Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
23(4): 651-661, Jul./Aug. 2013

Evaluation of antinociceptive and anti-inflammatory activities of extract and fractions of *Eugenia jambolana* root bark and isolation of phytoconstituents

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Abstract: *Eugenia jambolana* Lam., Myrtaceae, is a widely distributed and traditionally well known plant in India. The root bark of the plant was extracted with ethanol and then successively fractionated into petroleum ether fraction, chloroform fraction, *n*-butanol fraction and methanol fraction. The extract and fractions of the plant material were evaluated for the antinociceptive activity by acetic acid-induced writhing test and formalin-induced nociception test, and anti-inflammatory activity was screened by carrageenan-induced rat paw edema, cotton pellet induced granuloma formation and adjuvant induced arthritis in rat models. The test materials showed the antinociceptive and anti-inflammatory effect in dose dependent manner and the petroleum ether fraction was found to be most potent among the test materials. At 400 mg/kg b.w., *p.o*. dose petroleum ether fraction significantly inhibited 54.28% writhing response and 73.77% formalin induced nociception in mice. The fraction with same dose showed significant 79.31% inhibition of carrageenan-induced rat paw edema, 57.78% anti-proliferative effect and 77.93% inhibition of adjuvant induced arthritis. The bioactive petroleum ether fraction was then subjected to column chromatography which led to isolate three compounds, namely, β-sitosterol, stigmasterol and lupeol. These compounds were characterized and identified by IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopy.

Article

Received 7 Jun 2013
Accepted 24 Jul 2013
Available online 20 Aug 2013

Keywords:
adjuvant induced arthritis
anti-inflammatory
antinociceptive
formalin induced nociception
Eugenia jambolana
Myrtaceae

ISSN 0102-695X
DOI: 10.1590/S0102-695X2013005000055

Introduction

Eugenia jambolana Lam., Myrtaceae, is a known Indian medicinal plant for traditional uses to treat different ailments. This large evergreen tree is distributed through out India and commonly known as 'Jamun'. Root bark of the plant is pale brown at inner surface and from outside dark brown. Ayurveda and Unani system of medication have mentioned variety of therapeutic properties of the plant like astringent, carminative, diuretic, antidiabetic, anthelmintic, antibacterial, analgesic, anti-inflammatory, antioxidant, as well as gastro protective agents (Kirtikar & Basu, 2006). Several phytoconstituents belonging to category alkaloids, glycosides, flavonoids and volatile oil has been reported from different parts of plant. Betulinic acid, ß-sitosterol, friedelin, *epi*-friedelanol and fatty acid ester of epi-friedelanol (eugenin) has been isolated from stem bark of *E. jambolana* (Sengupta & Das, 1965). Quercetin, myricetin, myricitrin, myricetin 3-*O*-(4"-acetyl)-α-L-rhamnopyranoside and two acylated flavonol glycosides 3-*O*-(4"-*O*-acetyl)-α-L-rhamnopyranoside of mearnsetin (myricetin 4'-methyl ether) and myricetin 3-*O*-(4"-*O*-acetyl-2"-*O*-galloyl)-α-L-rhamnopyranoside were isolated from leaves of *E. jambolana*, in different studies (Mahmoud et al., 2001; Timbola et al., 2002). The plant is used in treatment of fevers, indigestion, diabetes urine related troubles, loss of appetite, loose stools and vomiting. The anti-diarrheal, gastroprotective, antidiabetic, hypoglycemic and nitric oxide scavenging effects by

different parts of this plant have also been reported earlier (Ratsimamanga et al., 1972; Mukherjee et al., 1998; Jagetia & Balinga, 2004; Sharma et al., 2006; Chaturvedi et al., 2007). Traditionally, barks of this plant are used for treatment of inflammation (Nadkarni, 2009). Earlier studies reported the anti-inflammatory activity by stem bark of *Eugenia jambolana* (Muruganandan et al., 2001). As per our knowledge, there are no scientific evidences available on antinociceptive and anti-inflammatory activity of root bark. Hence, in this study we have evaluated the antinociceptive and anti-inflammatory activity of ethanolic extract and fractions of root bark of *Eugenia jambolana* and isolated phytoconstituents from active fraction.

Materials and Methods

General methods

All the melting points were recorded in a Toshniwal melting point apparatus and were uncorrected. IR spectra of the compounds were recorded using the KBr pellet method on a Perkin Elmer 700 IR spectrophotometer. NMR spectra of the compound were taken on Bruker Avance II 400 NMR spectrometer in $CDCl_3$, at 400 MHz for ^{1}HNMR and 100.62 MHz for ^{13}CNMR with tetramethylsilane (TMS) as internal standard. EIMS (Electron impact mass spectrum) was taken on a Shimadzu LCMS-2010A. TLC was carried out using Silica gel 60 F_{254} plates (Merck). Column chromatography was carried out on neutral alumina of 70-300 mesh from S.D. fine chemicals Pvt. Ltd., Mumbai. All the chemicals and reagents used were obtained in high purity either from S.D. fine chemicals Pvt. Ltd; Bombay, India or E. Merck (India) Ltd., Mumbai.

Plant material

The root bark of *Eugenia jambolana* Lam., Myrtaceae, were collected from Derelakatte, Mangalore, during December 2009 and its botanical identity was confirmed by Dr. Noeline J. Pinto Head of Botany Department St. Agnes College, Mangalore. A voucher specimen (voucher no. 625e) has been deposited in NGSM Institute of Pharmaceutical Sciences, Derelakatte, Mangalore.

Extraction and fractionation of plant material

The shade dried powdered root barks (5 kg) were extracted with ethanol (95%) by cold maceration method for four times and the extract was concentrated by reduced pressure to yield 500 g of crude ethanolic extract. The ethanolic extract was suspended in distilled water (1:3, v/v) and successively partitioned with petroleum ether (60-80 °C, 8x500 mL), chloroform (8x500 mL), *n*-butanol (8x500 mL) and methanol (8x500 mL). The organic layers were brought to dryness to yield petroleum ether (35 g), chloroform (50 g), *n*-butanol (55 g) and methanol (70 g) fractions.

Animals

Studies were carried out by using male Swiss albino mice (18-25 g) and male albino Wistar rats (180-200 g). All animals were obtained from K.S. Hegde Medical Academy, Deralakatte, Mangalore. Animals were grouped and housed in polyacrylic cages and kept at ambient temperature (25±2 ºC), 60±5% relative humidity and 12 h light and dark cycle. They had been given standard pellet diet (Hindustan Lever Limited, Mumbai, India) and water *ad libitum* throughout the course of the study. The study protocols were approved by Institutional Animal Ethical Committee (KSHEMA /AEC/077/2008).

Chemicals and drugs

Carrageenan (Hi–Media Research Laboratories Pvt. Ltd., Mumbai), tween 80 (S.D. fine Chemicals Pvt. Ltd., Mumbai), Complete Freund's adjuvant (FCA), morphine hydrochloride (Morphine) and indomethacin (Indo) (Sigma Aldrich St. Louis, USA). The ethanolic extract (EJE) of root bark of *E. jambolana* and petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) of ethanolic extract of root bark of *E. jambolana* were used as a suspension in tween 80 (3 mL of 1% solution) to screen biological activity. Indomethacin and morphine hydrochloride were also used as a suspension in tween 80 (3 mL of 1% solution).

Acute toxicity study

Acute toxicity study was conducted to determine the median lethal dose (LD50) of test materials EJE, EJPE, EJCE, EJNB and EJME, as described in previous study, in adult male albino Wistar rats and by following up and down procedure of OECD guideline no. 425 (OECD 425, 2001; Ranawat et al., 2010). Animals were administered by test material orally and observed at half hour intervals for 4 h, then after 24 h. Test materials were found to be safe up to 2000 mg/kg b. w., *p.o*. dose.

Selection of dose

The test materials (EJE, EJPE, EJCE, EJNB and EJME) did not exhibit any toxic effect up to 2000 mg/kg b.w., *p.o*. dose in acute toxicity study. Hence, for biological screening the two doses of test materials were selected from the LD50 (2000 mg/kg) in such a manner that middle dose was approximately one tenth of the LD50, low dose was half of that one tenth dose, and a high dose was twice

of that one tenth dose, *i.e*. 200, 100 and 400 mg/kg *b.w*., *p.o.* doses, respectively. The antinociceptive and anti-inflammatory activity studies were carried out by 100, 200 and 400mg/kg doses of all the test materials EJE, EJPE, EJCE, EJNB and EJME.

Antinociceptive activity studies in mice

Acetic acid-induced writhing in mice

Antinociceptive activity of drugs was evaluated by the method described earlier (Collier et al., 1968). Male Swiss albino mice (18-25 g) were divided in eighteen groups (n=10) and respectively treated orally with vehicle (tween 80, 3 mL of 1% solution), indomethacin (Indo) 10 mg/kg b.w., *p.o*. and 100, 200 and 400 mg/kg b.w., *p.o*. of EJE, EJPE, EJCE, EJNB and EJME at 1 h prior to acetic acid injection. Morphine 1 mg/kg b.w. was administered intraperitoneally (*i.p*.) 30 min before the stimulus injection. All the animals were injected with 0.6% v/v acetic acid by intraperitoneal route and the numbers of writhe were counted over the period of 30 min. The percentage inhibition of number of writhings were calculated and compared with control group.

Formalin-induced nociception

This test was carried out as decribed by Hunskaar & Hole (1987). In this experiment mice of respective groups (n=10) were treated orally with indomethacin (Indo) 10 mg/kg b.w., *p.o*. and 100, 200 and 400 mg/kg b.w., *p.o*. of EJE, EJPE, EJCE, EJNB and EJME at 1 h prior to acetic acid injection. Animals of control group received vehicle (tween 80, 3 mL of 1% solution). Morphine 10 mg/kg b.w. was administered intraperitoneally (*i.p*.). After 1 h of drug treatment, 20 µL of 1% formalin (in 0.9% saline) were injected in dorsal surface of right hind paw of all animals. The animals were placed immediately in transparent observation chambers to observe the time spent by animals in licking the injected paw during early phase (0-5 min) and late phase (20-30 min) after formalin injection.

Anti-inflammatory activity studies

Carrageenan induced rat paw edema

Acute anti-inflammatory activity of test materials (EJE, EJPE, EJCE, EJNB and EJME) were evaluated by carrageenan induced rat paw edema study as described by Winter et al. (1962). Paw edema was induced by injecting 0.1 mL of 1% (w/v) carrageenan suspension in 0.9% (w/v) sterile saline into the plantar tissue of the left hind paw of albino wistar rats. Animals of respective groups (n=8) were administered orally with vehicle (tween 80, 3 mL of 1% solution), indomethacin (Indo) 10 mg/kg b.w., *p.o*. and 100, 200 and 400 mg/kg b.w., *p.o*. of EJE, EJPE, EJCE, EJNB and EJME at 1 h prior to the carrageenan induced paw edema. The right paw served as a reference to non-inflamed paw for comparison. Then every hour relative increase in the paw volume was measured by plethysmograph for all groups up to 4 h after carrageenan injection. The percentage inhibition of edema volume by test materials and standard drug treated groups were compared with control group. The percentage inhibition of edema volume was calculated using the following formula (Suleyman et al., 1991)

$$\text{Percentage inhibition} = (1-Vt/Vc) \times 100$$

Where *Vt* and *Vc* are the relative changes in the edema of the test and control respectively.

Cotton pellets induced granuloma in rats

The cotton pellets induced granuloma formation in albino wistar rats were carried out by following the method described by D' Arcy et al. (1960). All animals were anaesthetized with ether then shaved the fur and 10 mg of sterile cotton pellets were inserted, one in each axilla. Test drugs (EJE, EJPE, EJCE, EJNB and EJME) at 100, 200 and 400 mg/kg b.w., *p.o*. doses and standard drug (Indo) at 10 mg/kg b.w., *p.o*. dose were administered orally to animals of respective groups (n=8) for seven days. Animals of control groups received vehicle also for seven days. On eighth day animals were anaesthetized to remove cotton pellets surgically and made free from extraneous tissues. The moist pellets were weighed and then dried at 60 °C for 24 h, after that dried pellets were weighed again. Increment in dry weight of pellets was taken as measure of granuloma formation. The percentage inhibition of weight was calculated and anti-proliferative effect of drug treated groups was compared with the control group.

Adjuvant induced arthritis in rats

Arthritis was induced by the injection of 0.1 mL of Freund's Complete Adjuvant containing 1 mg/mL of heat killed *Mycobacterium tuberculosis* in paraffin oil and mannide monooleate (Sigma Aldrich St. Louis, USA) into the subplantar region of right hind paw of rat on day 0 of the experiment (Whittington & Green, 1970). On day 0, 2 h prior to induce arthritis all animals of respective groups (n=8) were treated orally with indomethacin (Indo) 10 mg/kg b.w., *p.o*. dose, 100, 200 and 400 mg/kg b.w., *p.o.* doses of EJE, EJPE, EJCE, EJNB and EJME and control group received vehicle. Treatments were continued till day 14. The volume of the paw was measured up to day 14 and the percentage inhibition was determined by using formula mentioned earlier.

Statistical analysis

Values were expressed as mean±S.E.M. Statistical significance was determined by ANOVA, followed by Dunnet's *t*-test; values with $p<0.05$ and $p<0.01$ were considered as statistically significant. GraphPad Prism version 4.0, GraphPad Software Inc., was used for statistical analysis.

Isolation and characterization of constituents

Since EJPE (petroleum ether fraction), was found to be most active fraction for antinociceptive and anti-inflammatory activity, used further for isolation of constituents. The petroleum ether fraction was dissolved in $CHCl_3$ (30 mL) and adsorbed onto neutral alumina (30 g). After evaporation of the solvent it was loaded onto a silica gel column (150 g) prepared in petroleum ether (60-80 ˚C). The column was eluted first with petroleum ether (60-80 ˚C) followed by petroleum ether (60-80 ˚C):chloroform graded mixtures (95:5, 90:10, 80:20, 70:30, 60:40 and 50:50), then with chloroform and finally chloroform:methanol (95:5, 90:10, 80:20, 70:30, 60:40 and 50:50). The elution was monitored by TLC (Silica gel-G: visualization by vanillin-sulphuric acid reagent heated at 110 ºC). Each time 10 mL were collected in a test tube and identical elutes (TLC monitored) were combined and concentrated to 10 mL and kept in a refrigerator. Three pure compounds was isolated respectively by petroleum ether (60-80 °C):chloroform grade mixtures (90:10), petroleum ether (60-80°C):chloroform (80:20) and petroleum ether (60-80°C):chloroform (60:40).

Structure elucidation of the isolated compounds from petroleum ether fraction was carried out by IR, ^{1}H and ^{13}C NMR and mass spectrometry (EIMS). By comparing the spectral data with the previously reported data these compounds were identified as β-sitosterol, stigmasterol and lupeol (Wenkert et al., 1978; Khalil & Ldler, 1980; Agarwal et al., 1985; Reynolds et al., 1986).

Results and Discussion

In the present study, root bark of *Eugenia jambolana* Lam., Myrtaceae, was extracted with ethanol and followed by successive fractionation of ethanolic extract with petroleum ether (60-80 °C), chloroform, *n*-butanol and methanol. All the extract (EJE) and fractions (EJPE, EJCE, EJNB and EJME) were evaluated for antinociceptive and anti-inflammatory activity by *in vivo* studies. In acute toxicity study the ethanolic extract (EJE) was found to be safe up to 2000 mg /kg b.w., *p.o*. dose. Thus, 100, 200 and 400 mg/kg b.w., *p.o*. doses were selected for all the test materials to carry out screening of biological activities. The studies to evaluate antinociceptive and anti-inflammatory effects of root bark of *Eugenia jambolana* were carried out for the first time.

Acetic acid induced writhing test is a widely employed visceral inflammatory pain model for screening of peripherally acting analgesics. In the study, injecting acetic acid into the peritoneal cavity of animals augments the level of mediators like cytokine, histamine, serotonin, bradykinin etc., which are activating peripheral nociceptors to persuade writhing response, *i.e.* episodes of stretching of abdomen (Gyires & Torna, 1984; Ikeda et al., 2001; Lima et al., 2011). Furthermore, acetic acid induces prostaglandin biosynthesis which is also the possible mechanism for the writhing response (Duarte et al., 1988). During the study, analgesic activity of drugs is measured by the ability of inhibition of writhing response. In this study, test drugs showed dose dependent response. As shown in Figure 1, 400 mg/kg b.w., *p.o.* dose of EJPE and EJCE showed the significant ($p<0.05$) 54.28 and 48.57% peripheral antinociceptive effect, respectively, and 400 mg/kg dose of EJPE was found to be most potent among the test materials. Indo 10 mg/kg b.w., *p.o*. and morphine 1 mg/kg *i.p*. doses showed respectively 71.42% and 91.42% significant ($p<0.01$) inhibition of writhing response. Hence, the result suggests that the possible mechanism for antinociceptive effect of test drug is the inhibition of the prostaglandin biosynthesis and the release of those inflammatory mediators. The antinociceptive effect of test materials were corroborated by formalin induced paw licking test. Formalin injection at mice paw stimulates the nociception effect by two distinct phases. In first phase, formalin directly acts on C-fibers which cause acute nociceptive neurogenic pain. Where as the late phase correspond inflammatory pain due to release of nociceptive mediators and persists for longer period (Tjolsen et al., 1992). The late phase is inhibited by peripherally acting drugs; where as, drugs act on opioid system inhibit the first phase (Shibata et al., 1989). In this study, Indo (10 mg/kg b.w., *p.o*.) showed significant 77.51% inhibition ($p<0.01$) of late phase (Figure 2). Among the test materials EJE, EJPE and EJCE at 400 mg/kg b.w., *p.o*. dose inhibited significantly ($p<0.01$) the late phase of nociception by 65.47, 73.77 and 69.87%, respectively, and EJPE at 400 mg/kg dose was found to be most potent among the test materials. But the test materials did not show any significant inhibition of first phase of nociception. It has been seen that drugs which act primarily on central nervous system can inhibit both phases (Dubuisson & Dennis, 1977; Rosland et al., 1990). In this study, morphine 10 mg/kg *i.p.* dose showed the significant inhibition of early and late phase of formalin induced nociception by 91.14% and 98.36% ($p<0.01$), respectively. Hence, the result of the present study confirmed that the antinociceptive effect of test material was due to inhibition of inflammatory pain.

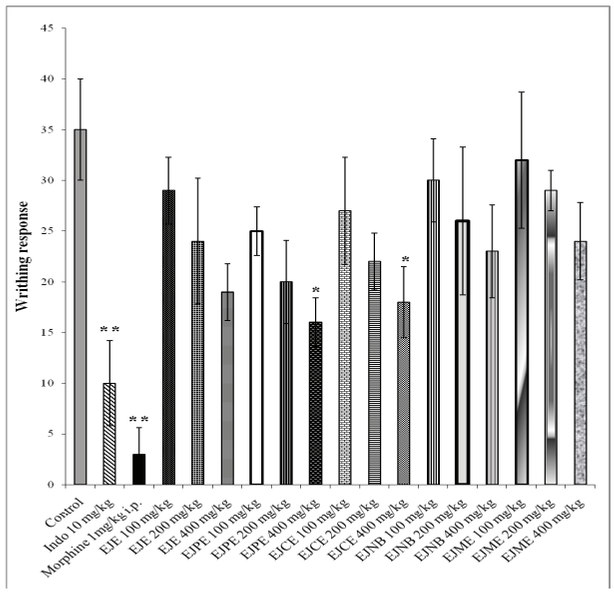

Figure 1. Effects of extract and fractions of *Eugenia jambolana* root bark on acetic-acid induced writhing response in mice model. Mice were treated with vehicle (control), indomethacin (Indo) 10 mg/kg b.w., *p.o*., morphine 1 mg/kg b.w. intraperitoneally (*i.p*.), ethanolic extract (EJE), petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) at 100, 200 and 400 mg/kg b.w., *p.o.* doses. Each value represents as mean±SEM. **p*<0.05 and ***p*<0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test).

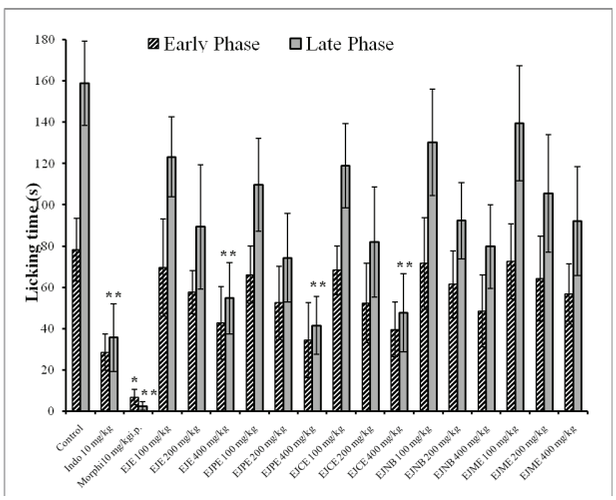

Figure 2. Effects of extract and fractions of *Eugenia jambolana* root bark on formalin induced nociception study in mice. Mice were treated with vehicle (control), indomethacin (Indo) 10 mg/kg b.w., *p.o.*, morphine (Morphi) 10 mg/kg b.w. intraperitoneally (*i.p*.), ethanolic extract (EJE), petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) at 100, 200 and 400 mg/kg b.w., *p.o.* doses. Antinociceptive effects were observed in early phase (0-5 min) and late phase (20-30 min). Each value represents as mean±SEM. **p*<0.05 and ***p*<0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test).

The results of antinociceptive activity were indicative for anti-inflammatory properties of test drugs. The hypothesis were evaluated by commonly used models for screening of anti-inflammatory activity of drugs, such as, carrageenan-induced rat paw edema, cotton pellet induced granuloma formation and adjuvant induced arthritis in rat. The carrageenan induced rat paw edema is an experimental model for acute inflammation study and which is widely used to screen most of the anti-inflammatory agents. The carrageenan induced rat paw edema development is a biphasic response, where the initial stage is dependent on the release of signature mediators like histamine, kinin and bradykinin, and the last phase is attributed to the synthesis of prostaglandins (Vinegar et al., 1969; DiRosa et al., 1971). Anti-inflammatory drugs are regulating these mediators to reduce edema. Mostly, the anti-inflammatory drugs are found to be effective during late phase, as in this study indomethacin showed the inhibition of rat paw edema in third and fourth hour. As shown in Table 1, indomethacin 10 mg/kg showed significant 82.75% (p<0.01) inhibition of rat paw edema at 4 h and 71.79% (p<0.05) at 3 h in the present study. Like indomethacin, EJPE and EJCE 400 mg/kg b.w., *p.o*. doses also respectively exhibited significant 79.31% (p<0.01) and 72.41% (p<0.01) inhibition of rat paw edema at 4 h. During 3 h EJPE 400 mg/kg *p.o*., b.w. dose inhibited edema by 61.53% (p<0.05). Though, EJE 400 mg/kg b.w., *p.o*. dose also showed significant 68.1% (p<0.05) inhibition at 4 h, EJPE 400 mg/kg dose was found to be most potent among all the test materials (Figure 3), and this response may be due to the inhibition of biosynthesis of prostaglandins (Niemegeers et al., 1964).

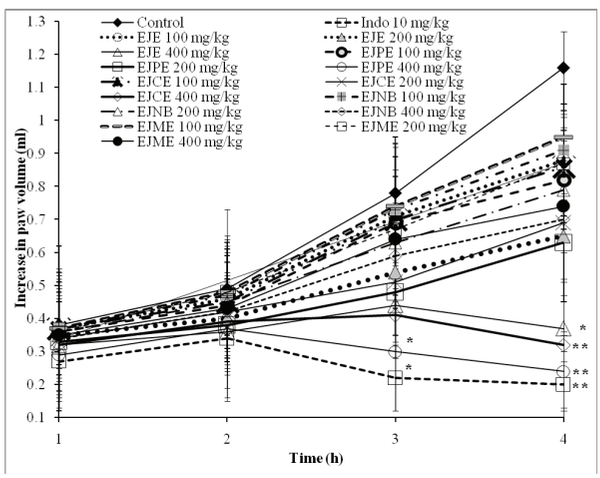

Figure 3. Anti-inflammatory effect of extract and fractions of Eugenia jambolana root bark on carragennan-induced rat hind paw edema. Effects of indomethacin (Indo) 10 mg/kg b.w., *p.o*., ethanolic extract (EJE), petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) at 100, 200 and 400 mg/kg b.w., *p.o.* doses were evaluated in the study. Each value represents as mean±SEM. **p*<0.05 and ***p*<0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test).

Table 1. Anti-inflammatory activity of *Eugenia jambolana* root bark extracts in carrageenan induced rat paw edema model.

Groups	Dose (mg/kg b.w., *p.o.*)	Increase in paw volume (mL)			
		1 h	2 h	3 h	4 h
Control	-	0.38±0.13	0.49±0.24	0.78±0.14	1.16±0.11
Indo	10	0.27±0.12 (28.94)	0.34±0.16 (30.61)	0.22±0.13 (71.79)*	0.20±0.17 (82.75)**
	100	0.37±0.16 (2.63)	0.46±0.18 (6.12)	0.71±0.14 (8.97)	0.88±0.10 (24.13)
EJE	200	0.35±0.10 (7.89)	0.4±0.12 (18.36)	0.54±0.19 (30.76)	0.65±0.20 (43.96)
	400	0.32±0.15 (15.78)	0.36±0.21 (26.53)	0.44±0.11 (43.58)	0.37±0.10 (68.10)*
	100	0.36±0.18 (5.26)	0.44±0.13 (10.20)	0.69±0.26 (11.53)	0.82±0.15 (29.31)
EJPE	200	0.33±0.13 (13.15)	0.38±0.19 (22.44)	0.48±0.2 (38.46)	0.63±0.12 (45.68)
	400	0.29±0.16 (23.68)	0.37±0.14 (24.48)	0.3±0.18 (61.53)*	0.24±0.11 (79.31)**
	100	0.37±0.14 (2.63)	0.45±0.18 (8.16)	0.70±0.23 (10.25)	0.86±0.17 (25.86)
EJCE	200	0.34±0.18 (10.52)	0.42±0.12 (14.28)	0.51±0.16 (34.61)	0.69±0.33 (40.51)
	400	0.32±0.17 (15.78)	0.39±0.23 (20.40)	0.41±0.08 (47.43)	0.32±0.20 (72.41)**
	100	0.37±0.25 (2.63)	0.47±0.12 (4.08)	0.73±0.1 (6.41)	0.91±0.20 (21.55)
EJNB	200	0.36±0.14 (5.26)	0.45±0.16 (16.32)	0.63±0.22 (19.23)	0.79±0.15 (31.89)
	400	0.34±0.16 (10.52)	0.42±0.11 (14.28)	0.59±0.14 (24.35)	0.70±0.06 (39.65)
	100	0.37±0.11 (2.63)	0.48±0.13 (2.04)	0.74±0.15 (5.12)	0.95±0.10 (18.10)
EJME	200	0.36±0.14 (5.26)	0.48±0.17 (2.04)	0.67±0.11 (14.10)	0.87±0.14 (25.00)
	400	0.35±0.19 (7.89)	0.43±0.12 (12.24)	0.64±0.15 (17.94)	0.74±0.13 (36.20)

All the result are expressed in term of mean±SEM; n=8 animals in each group; number in parenthesis indicates percentage inhibition in increase in paw volume. Statistical significance was determined by ANOVA, followed by Dunnet's t-test. *p<0.05, **p<0.01, statistically significant.

The cotton pellet induction in rat develops granuloma as a sign of chronic inflammation by accumulation of macrophages and lymphocytes around the foreign particles together with epitheloid and giant cells which are derived from macrophages (Swingle & Shideman, 1972; Snedegard, 1985; Serhan & Savil, 2005). The inhibition of granulomatous tissue formation in rat indicates the anti-proliferative effect of drugs, and which is measured by percentage inhibition of cotton pellet weight. The wet weight of the pellet is influenced by absorption of fluid while the dry weight correlates with the amount of granulomatous tissue formed (Spector, 1969). As shown in Table 2, Indo 10 mg/kg significantly inhibited wet cotton pellet weight (62.68%, p<0.01). EJE, EJPE and EJCE 200 mg/kg b.w., *p.o.* doses respectively inhibited 31.62, 35.98 and 24.74% (p<0.05) weight of wet cotton pellet as compared to control group (Figure 4). EJE and EJNB 400 mg/kg b.w., *p.o*. doses showed 34.57 and 22.63% (p<0.05) inhibition of weight of wet cotton pellet, respectively. Where as, EJPE and EJCE 400 mg/kg b.w., *p.o*. doses showed significant 57.78 and 50.49% (p<0.01) inhibition of wet cotton pellet weight, respectively. Indomethacin (10 mg/kg) also showed significant inhibition of the weight of dry cotton pellets (68.03%, p<0.01) (Table 2). EJPE 400 mg/kg b.w., *p.o.* dose showed significant 60.35% (p<0.01) inhibition of dry cotton pellets weight, which was closer to the result showed by indomethacin (Figure 4). EJE and EJCE 400 mg/kg b.w., *p.o*. doses also significantly inhibited 48.41 and 52.66% (p<0.01) weight of dry cotton pellets, respectively. Hence, the findings suggested that the test drugs significantly (p<0.05 and p<0.01) inhibited the proliferative phase of inflammation.

The FCA induced arthritis assessed by formation of rat paw edema. The chronic inflammation induced by adjuvant involved polyartritis in 14th day study (Ward & Cloud, 1996). The arthritis was induced in this study by a sub-cutaneous injection of FCA, *i.e*. heat killed Mycobacterium tuberculosis in paraffin oil and mannide monooleate, in the rat paw's plantar surface. The arthritis was developed in the joints of hind limbs of rats by reduction of motor activity and increased paw diameter was observed due to inflammation and edema (Calvino et al., 1987; Cain et al., 1997). The initial inflammatory response was observed within hours, but more critical signs of inflammation were developed with days and observed till the 14th day of the study. EJPE and EJCE 400 mg/kg b.w., *p.o.* doses on day 14th significantly inhibited rat paw swelling by 77.93 and 68.27% (p<0.01) respectively (Figure 5). Indo 10 mg/kg b.w., *p.o*. dose significantly inhibited the edema by 84.82% (p<0.05) on 14th day of study (Table 3). EJE 200 and 400 mg/kg b.w., *p.o*. doses on day 14th showed respectively 46.2 and 64.82% (p<0.05) inhibition of edema (Figure 5). EJPE, EJCE and EJNB 200mg/kg b.w., *p.o.* doses also showed significant (p<0.05) 55.17, 50.34 and 40.68% inhibition of adjuvant induced rat paw edema, respectively (Figure 5). The test drugs

Table 2. Effects of *Eugenia jambolana* root bark extracts on cotton pellets induced granuloma formation in rats.

Groups	Dose (mg/kg b.w., *p.o.*)	Moist cotton pellet		Dry cotton pellet	
		Weight (mg)	% inhibition	Weight (mg)	% inhibition
Control	-	295.63±14.53	-	84.37±3.00	-
Indo	10	110.32±9.07	62.68**	26.97±1.34	68.03**
	100	262.74±16.71	11.12	72.53±1.56	14.03
EJE	200	202.15±10.35	31.62*	59.24±2.03	29.78**
	400	193.43±11.63	34.57*	43.52±1.80	48.41**
	100	258.29±8.11	12.63	68.11±2.35	19.27
EJPE	200	189.26±17.66	35.98*	48.25±2.41	42.81**
	400	124.81±19.81	57.78**	33.45±3.26	60.35**
	100	265.13±14.32	10.31	70.84±1.78	16.03
EJCE	200	222.49±18.26	24.74*	61.23±2.05	27.42**
	400	146.35±13.74	50.49**	39.94±1.93	52.66**
	100	272.48±12.60	7.83	75.27±1.22	10.78
EJNB	200	244.69±15.47	17.23	73.42±1.63	12.97
	400	228.72±16.93	22.63*	66.98±2.11	20.61*
	100	283.14±23.58	4.22	78.51±1.88	6.94
EJME	200	263.34±19.64	10.92	75.82±1.54	10.13
	400	246.58±10.33	16.59	71.57±2.47	15.17

All the result are expressed in term of mean±SEM. n=8 animals in each group; Statistical significance was determined by ANOVA, followed by Dunnet's t-test. *p<0.05, **p<0.01, statistically significant.

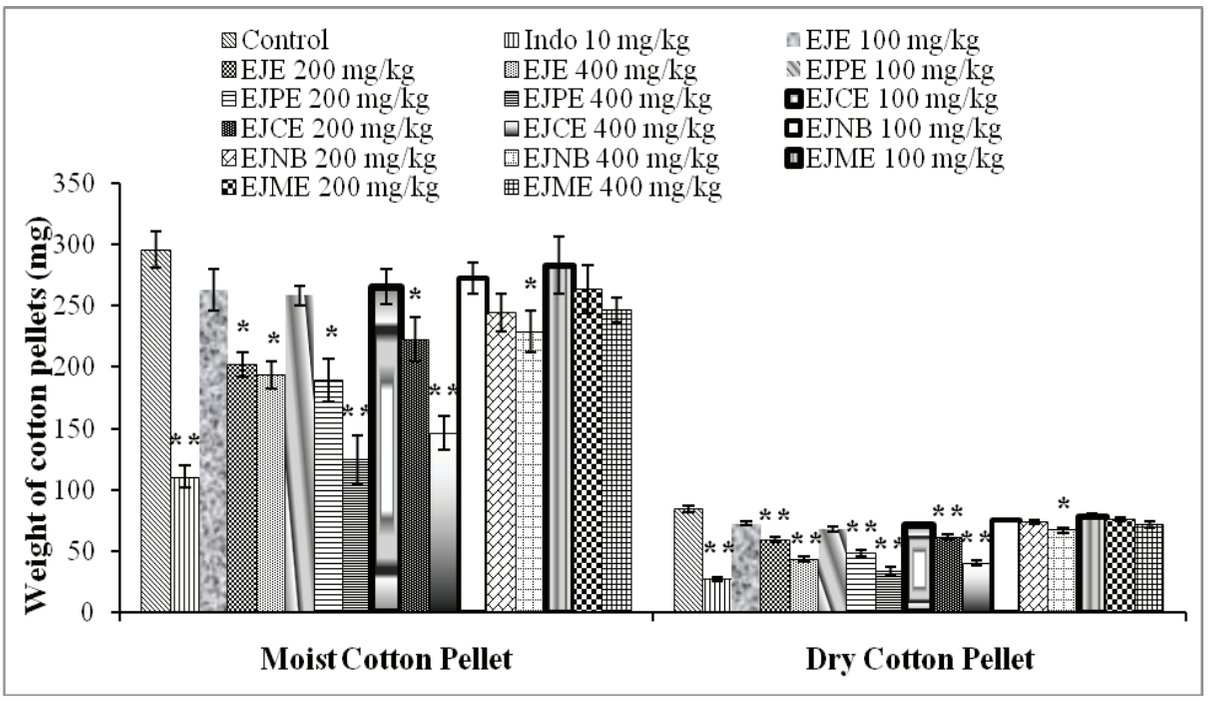

Figure 4. Effects of extract and fractions of *Eugenia jambolana* root bark on cotton pellet induced granuloma in rat. Anti-proliferative effect of indomethacin (Indo) 10 mg/kg b.w., *p.o.*, ethanolic extract (EJE), petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) at 100, 200 and 400 mg/kg b.w., *p.o.* doses were evaluated by weighing moist and dry cotton pellets. Each value represents as mean±SEM. *p<0.05 and **p<0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test).

Table 3. Anti-inflammatory activity of *Eugenia jambolana* root bark extracts in adjuvant induced arthritis in rats.

Groups	Dose (mg/kg b.w., *p.o.*)	Increase in paw volume (mL)			
		0 day	3 day	7 day	14 day
Control	-	0.42±0.20	0.57±0.13	0.82±0.19	1.45±0.12
Indo	10	0.38±0.16 (9.52)	0.46±0.12 (19.29)	0.37±0.13 (54.87)	0.22±0.11 (84.82)**
EJE	100	0.42±0.15 (0.00)	0.56±0.27 (1.75)	0.77±0.14 (6.09)	1.09±0.09 (24.82)
	200	0.40±0.12 (4.76)	0.53±0.1 (7.01)	0.74±0.19 (9.75)	0.78±0.15 (46.2)*
	400	0.39±0.21 (7.14)	0.51±0.15 (10.52)	0.61±0.11 (25.61)	0.51±0.14 (64.82)*
EJPE	100	0.42±0.1 (0.00)	0.55±0.23 (3.5)	0.76±0.17 (7.31)	0.93±0.13 (35.86)
	200	0.41±0.19 (2.38)	0.54±0.13 (5.26)	0.74±0.2 (9.75)	0.65±0.16 (55.17)*
	400	0.39±0.14 (7.14)	0.5±0.16 (12.28)	0.56±0.18 (31.7)	0.32±0.10 (77.93)**
EJCE	100	0.42±0.15 (0.00)	0.56±0.24 (1.75)	0.77±0.13 (6.09)	0.97±0.12 (33.1)
	200	0.42±0.12 (0.00)	0.55±0.18 (3.5)	0.76±0.16 (7.31)	0.72±0.18 (50.34)*
	400	0.4±0.23 (4.76)	0.52±0.17 (8.77)	0.60±0.08 (26.82)	0.46±0.12 (68.27)**
EJNB	100	0.42±0.08 (0.00)	0.56±0.06 (1.75)	0.79±0.13 (3.65)	1.17±0.29 (19.31)
	200	0.42±0.16 (0.00)	0.55±0.14 (3.5)	0.77±0.22 (6.09)	0.86±0.15 (40.68)*
	400	0.41±0.11 (2.38)	0.54±0.16 (5.26)	0.64±0.14 (21.95)	0.79±0.09 (45.51)*
EJME	100	0.42±0.15 (0.00)	0.57±0.13 (0.00)	0.80±0.26 (2.43)	1.23±0.30 (15.17)
	200	0.42±0.17 (0.00)	0.56±0.14 (1.75)	0.77±0.11 (6.09)	0.91±0.14 (37.24)
	400	0.41±0.14 (2.38)	0.55±0.09 (3.5)	0.68±0.17 (17.07)	0.82±0.19 (43.44)*

All the result are expressed in term of mean±SEM.; n=8 animals in each group; number in parenthesis indicates percentage inhibition in increase in paw volume. Statistical significance was determined by ANOVA, followed by Dunnet's t-test. *p<0.05, **p<0.01, statistically significant.

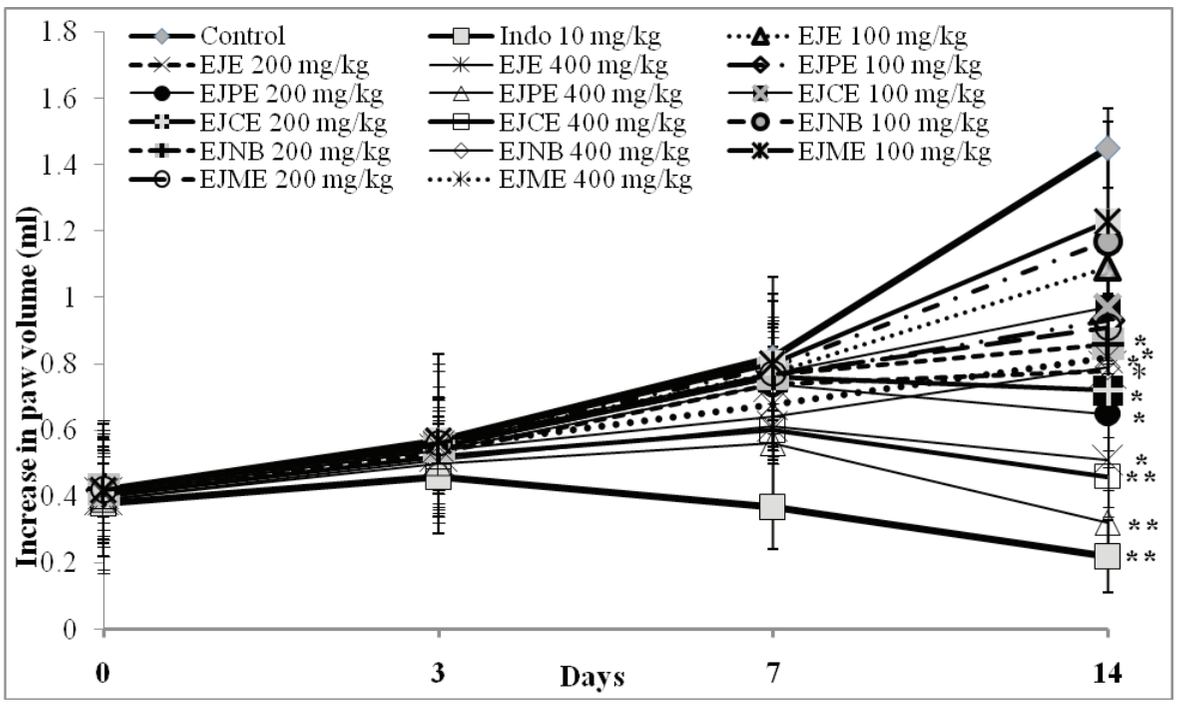

Figure 5. Effects of extract and fractions of *Eugenia jambolana* root bark on adjuvant induced arthritis in rat. The anti-arthritic activity of indomethacin (Indo) 10 mg/kg b.w., *p.o*., ethanolic extract (EJE), petroleum ether fraction (EJPE), chloroform fraction (EJCE), *n*-butanol fraction (EJNB) and methanol fraction (EJME) at 100, 200 and 400 mg/kg b.w., *p.o*. doses were evaluated by reduction of adjuvant induced rat hind paw edema. Each value represents as mean±SEM. *p<0.05 and **p<0.01 as compared with the control group (one-way ANOVA followed by Dunnet's t-test).

showed the efficacy during the study and EJPE 400 mg/kg b.w., *p.o.* dose showed the maximum reduction of rat paw edema among the other doses (Table 3) which was also comparable to the effect of standard drug. The inhibition of rat paw edema was indicated the therapeutic efficacy of the test drug in chronic inflammatory state and the effect was observed may be due to the inhibition of prostaglandin biosynthesis (Babu et al., 2009).

Therefore, these results suggested that the test materials exhibited significant antinociceptive and anti-inflammatory effect and EJPE (400 mg/kg b.w., *p.o*. dose) was found to be most potent among all the test materials. Hence, the current study has qualified the traditional claim for anti-inflammatory effect by *Eugenia jambolana.* Furthermore, the bioactive petroleum ether fraction of ethanolic extract of root bark of *Eugenia jambolana* (EJPE) was subjected to column chromatography which led to isolate three pure compounds. These compounds were characterized by IR, ^{1}H and ^{13}C NMR and mass spectroscopy. These compounds were identified as β-sitosterol, stigmasterol and lupeol by comparing with previously reported spectroscopical data. Nevertheless, by comparison with the previous reports the findings suggested that the antinociceptive and anti-inflammatory activities of petroleum ether fraction of ethanolic extract of root bark of *Eugenia jambolana* may be due to the presence of these compounds (Garcia et al., 1999; Navarro et al., 2001; Geetha & Varalakshmi, 2001; Backhouse et al., 2008). Therefore, the further studies on antinociceptive and anti-inflammatory activities need to be performed with these isolated compounds.

Conclusion

The antinociceptive and anti-inflammatory activity of ethanolic extract and different fractions of root bark of *Eugenia jambolana* were evaluated in the present study. The bioassay potential petroleum ether fraction was led to isolate β-sitosterol, stigmasterol and lupeol. Hence, these findings suggested the significant antinociceptive and anti-inflammatory effects of root bark of *Eugenia jambolana*. However, the mechanisms underlying the antinociceptive and anti-inflammatory effects of Eugenia jambolana is still needed to be determined.

Acknowledgements

Authors are thankful to Nitte Gulabi Shetty Memorial Institute of Pharmaceutical Sciences for providing the necessary facilities and infrastructure for the study. Authors are also grateful to Nitte University for the financial support.

Authors' contributions

SS (PhD student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, extraction of plant material and isolation of compound, biological studies, analysis of the data and drafted the paper. EVSS guided and supervised the research work and contributed to critical reading of the manuscript. CK contributed to chromatographic analysis, supervised the isolation of compounds and spectroscopical data analysis of compounds. SCM contributed to critical reading of the manuscript. SCS provided and made the availability of all the necessary facilities to carry out the research work. All the authors have read the final manuscript and approved the submission.

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