in the methanolic extract (EC-ME) (Table 1).

Total phenolic and flavonoid contents

The total phenolic content and flavonoid content of *E. caudata* extracts increased in a concentration dependent manner (Fig. 1A & B). At 4 mg/mL, EC-AE has significantly higher (P <0.001) total phenolic content (259.32 ± 5.18 mg gallic acid equivalent/g of dry extract) than that of EC-ME (157.92 ± 4.1 mg gallic acid equivalent/g of dry extract). Similarly, EC-AE has significantly higher (P <0.001) total flavonoid content (1869.25 ± 25.9 mg quercetin equivalent/g of dry extract) compared to that of EC-ME (1000 ±12.93 mg quercetin equivalent/g of dry extract).

In vitro antioxidant assay

DPPH radical scavenging activity

Elaeagnus caudata extracts showed a dose-dependent increase in DPPH radicals scavenging activity as indicated by the discoloration of DPPH.

Table 1 — Phytochemical screening of various extracts of *E. caudata.* ('+' indicates presence of phytochemicals and '−' indicates absence of phytochemicals). EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract

Phytochemicals	Reagent	Colour Indication	ECME	ECAE
Alkaloids	Dragendorff's Reagent	Reddish brown precipitate	+	+
Cardiac glycosides	Glacial Acetic Acid Ferric Chloride Sulphuric Acid	Brown ring	−	+
Saponins	Olive oil	Whitish Emulsion	−	+
Steroids	Sulphuric Acid	Red Colour	−	−
Tannins	Ferric Chloride	Brownish Green or blue-black	+	+
Terpenoids	Sulphuric acid	Reddish Brown	−	+
Phlobatannins	Hydrochloric acid	Red precipitate	−	+
Resins	Copper Sulphate	Green precipitate	+	+

Maximum scavenging was observed at the dose of 200 μg/mL for both methanolic and aqueous extracts that declined thereafter. Log-doses of *E. caudata* extracts and standard ascorbic acid (ASA) were plotted against DPPH inhibition (%) for the calculation of IC_{50} (Fig. 2A). EC-AE possessed a higher scavenging activity than that of EC-ME. However, the standard ascorbic acid still possessed significantly higher scavenging activity (P <0.001) than that of *E. caudata* extracts (Fig. 2B).

ABTS radical scavenging activity

$ABTS^{\bullet+}$ radical scavenging activity of *E. caudata* extracts increased in a dose-dependent manner as indicated by discoloration of the $ABTS^{\bullet+}$**,** which was measured spectrophotometrically at 745 nm. Maximum scavenging activity was observed at the dose of 1000 μg/mL for aqueous extract. Log-doses of *E. caudata* extracts and the standard ascorbic acid (ASA) were plotted against $ABTS^{\bullet+}$ inhibition (%) for the calculation of IC_{50} (Fig. 3A). EC-AE possessed higher $ABTS^{\bullet+}$ scavenging activity than the EC-ME. The $ABTS^{\bullet+}$scavenging activity of the standard ascorbic acid was not significantly different from the aqueous extract of *E. caudata* (Fig. 3B).

Superoxide radical scavenging activity

Elaeagnus caudata extracts also showed a dose-dependent inhibition of superoxide radical ($O2^{\bullet-}$) generation. Maximum $O2^{\bullet\bullet}$scavenging activity was observed at a concentration of 1000 μg/mL for EC-AE. Log-doses of *E. caudata* extracts and the standard ascorbic acid (ASA) were plotted against $O2^{\bullet-}$ inhibition (%) for the calculation of IC_{50} (Fig. 4A). EC-AE possessed a higher scavenging activity than that of EC-ME. Moreover, scavenging activity of ASA was not significantly different from the aqueous extract of *E. caudata* (Fig. 4B).

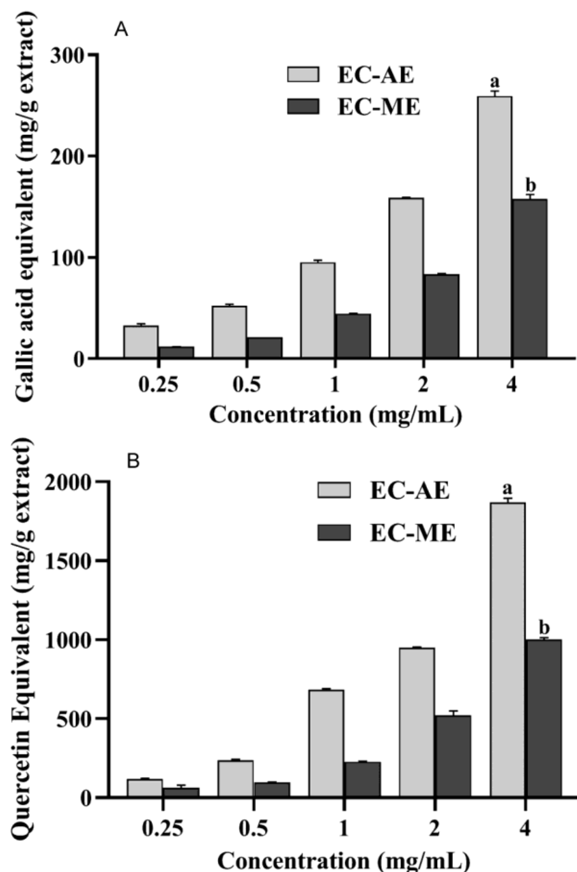

Fig. 1 — (A) Phenolic content of various extracts of *E. caudata* determined as gallic acid equivalent. (B) Flavonoid content of various extracts of *E. caudata* determined as quercetin equivalent. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation

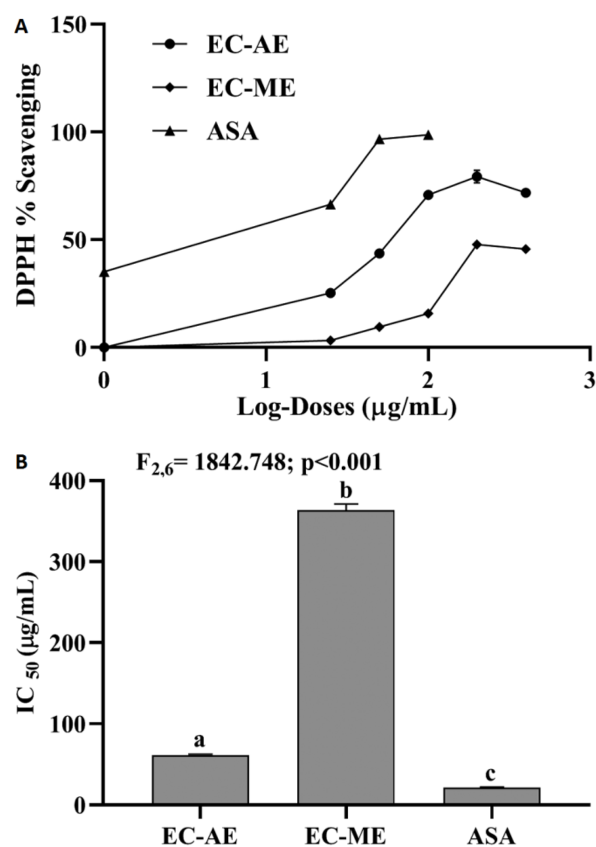

Fig. 2 — (A) Plots of log-doses of *E. caudata* extracts and the standard ascorbic acid against DPPH inhibition (%) for the calculation of IC_{50}; (B) IC_{50} (μg/mL) for DPPH of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation

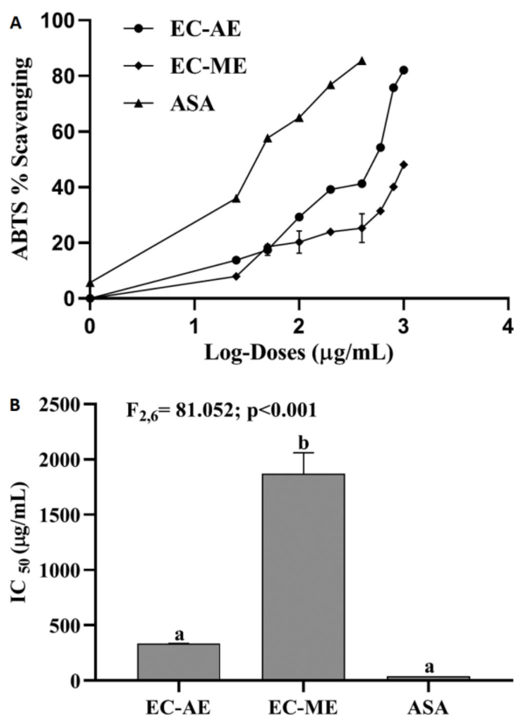

Fig. 3 — (A) Plots of log-doses of *E. caudata* extracts and the standard ascorbic acid against ABTS inhibition (%) for the calculation of IC_{50}. (B) IC_{50} (µg/mL) for ABTS of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation

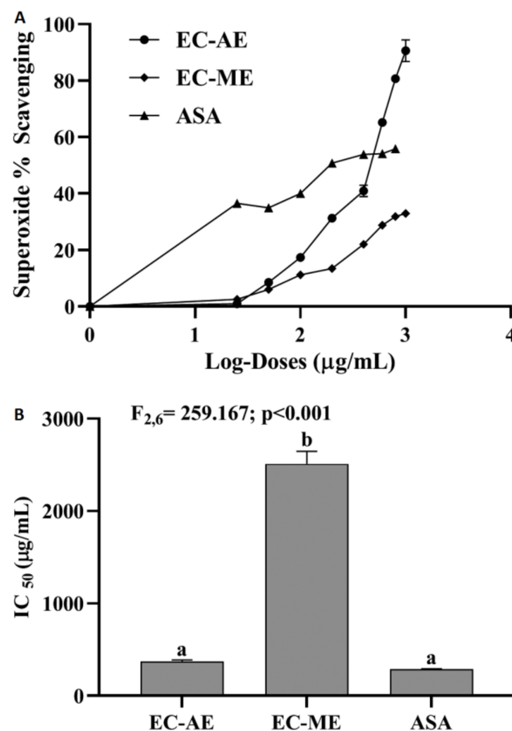

Fig. 4 — (A) Plots of log-doses of *E. caudata* extracts and the standard ascorbic acid against superoxide radical inhibition (%) for the calculation of IC_{50}. (B) IC_{50} (µg/mL) for superoxide radical inhibition of *E. caudata* extracts and standard ascorbic acid. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract; ASA: standard ascorbic acid. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation

Reducing power

The reducing power of *E. caudata* extracts was determined by measuring the transformation of Fe^{3+} to Fe^{2+}. The reducing activity of *E. caudata* extracts increased in a concentration dependent manner (Fig. 5). At 1000 µg/mL, EC-AE (2.291 ± 0.044) showed a significantly higher (P <0.001) reducing activity than that of EC-ME (0.671 ± 0.021).

***Ex-vivo* antioxidant assay**

Anti-haemolytic activity

The anti-haemolytic activity was determined using 0.5 mg/mL of *E. caudata* extracts. Significant variation was observed in the anti-haemolytic activity of the two extracts (P <0.005). EC-AE showed a significantly higher inhibitory activity against erythrocyte hemolysis with an inhibition rate of 80.77% compared to that of EC-ME (43.48%) (Fig. 6A).

Lipid peroxidation inhibition

The lipid peroxidation inhibition potential of *E. caudata* extracts was estimated in mice liver homogenate. Significant variation was observed between the two extracts in their inhibitory activity against lipid peroxidation (P <0.001).EC-AE possessed a higher inhibitory activity with an inhibition rate of 81.58% compared to that of EC-ME (61.63%) (Fig. 6B).

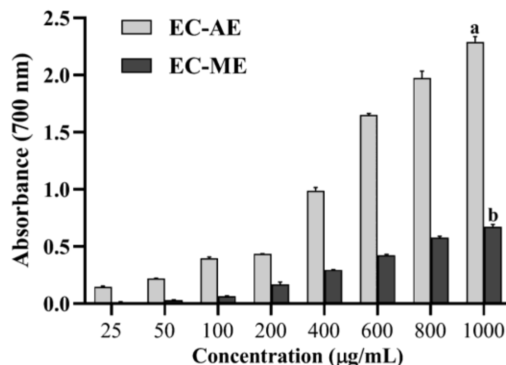

Fig. 5 — Reducing power of *E. caudata* extracts. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation

Cytotoxic effects of EC-AE in A549 cells

The cytotoxic effect of *E. caudata* was expressed as % inhibition of A549 cells and was plotted against

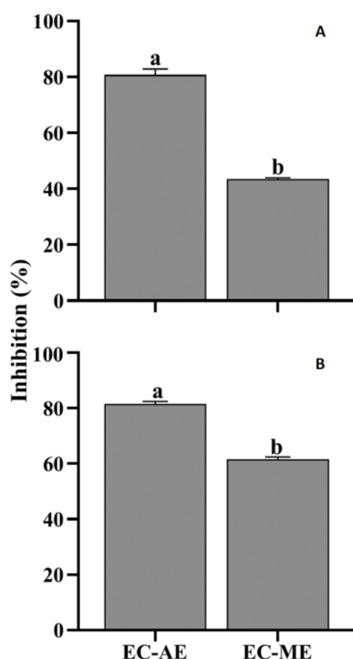

Fig. 6 — (A) Anti-haemolytic activity; and (B) Lipid peroxidation inhibition of *E. caudata* extracts at a concentration of 0.5 mg/mL. EC-AE: *E. caudata* aqueous extract; EC-ME: *E. caudata* methanolic extract. Values are expressed as Mean ± SEM, n=3. Different letters indicate significant variation.

log-doses for the calculation of IC_{50}. Treatment of cells with EC-AE showed a dose and time-dependent increase in cytotoxicity (Fig. 7). The IC_{50} of EC-AE against A549 cells after 24 h and 48 h treatment were found to be 81.63 ± 4.51 µg/mL and 49.84 ± 3.05 µg/mL, respectively.

Morphological evidence of apoptosis induced by EC-AE

Acridine orange is nucleic acid fluorescent dye that permeates both live and dead cells, and makes the nuclei appear green. Ethidium bromide is taken up only by dead cells whose cytoplasmic membrane integrity is lost and stains the nuclei yellowish orange. For this reason, live cells have green nuclei whereas, apoptotic cells that incorporated ethidium bromide exhibit condensed and fragmented orange chromatin (Fig. 8A). Dual AO/EtBr staining revealed that exposure of A549 cells to 50 µg/mL of EC-AE for 48 h resulted in significant increase in the number of apoptotic cells. Fluorescence microscopic image shows that EC-AE was able to induced morphological alterations in A549 cells such as membrane blebbing, nuclear condensation and nuclear fragmentation which are the distinct characteristics of apoptotic cells. The percentage of dead cells in EC-AE treated A549 cells was 86.7% while it was only 1.2% in untreated control (Fig. 8B).

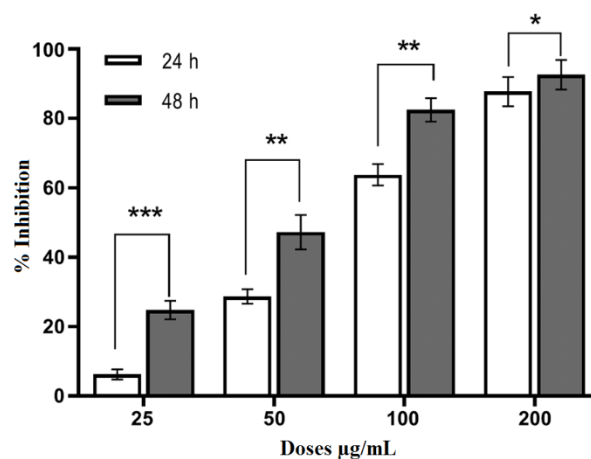

Fig. 7 — Effects of treatment duration (24 h and 48 h) with different concentration of aqueous extract of *E. caudata* (EC-AE; 25, 50, 100 and 200 µg/mL) on A549 cells. Values are expressed as Mean ± SE, n=3. *, ** and *** indicate significant variation at P <0.05, P <0.01 and P <0.001 respectively.

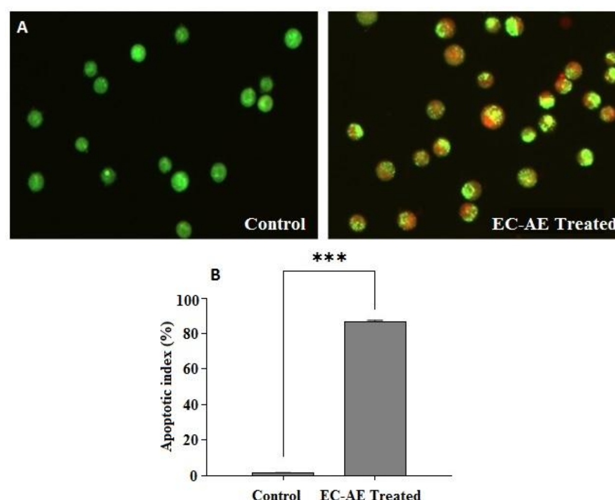

Fig. 8 — (A) Acridine orange/Ethidium bromide (AO/EtBr) dual staining of A549 cells after treatment with 50 µg/mL of aqueous extract of *E. caudata* for 48 h. (B) Percentage of apoptotic cells after treatment of A549 with aqueous extract of *E. caudata*. Control: A549 cells without treatment. EC-AE: Aqueous extract of *E. caudata*. Values are expressed as Mean ± SEM. ***P <0.001 compared with the untreated control

Effects of EC-AEon antioxidants/oxidant status of A549 cells

In order to assess whether *E. caudata* alter the antioxidant levels in A549 cells, cells were treated with 50 µg/mL of EC-AE and the levels of GSH, GST and SOD were assessed. Glutathione content of A549 cells in treatment group was significantly (P <0.01) higher than the untreated control (Fig. 9A). A549 cells treated with EC-AE for 48 h also resulted in significant increase in GST and SOD activities when compared to untreated control (Fig. 9B & C). In an effort to investigate whether

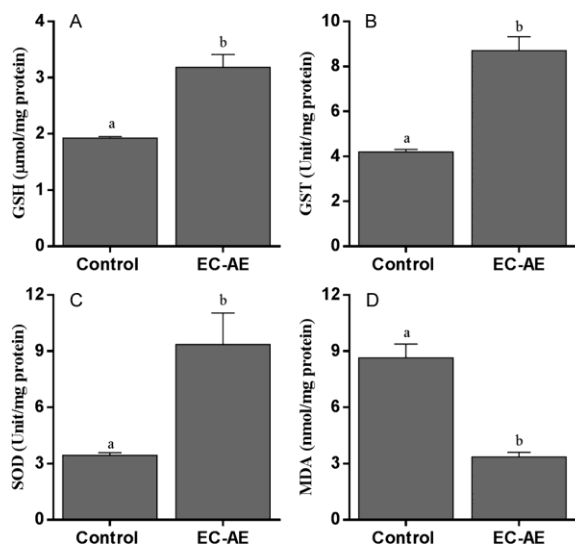

Fig. 9 — Effects of aqueous extract of *E. caudata* (EC-AE; 50 µg/mL) on: (A) glutathione (GSH) level; (B) glutathione-s-transferase (GST) activity; (C) superoxide dismutase (SOD) activity; (D) malondialdehyde (MDA) level to assess lipid peroxidation. Values are expressed as Mean ± SEM. Means not sharing the same letter are significantly different

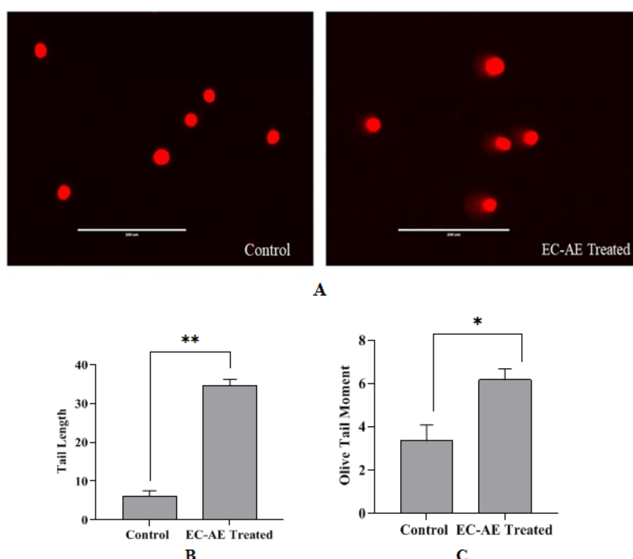

Fig. 10 — (A) Florescence images of Comets observed in control and A549 cells treated with 50 µg/mL of aqueous extract of *E. caudata*. (B & C) The extent of DNA damage expressed in terms of Tail length and Olive tail moment. Control: A549 cells without treatment; EC-AE treated: A549 cells treated with 50 µg/mL of aqueous extract of *E. caudata.* * and ** indicates significant variations at $P<0.05$ and $P<0.01$, respectively

EC-AE treatment induces rise in intracellular oxidant level, the level of lipid peroxidation (LPO) as a biomarker of oxidative stress was assessed. Treatment of A549 cells with 50 µg/mL of EC-AE resulted in reduction of lipid peroxidation (Fig. 9D).

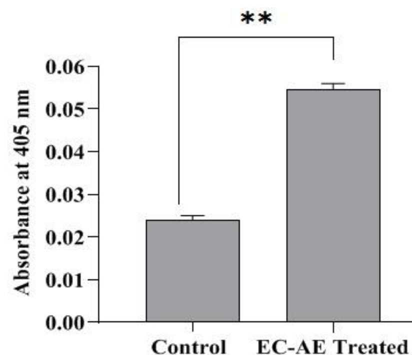

Fig. 11 — Effects of the aqueous extract of *E. caudata* on caspase-6 activity in A549 cells after 48 h treatment. Control: A549 cells without treatment; EC-AE treated: A549 cells treated with 50 µg/mL of aqueous extract of *E. caudata.* **$P<0.01$ compared with the untreated control

Induction of DNA strand breaks by EC-AE

DNA damaging effect of EC-AE in A549 cells was assessed by employing the alkaline comet assay. Treatment of A549 cells with 50 µg/mL of EC-AE induced significant DNA damage as indicated by increased tail length and olive moment in EC-AE treated group when compared to the untreated control (Fig. 10A-C).

Activation of caspase-6 by EC-AE on A549 cells

Caspase-6 plays an important role in execution of apoptosis in cancer cells. Effect of EC-AE on A549 cell apoptosis was assessed by caspase-6 activity. Treatment of A549 cells with 50 µg/mL of EC-AE for 48 h resulted in 2.3 folds increase in the activity of caspase-6 when compared to the untreated control (Fig. 11).

Discussion

Free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in our body by various endogenous systems. A balance between free radicals and antioxidants is necessary for maintaining proper physiological functions of the body[34]. Excessive generation of these radicals leads to a condition called 'oxidative stress'. Free radicals thus adversely alter various biomolecules including lipids, proteins and DNA triggering certain human diseases[2]. Hence application of external sources of antioxidants can assist in coping the oxidative stress. Phytochemicals are biologically active, naturally occurring compounds found in plants, providing certain health benefits for humans more than those attributed to macronutrients and micronutrients[35].The present study revealed the presence of various naturally occurring compounds

such as alkaloids, cardiac glycosides, saponins, tannins, terpenoids, phlobatannins and resins. These phytochemicals belong to polyphenolic compounds and have been reported to possess numerous pharmacological values including anti-malarial[36], anti-inflammatory[37], anti-ulcer and antimicrobial activities[38].

Quantitative phytochemical analysis also revealed the presence of significant amounts of phenolic and flavonoid compounds in *E. caudata* extracts. Phenolic compounds have been reported to show antioxidant activity by scavenging or stabilizing free radicals due to their conjugated ring structures and presence of hydroxyl groups[39]. Phenolic compounds have also been reported to exhibit antiallergenic, antimicrobial, anti-artherogenic, antithrombotic, anti-inflammatory, vasodilatory and cardioprotective effects[40]. Similarly, flavonoids are also reported to have antioxidative action through scavenging or chelating processes[41]. In addition, flavonoids have been reported to serve as health-promoting compound and protect humans against several diseases like atherogenesis, carcinogenesis, hepatotoxicity, and thrombosis[42].

The reduction of methanolic DPPH solution to non-radical form DPPH-H has been widely used to evaluate antioxidative property of certain compounds and plant extracts. *E. caudata* extracts effectively reduced the stable DPPH radical to a yellow-colored diphenyl-picrylhydrazine, probably due to presence of certain active compounds that are capable of donating hydrogen to a free radical in order to remove odd electron. Cysteine, glutathione, ascorbic acid, tocopherol, poly-hydroxyl aromatic compounds have been reported to reduce DPPH due to their hydrogen donating ability[43]. The anti-oxidative property of *E. caudata* extracts was also determined by measuring their ability to convert the blue colored $ABTS^{\bullet+}$, which is formed by the interaction of ABTS and potassium ferricyanide, to ABTS. The effectiveness of this conversion depends on the molecular weight of phenolic compounds, the number of aromatic rings and nature of hydroxyl group's substitution than the specific functional groups[44]. The study demonstrates aqueous extract of *E. caudata* as a potent antioxidant and their $ABTS^{\bullet+}$scavenging activity could be due to their high phenolic contents. Interestingly, scavenging activity of EC-AE was not significantly differed from the standard ascorbic acid.

Superoxide ($O_2^{\bullet-}$) radical produced as a result of incomplete metabolism of oxygen serve as a precursor of more reactive oxygen species, contributing to the tissue damage and various diseases. Superoxide can decompose to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, which are very harmful to the cellular components and initiate lipid peroxidation[45]. Thus, neutralization of superoxide radical is necessary to inhibit the chain of ROS generation and protect the cells from oxidative stress. It has also been reported that anti-oxidant properties of some flavonoids are effective mainly through scavenging of superoxide anion radical[46]. Thus, the presence of significant amounts of flavonoids in *E. caudata* extracts might be responsible for their scavenging activity against superoxide radical. The reducing power of a compound may serve as a significant indicator of its potential anti-oxidant activity. However, the activity of anti-oxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging ability[47]. The dose dependent increase in the reducing power of the *E. caudata* extracts also suggested their potent antioxidant activity.

Haemolysis occurs due to membrane damage caused by the activity of free radicals on erythrocytes; the major target of free radicals[48]. Lipid peroxidation generates certain degradation products such as malondialdehyde (MDA) that are considered to be an important cause of cell membrane destruction and cell damage[26]. In the present study, lipid peroxidation in mice liver homogenate was induced by $FeCl_2$-H_2O_2. Formation of MDA is used as an indicator of lipid peroxidation, and subsequently oxidative stress. *E. caudata* extracts showed significant anti-haemolytic activity and lipid peroxidation inhibition potential which could be due to the presence of numerous amounts of phenols and flavonoids. Certain phenolic compounds have been reported to participate in the cell membrane, hindering the diffusion of free radicals and consequently decreased the chain reaction of free radicals[49]. Flavonoids have also been reported to inhibit lipid peroxidation in the erythrocytes membrane and improved their integrity against lyses by binding to the membrane[50].

To enhance the effectiveness of cancer treatment, recent studies have been focused on drugs that have been used in traditional medicine[51]. Despite the availability of numerous reports on the medicinal benefit of *E. caudata*, investigations that look into the

scope of *E. caudata* for cancer therapy is still limited. Therefore, this study was undertaken to examine growth inhibitory and cytotoxic effects of *E. caudata* in human lung adenocarcinoma A549 cells. The goal of targeting cell proliferation in cancer is to induce cell death or arrest the cell cycle using cytotoxic compounds. MTT assay is a rapid and standard method for testing cytotoxicity of drugs in various cultured cells wherein reduction of MTT can occur only in metabolically active cells. Treatment of A549 cells with aqueous extract of *E. caudata* resulted in a dose dependent increased in cytotoxicity (Fig. 7).

Knowing the precise mechanisms by which anticancer agent exerted their actions has become an important approach for the evaluation and development of anticancer drug. Apoptosis is an essential and highly regulated cell death mechanism that serves to eliminate ailing cells without causing injury to the normal cells, and loss of its regulation underlies numerous pathologies including cancer[52]. Any compound that induces apoptosis is considered to be a promising cancer chemotherapeutic treatment[53]. To investigate whether EC-AE-induced inhibition of A549 cell growth is *via* apoptosis, AO/EtBr dual staining was used to identify and quantify the apoptotic morphology. A549 cells treated with 50 µg/mL of EC-AE exhibited characteristic apoptotic morphology with brightly orange red and condensed nuclei compared to the untreated control cells which showed round and intact green nucleus representing the live cells (Fig. 8A). Several plant derived anti-cancer drug have been reported to show similar effects in various cancer types[54-57].

Maintenance of redox balance is important as excessive ROS production have been associated with pathophysiology of various diseases including cancer[58]. Many cancer cells are shown to have higher ROS levels as compared to their normal counterpart due to enhanced metabolism and mitochondrial dysfunction[59]. Since high ROS ($O_2^{\cdot-}$, H_2O_2 and $^{\cdot}OH$) levels have close association with tumor initiation, angiogenesis, cell invasion, metastasis and chemoresistance in different cancer models[60]; use of antioxidants or agents that enhance antioxidant system may provide an opportunity to reduce intracellular ROS-mediated tumorigenesis and cancer progression. In fact, natural products including plant extract have demonstrated antioxidant efficacy such as sesamol, curcumin, ascorbic acid, vitamin E and melatonin for cancer treatment both *in vitro* and *in vivo*[60]. Consistently, in the present study, augmentation of GSH level and activities of GST and SOD, and decreased lipid peroxidation as evidenced by the significant decrease in MDA levels after EC-AE treatment clearly demonstrates its antioxidant nature which may be responsible for its anti-cancer activity in A549 cell. Over-expression of antioxidant enzymes such as SOD1, SOD2 SOD3, GPx3 and Prx6 have been reported to induce cell death, decrease survival time and suppressed metastasis in various cancer cells[60]. Plants such as *Hypericum hookerianum*[61], *Cyathula prostrata*[62] and *Cocculus hirsutus*[63] have been reported to possess anti-cancer activities *via* elevation of antioxidant defence system and reduction of lipid peroxidation.

DNA damage in response to anti-cancer agents is an important factor in anti-cancer therapy. To check for DNA strand breaks as a result of EC-AE treatment, single-cell gel electrophoresis assay (Comet assay) was performed. Our results showed that EC-AE possessed DNA-damaging effect as evident from the increased comet tail length and olive moment in A549 cells treated with EC-AE (Fig. 10). Several plant-derived anti-cancer drugs have also been reported to show similar effects in various cancer types[55,64].

Caspase-6 is one of the executor caspase that plays a central role in the execution of both the intrinsic (the mitochondrial mediated) and extrinsic (the death receptor mediated) apoptotic pathways by cleaving several key proteins including intra-nuclear proteins. This cleavage mediates disassembly of the cell into the apoptotic morphological changes including cell shrinkage, chromatin condensation, and nuclear fragmentation[52]. Thus, activation of caspase-6 is a strong biomarker for cells undergoing apoptosis. Intense up-regulation of caspase-6 has been demonstrated to be an important phenomenon associated with lung carcinoma cells undergoing apoptosis[65]. Increased caspase-6 activity in A549 cells following EC-AE treatment strongly indicated that EC-AE-induced apoptosis was executed through a caspase-dependent pathway.

Conclusion

Our study revealed the presence of significant amounts of phenolic and flavonoid compounds in *E. caudata* extracts. This study also demonstrates that *E. caudata* extracts possessed anti-haemolytic and inhibitory action against lipid peroxidation. *E. caudata* extracts was also found to exhibit a concentration dependent inhibition of free radicals

such as DPPH, ABTS and superoxide along with ferric reducing power which might be due to presence of significant amounts of phenols and flavonoids. Our study also demonstrates a novel finding showing the role of the aqueous extract of *E. caudata* as a potential anti-cancer agent. Insights into the detailed mechanism of action exerted by *E. caudata* in free radical scavenging and anti-cancer activities however remain to be explored.

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Conflicts of interest

All authors declare no conflict of interest.

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