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Antioxidant and immunomodulatory activities of the ethanolic extract of *Eugenia jambolana* Lam. root bark assessed using *in vitro* and *in vivo* methods

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ABSTRACT

Introduction: Natural products are a rich source of antioxidants, effective on immunological disorders. The present study was planned to evaluate the antioxidant activity of ethanolic extract of root bark of an Indian medicinal plant, *Eugenia jambolana, by in vitro* methods and immunomodulatory activity by *in vivo* methods.
Methods: The ethanolic extract of the collected root bark of *E. jambolana* (EJE) was prepared and tested *in vitro* for antioxidant activity using concentrations 10-50 µg/mL through 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and superoxide anion scavenging assays. Further, the immunomodulatory effects of EJE were assessed in animal studies against the experimental antigen, sheep RBC, through oral administration at doses of 100, 200, and 400 mg/kg, measuring its impact on antibody titer values in the antigen-antibody reaction and on paw thickness in mice (delayed-type hypersensitivity [DTH] study) to evaluate humoral and cell-mediated immune responses, respectively.
Results: EJE exhibited scavenging properties against DPPH free radicals and superoxide anion with IC_{50} values of 43.79 ± 0.24 µg/mL and 47.64 ± 0.18 µg/mL in the respective studies. EJE at 400 mg/kg oral dose demonstrated a significant ($P<0.05$) antibody titer value and DTH response in the *in vivo* immunomodulatory activity studies. These studies showed that the 400 mg/kg oral dose of EJE produced 29.36% and 39.06% immunomodulatory effects in humoral and cell-mediated immunity studies, respectively.
Conclusion: The experiments of this study demonstrated the antioxidant and immunomodulatory potential of the ethanolic extract of *E. jambolana* root bark.

Implication for health policy/practice/research/medical education:
Eugenia jambolana root bark's ethanolic extract showed free radical scavenging potentials in the *in vitro* experiments and immunomodulatory activity in the *in vivo* experiments of humoral and cell-mediated immunity studies. Hence, the root bark was identified as a source of natural antioxidants and may serve as an immunomodulator for the treatment of any immunological diseases. However, further studies are required to elucidate its mechanism of action and confirm its potential as a future therapeutic drug.

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Introduction

The approach of using natural products as medicine is pluralistic and ancient. Natural products have shown their therapeutic efficiency for various diseases. The wide range of efficacy of natural products is attributed to the presence of a broad array of chemical constituents. The presence of these constituents is not only responsible for their biological activities but also confirms their safety profile (1). Over the years, the potential of natural products has been utilized to develop medicines. These natural products, especially plant-based medicines are often found to be widely used as antioxidants (2).

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Antioxidants are agents used to reduce the excessive amounts of free radicals produced in the human body (3). The production of free radicals is a natural biological process. Normally, free radicals are utilized by the immune system to either kill the invading microorganisms or clear damaged cells at the site of tissue injury (4). However, during infection, diabetes, or any such chronic disease, the overproduction of free radicals surpasses the body's antioxidant defense. The oxidative stress causes uncontrolled tissue damage, which exacerbates the disease condition. Additionally, due to excess free radicals immunological homeostasis is also disrupted and that may cause the development of immunologically compromised diseases, like HIV/AIDS, rheumatoid arthritis, cancer, autoimmune disease, and inflammatory bowel diseases (5). Antioxidant agents control oxidative stress in such conditions and are found to be effective in modulating the immune system to maintain the body's defense mechanism (6).

The immune system is a defense paradigm of the human body to maintain health and prevent infection. There are non-specific and antigen-specific, two interdependent immune mechanisms. Innate immunity, the non-specific immune system, is activated during any pathogenic invasion or injury. Whereas, adaptive immunity is activated by the innate immune system to respond to any antigens by generating antibodies (Humoral immune system) and/or cell-mediated (Delayed type hypersensitivity) response (7,8). During the presence of antigens, the humoral immune systems respond immediately by producing antibodies to neutralize the antigens. In contrast, the cell-mediated response is observed to be late but assists the immunological cells in responding to specific antigens for a longer duration (9,10). In healthy conditions, the immunological mechanisms help the body to respond effectively to diseases. Nevertheless, in chronic disease states the defense mechanism fails to function properly, and in such conditions, immunomodulators help to modulate the immune system to restore homeostasis (11). Immunomodulators are agents that can modify the immune response, either by stimulating it, as immunostimulators, or by suppressing it, as immunosuppressants. The effectiveness of immunomodulatory drugs in treating numerous chronic immunological disorders has been well recognized, as they restore the immune system, which in turn supports and facilitates a synergistic approach in the recovery process (12). Several synthetic drugs are available and despite their effective immunomodulating effects, using these drugs is limited due to their associated side effects (13). Alternatively, natural immunomodulators have proved their potential and have also been found to be safe to use (14). Over the years, the therapeutic potential and safety profile of natural compounds have motivated scientists worldwide to research and identify novel natural immunomodulatory agents. In this regard, this current research work was structured to determine the immunomodulatory potential of the root bark of an Indian medicinal plant, *Eugenia jambolana* Lam. Furthermore, earlier research has suggested that antioxidant activity may be a possible mechanism for the immunomodulatory effects of drugs (15). Hence, this study also aims to assess the antioxidant potential of the plant drug.

Eugenia jambolana, of the Myrtaceae family, is commonly known as jamun, Indian blackberry, black plum, and Java plum. The phytochemical analysis studies of the different parts of this plant reported the presence of diverse groups of phytoconstituents, including flavonoids, tannins, triterpenoids, glycosides, volatile oils, and alkaloids (16,17). In India, the plant traditionally has been used for anti-inflammatory, antidiabetic, analgesic, diuretic, and hepato-protective properties. Previous research works reported the anti-inflammatory, analgesic, antidiabetic, and antineoplastic properties of different parts of this plant (16,18,19). However, no research works have been reported so far on the antioxidant and immunomodulatory activities of the root bark of *E. jambolana*. Hence, the present work was intended to assess the antioxidant potential of ethanolic extract of root bark of *E. jambolana* by *in vitro* studies and the humoral and cell-mediated immunomodulatory activities by *in vivo* studies.

Materials and Methods

Reagents and chemicals

Chemicals and reagents of analytical grade were used in this research. Ascorbic acid was procured from Himedia Laboratories Pvt. Ltd., Mumbai. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) of Loba Chemie (Mumbai) company was collected from a local supplier. Dicaris, the Levamisole tablets as a standard immunostimulating drug, was procured from Encore Healthcare Pvt. Ltd., Paithan. Instruments like, the UV-Vis spectrophotometer (Shimadzu UV-1800), and the Rotary vacuum evaporator (Remi, India) were used for the study.

Collection and authentication of plant material

The root bark was collected from the wildly grown *E. jambolana* plant in Mangaluru, Karnataka, India (Latitude: 12°55'2" N and Longitude: 74°51'21.7" E). The identification and authentication of this plant were carried out at the Department of Botany of St. Agnes College, Mangaluru. A voucher specimen (625e) was submitted to the institution.

Preparation of the extract of plant material

The collected plant materials were shade-dried and coarsely powdered. The powdered plant material was extracted with 95% ethanol by maceration method. The ethanolic extract was then concentrated by a rotary flash evaporator, and dried fully by heating on a water bath. The

dried extract of *E. jambolana* root bark (EJE) was used further in the pharmacological studies to evaluate their medicinal properties.

Antioxidant activity study

DPPH free radical scavenging potential

The experiment was used to evaluate the antioxidant activity of EJE (20). The DPPH stock solution of 0.2mM concentration was prepared using methanol. Ascorbic acid, the standard drug, and EJE were dissolved in methanol to prepare a series of concentrations of 10-50 µg/mL. An amount of 100 µL of DPPH from the prepared stock solution was taken out and mixed with each concentration of test and standard drugs. The mixture solution was stored in a shady place for 30 minutes to allow the reaction. The absorbance was measured for all mixture samples at 517 nm. The following formula was used to calculate the antioxidant activity in percentage for the test and standard drugs, which represented the potential of samples of scavenging free radicals.

$$Percentage\ of\ activity = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

Where Abs= Absorbance.

Superoxide anion scavenging activity

The experiment was used to evaluate the antioxidant activity of EJE by scavenging the superoxide anion, which was produced by the reaction of test reagents, nitro blue tetrazolium (NBT), nicotinamide adenine dinucleotide coenzyme (NADH), and phenazine methosulphate (PMS) (21). In the beginning, a solution of each test reagent was prepared by adding NBT (79µM), NADH (93µM), and PMS (24µM) in a phosphate buffer (100mM) solution of pH 7.4, individually. The reaction mixture was prepared by adding solutions of EJE and standard drugs (Ascorbic acid) of the concentration 10-50 µg/mL and 1 mL of each test reagent. Then, the mixture was incubated for 5 minutes at optimal temperature and measured the absorbance at 560 nm. The percentage of the activity of EJE was calculated using the above-mentioned formula.

Animals

In vivo experiments were carried out by using both gender variants, male and female Swiss albino mice of 4-5 weeks old with 15-20 g of weight. After procurement, all these animals were divided into groups and kept in different cages. These animals were maintained with a 12-hour light-dark cycle and a temperature of 24 ± 2 °C. The standard diet pellets (Hindustan Lever Co., Bombay, India) and water were provided and allowed animals to take as per their desire. The institutional animal ethics committee guidelines were followed in experiments with animals.

Selection of dose by acute toxicity study

Guideline no. 425 of the Organization for Economic Cooperation and Development (OECD) was followed to carry out the toxicity study of EJE using female Swiss albino mice (22). The test drug showed the safety of using up to a high dose during the study. Doses for EJE were selected based on this study, and depending on the body weight of each animal, 100, 200, and 400 mg/kg for the immunomodulatory activity studies.

Immunomodulatory activity studies

The following humoral and cell-mediated immunity experiments in mice were used to assess the immunomodulatory activity of EJE.

Experimental antigen

The sheep red blood cell (SRBC) was used as an antigen in the humoral and cell-mediated immunity studies. The collected SRBC was centrifuged and diluted with phosphate buffer saline (PBS of pH 7.4) to make the final concentration of 5 × 10^9 cells/mL to use as an antigen in both immunomodulatory studies.

Grouping of animals

During the immunomodulatory studies, Swiss albino mice were divided into five groups and six animals for each group (n=6) as follows:

- Control: Animals were immunized with antigens and administered 1 mL vehicle, 2% Carboxy methyl cellulose (CMC) solution.
- Standard: Animals were immunized with antigens and treated per day with the administration of 2.5 mg/kg dose of standard drug (Levamisole) as a suspension in the vehicle.
- EJE 100, 200, and 400 mg/kg: Animals of all these three groups were immunized with antigens and treated per day with the administration of the suspension of EJE in the vehicle with 100, 200, and 400 mg/kg doses, respectively.

Humoral study

The humoral immunity of the test drug was assessed by a haemagglutination reaction (antigen-antibody titer) study model by immunizing all animals with SRBC (5 × 10^9 cells/mL) (23). The 0.2 mL antigen was injected intraperitoneally (i.p) in all animals on day 0. Animals of all groups were treated orally with respective drugs, as per the group divisions, from day 1 to day 14. On the 15th day, the blood was collected by puncturing the retro-orbital sinus of animals with the purpose of checking the haemagglutination reaction to ascertain humoral immune responses. The serum was separated by centrifugation of collected blood samples at 2000 rpm and then mixed with phosphate-buffered saline (PBS) to obtain a stock solution of serum samples. These stock solutions of serum

samples were used to carry out the antigen-antibody titer to study the humoral immune response. In the study, 25 µL serum was placed in the first well of 96-well microtitre plates (round bottom), and in the following wells two-fold serial dilutions of the serum samples were added with PBS. Further, 25 µL of SRBC (5×10^9 cells/mL) was added to all wells, and plates were allowed for incubation (at 37 °C) for an hour to initiate the antigen-antibody reaction. The reaction of antigen-antibody that was observed in the well with the lowest dilution was considered and noted as a titer value for each group of this study. The percentage activity of immunomodulation for the groups treated with drugs (standard drug and EJE) was calculated by comparing their titer values with the control group.

Delayed type hypersensitivity (DTH) study

The cell-mediated immune response of EJE was assessed during the study by the DTH experiment (24). During the study, on day 0, the antigen (5×10^9 cells/mL) was administered by subcutaneous injection in the left hind paw of mice, and saline to the right hind paw. Animals of the individual group received treatment with respective drugs, as per the group divisions, for 14 days. On day 15, all animals were challenged by injecting antigens in the respective footpads of animals and kept under observation for 24 hours. The swelling or thickness of the paw was measured using digital vernier calipers and the percentage activity of immunomodulation for the groups treated with drugs (standard drug and EJE) was calculated by comparing the results of the control group.

Statistical analysis

The results were presented here as mean ± standard error of mean (SEM). The statistical significance was analyzed for the results of the groups treated with drugs (standard drug and EJE) by comparing with the control group of immunomodulatory activity studies using ANOVA followed by Dunnet's *t*-test and their results with *P* value <0.05 were considered significant.

Results

Antioxidant activity study

DPPH free radical scavenging potential

The percentage of DPPH free radicals scavenging potential of the plant extract (EJE) increased with the concentration. EJE showed the most potent effect at the concentration of 50 µg/mL. Ascorbic acid (standard drug) also showed the most potent free radical scavenging effect at the concentration of 50 µg/mL among all the concentrations. The IC_{50} values of EJE and the standard drug were found to be 43.79 ± 0.24 µg/mL and 17.43 ± 0.22 µg/mL, respectively (Table 1).

Superoxide anion scavenging activity

The result of the study showed that the superoxide anion scavenging potential of EJE increased with the concentration. EJE showed the most potent superoxide scavenging effect at the concentration of 50 µg/mL. Ascorbic acid (standard drug) showed the most potent superoxide scavenging effect at the concentration of 50 µg/mL among all the concentrations. The IC_{50} values of EJE and the standard drug were found to be 47.64 ± 0.18 µg/mL and 21.45 ± 0.27 µg/mL, respectively (Table 1).

Humoral study

In this study, the effect of EJE on antigen-antibody reaction (haemagglutination reaction) was considered as immunomodulatory activity. The effect of the standard drug and three different oral doses of EJE on antibody titer, along with their respective percentages of immunomodulatory activity, are presented in Table 2. The study suggested that the immunomodulatory effect of EJE increased with its doses. EJE with 400 mg/kg dose showed the most potent and statistically significant ($P < 0.05$) antibody titer value among all its doses. A significant ($P < 0.01$) antibody titer value was observed for the group that received the standard drug.

Delayed type hypersensitivity study

In this study, the effect of EJE on the paw thickness of mice (cell-mediated immune response) was considered as immunomodulatory activity. The effect of the standard drug and three different oral doses of EJE on paw thickness as a measure of DTH responses, along with their respective percentages of immunomodulatory activity, are presented in Table 3. This study also suggested that the immunomodulatory activity of the EJE increased with its doses. EJE with 400 mg/kg dose showed the most potent and statistically significant ($P < 0.05$) effect on the paw thickness of animals among all its doses. A significant

Table 1. The IC_{50} values of the standard drug and ethanolic extract of *Eugenia jambolana* root bark (EJE) in the assays of scavenging DPPH free radical and superoxide anion for the evaluation of antioxidant activity

Groups	IC_{50} values (µg/mL)	
	DPPH free radical scavenging assay	Superoxide anion scavenging assay
Standard drug	17.43 ± 0.22	21.45 ± 0.27
EJE	43.79 ± 0.24	47.64 ± 0.18

Data are presented here as mean ± SEM by carrying out experiments in triplicate. A standard drug (Ascorbic acid) and EJE were used in both assays with 10-50 µg/mL concentration. The linear regression was used to analyze the IC_{50} values (µg/mL). Standard drug: Levamisole (2.5 mg/kg).

Table 2. Immunomodulatory activity of ethanolic extract of *Eugenia jambolana* root bark (EJE) by an *in vivo* model for humoral immune response study

Groups	Antibody titer (mean ± SEM)	Immunomodulatory activity (%)
Control	4.36 ± 0.18	NA
Standard	6.21 ± 0.29	42.43**
EJE 100 mg/kg	4.89 ± 0.35	12.16
EJE 200 mg/kg	5.19 ± 0.47	19.04
EJE 400 mg/kg	5.64 ± 0.22	29.36*

Data are presented here as mean ± SEM (n = 6). * indicates $P < 0.05$ and ** $P < 0.01$ compared to the control group (ANOVA followed by Dunnett's t-test). Standard: Levamisole (2.5 mg/kg).

Table 3. Immunomodulatory activity of ethanolic extract of *Eugenia jambolana* root bark (EJE) by an *in vivo* model for cell-mediated immune response study

Groups	Paw thickness (mm)	Immunomodulatory activity (%)
Control	0.64 ± 0.05	NA
Standard	1.16 ± 0.07	81.25**
EJE 100 mg/kg	0.71 ± 0.03	11.00
EJE 200 mg/kg	0.78 ± 0.07	21.88
EJE 400 mg/kg	0.89 ± 0.06	39.06*

Data are presented here as mean ± SEM (n = 6). * indicates $P < 0.05$ and ** $P < 0.01$ compared to the control group (ANOVA followed by Dunnett's t-test). Standard: Levamisole (2.5 mg/kg).

($P < 0.01$) effect on the paw thickness of animals was observed for the group receiving the standard drug.

The percentage of immunomodulatory effects of the standard drug and the three different oral doses of EJE in both humoral and cell-mediated immune response studies are presented in Figure 1.

Discussion

Free radicals (Reactive oxygen species/reactive nitrogen species) are highly reactive chemical molecules due to the presence of unpaired electrons. These free radicals are generated during cellular activities and accumulate in cells that participate in regular biomolecular reactions (25). Under the influence of abnormal conditions, excess oxidants are produced. The excessive production of oxidants, combined with their highly reactive characteristics, causes uncontrolled cellular or biomolecular damage, which leads to the development of diseases (26).

Antioxidants are a natural mechanism of the human body that neutralizes and removes reactive molecules. In the oxidative stress condition, this natural process fails to functionalize properly and that leads to the disruption of the redox homeostasis. An antioxidant agent can help overcome these conditions (27). Natural products are found to be rich sources of antioxidants. In the present study, the antioxidant activity of the ethanolic extract of the root bark of *E. jambolana* (EJE) was evaluated by the *in vitro* method, DPPH free radical, and superoxide anion scavenging assays.

DPPH produces a violet color in the reaction solution. The antioxidant agents decolorize the solution, which is their free radicle scavenging property (28). In this study, EJE exhibited antioxidant properties with an IC_{50} value of 43.79 ± 0.24 µg/mL (Table 1). In the study, a potent effect was displayed by the standard drug (Ascorbic acid).

The superoxide scavenging assay corroborated the antioxidant potential of EJE. In the *in vitro* study, the superoxide anion is produced by the reaction of phenazine methosulphate and the coenzyme nicotinamide adenine dinucleotide. This superoxide anion production is

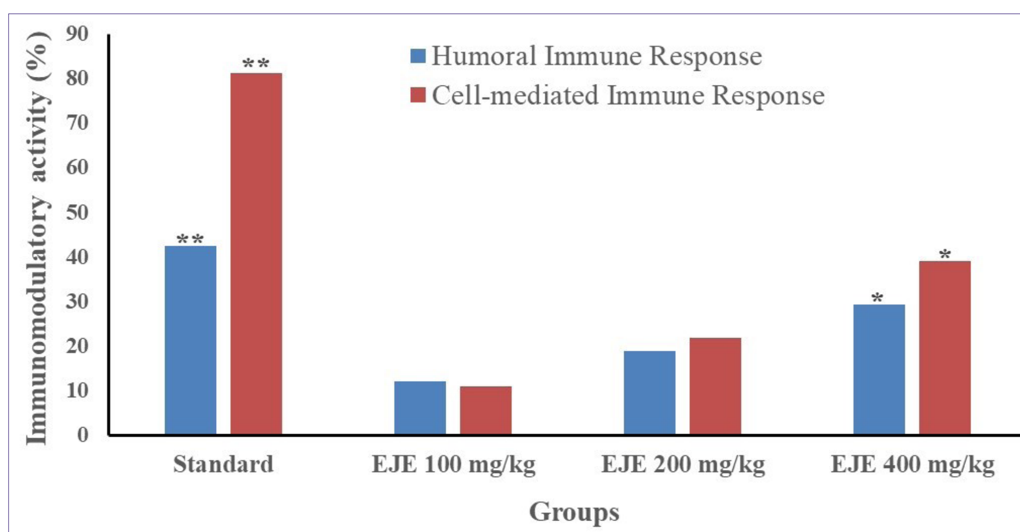

Figure 1. The percentage of immunomodulatory activity of ethanolic extract of *Eugenia jambolana* root bark (EJE) by *in vivo* models for humoral (Haemagglutination reaction) and cell-mediated (Delayed-type hypersensitivity) immune response study. Data are presented here as mean ± SEM (n=6). *indicates $P < 0.05$ and ** $P < 0.01$ compared to the control group (ANOVA followed by Dunnett's t-test). Standard: Levamisole (2.5 mg/kg).

indicated by a color change of the reaction mixture to blue, due to the formation of formazan through reducing nitro blue tetrazolium reagent. The antioxidant activity of a chemical compound is assessed based on its potential to inhibit the formation of blue color in the reaction mixture (29). In this study, the plant extract (EJE) showed the superoxide anion scavenging potential with an IC_{50} value of 47.64 ± 0.18 μg/mL (Table 1). The study proved the antioxidant activity of EJE. Hence, both studies confirmed the antioxidant potential of the root bark of *E. jambolana*. Moreover, the potential of an antioxidant drug to scavenge superoxide anions may mimic the activity of the natural antioxidant enzyme superoxide dismutase, which is present in the human body (30). Consequently, the study confirmed that EJE could imitate the effect of this natural antioxidant enzyme to scavenge the excess free radicals produced in the body during disease conditions, thereby helping to treat the associated diseases (5,31).

The experiments of humoral cell-mediated immunity were conducted to determine the immunomodulatory activity of EJE. The immune system responds to antigens by producing antibodies; the process is known as humoral immunity. The role of immunological cells in antigen-specific defense mechanisms is immense. The B lymphocyte cells produce antibodies for humoral immune response (32). Whereas, the T lymphocyte cells are found to be responsible for cell-mediated response. The response of the T-cells to any pathogens or infections is done by cytotoxic effect or by releasing cytokines to achieve the desired immunological effects. Due to these versatile approaches, cell-mediated immunity is essential for the treatment of various chronic diseases such as cancer, autoimmune diseases, rheumatoid arthritis, and neurodegenerative diseases (33). However, the body's natural defense mechanism fails to respond to any disease due to an imbalance in immune homeostasis, which can be restored by using immunomodulating agents. Immunomodulators can regulate the immune system by stimulating or suppressing it to re-establish homeostasis (34).

The present study was carried out to evaluate the effect of EJE on the adaptive immune response by introducing sheep RBC as an antigen in animals. In the study, the potential of the plant extract to enhance humoral immunity in the presence of experimental antigens (sheep RBC), as part of the adaptive immune response, was assessed based on its effect on increasing antibody production, as reflected in the antigen-antibody reaction. The immunomodulatory effect of EJE in the study was found to be directly proportional to its doses. The 400 mg/kg dose of EJE produced the highest and most significant ($P < 0.05$) hemagglutination value (antibody titer) among all its doses tested.

The immunomodulatory effect of EJE, as observed in the humoral study results, was further supported by its response in the DTH study, which was considered as a cell-mediated immunity of the drug. The results of humoral and cell-mediated immunity studies confirmed the immunomodulatory potential of the ethanolic extract of *E. jambolana* root bark.

Altogether, the findings of the present studies on free radical scavenging potential and immunomodulatory effects of the plant extract provided an understanding of its previously reported therapeutic potentials related to inflammation, arthritis, cancer, tumors, and diabetes (17). Nevertheless, the results of the present research work are primary responses and further detailed research needs to be performed to confirm the therapeutic properties and also to understand their mechanism of action.

Conclusion

In this study, the plant material showed antioxidant potential by scavenging DPPH free radicals and superoxide in *in vitro* methods. Furthermore, the test drugs showed significant immunomodulatory effects in *in vivo* studies on humoral immunity and DTH. The findings confirmed the proposed therapeutic activities of the plant extract, and that supported the hypothesis of this present study. The outcome of the study provides preliminary responses about the biological activities of EJE. Moreover, in the future, thorough studies need to be conducted to ascertain the mechanism of the biological activities of this plant extract and also to identify the therapeutically active phytoconstituents.

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Authors' contribution

Conceptualization: Santanu Saha, EVS Subrahmanyam.
Data curation: Santanu Saha.
Formal analysis: Santanu Saha.
Investigation: Santanu Saha.
Methodology: Santanu Saha.
Software: Santanu Saha.
Writing–original draft: Santanu Saha, EVS Subrahmanyam.
Writing–review & editing: Santanu Saha, EVS Subrahmanyam.

Conflict of interests

The authors of the present study announce that there is no conflict of interest to declare.

Ethical considerations

All experiments using animals in this study were carried out by following the guidelines and instructions of the institutional animal ethics committee (AEC). Permission was procured from the committee (AEC) to carry out the experiments with animals and the registration number was KSHEMA / AEC/077/2008.

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