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Pharmacognostic and analgesic screening studies of *Eugenia jambolana* seeds

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Abstract

Analgesic activity of some indigenous medicinal plants, the medicinal flora of our country still remains virtually unexplored. Thus, in an attempt to develop potent medicinal agent to fight against infectious diseases and related pain, in the present study, evaluation of analgesic activity of seeds of Jamun (*Eugenia Jambolana* Lam.) *E. jambolana* Linn. has been traditionally used for the treatment of various ailments. Thus, this plant was selected for the analgesic activities. Properly identified *E. jambolana* seeds were subjected to analgesic activity using models (Eddy's hot plate and Tail immersion method). Amongst various extracts (EJPE, EJC, EJM and EJAQ) of *E. jambolana* tested, only methanol extract (EJM) exhibit significant analgesic activity. Finally, bioactive extract was subjected to the chromatographic separation of chemical compound which was further characterized by using spectroscopic method and the isolated compound was found to be 3-(2,2,3,3,4,5,5,6,6-nonamethylcyclohexyloxy)-3,4-dihydro-2-(4methoxyphenyl)-2H-chromene-7,8-diol. It is concluded that the seeds of *E. jambolana* showed good analgesic activities which could be due to the presence of anthocyanin compounds.

Keywords: *Eugenia jambolana* seeds, Pharmacognostic, Analgesic study, Medicinal plants

Introduction

The great Northern region of India and its herbal richness cater to the needs of modern medicine. This diversity of the Northern region, if used effectively and scientifically, it can provide a new drug molecule which may combat the adverse effects of the synthetic drugs used worldwide as well as reduce the cost of the medication. So, these facts if used further can be useful in treating many dangerous diseases. Especially, herbal plants have proven themselves a strong contender in acting as natural resource for bioactive compounds, such as *Taxus brevifolia* (Houghton *et al*., 2007), *Gingko biloba* (Goktas *et al*., 2007). Therfore, the herbal plants got importance and became essential ingredients of the various medicines of traditional Indian system of medicine such Ayurveda, Unani and Homeopathy. Besides the availability of reports on analgesic activity of some indigenous medicinal plants, the medicinal flora of our country still remains virtually unexplored. Thus, in an attempt to develop potent medicinal agent to fight against infectious diseases and related pain, in the present study, evaluation of analgesic

activity of seeds of Jamun (*Eugenia Jambolana* Lam.) is carried out. It belongs to the family Myrtaceae and is native to the Indian subcontinent. Jamun tree is famous for its different names and for its fruit called berries. Annually, this tree produces oblong and ellipsoid fruits called berries. They are green, when raw and purplish black, when fully ripe and are sweetish sour to taste and contains high percentage of nutrients such are minerals, vitamins, tannins and protein content in adequate amount (Paul and Shaha, 2004). The pharmacologically active phytoconstituents present are flavanoids, terpenoids, anthocyanins and tannins (Li, Zhang and Seeeram, 2009). Jamun is a plant with known ethnomedicinal uses. Before the discovery of Insulin, jamun was highly useful in the treatment of diabetes and is an integral part in various system of medicine (Helmstadter, 2007). The plant has gained importance as an herbal drug to cure several diseases such as viral infections, inflammatory disorders, allergic disorders, gastric ulceration, heart diseases, cancer, liver infections, diarrhoea and diabetes. But the analgesic activity of *Eugenia jambolana* is not yet explored. So, it was decided to study this activity with the seeds of *Eugenia jambolana* using various animal and experimental models.

Materials and methods

Plant Material

The seeds of *E. jambolana*, commonly known as Jamun, were collected from the botanical garden of Guru Nanak Dev University (GNDU) campus in the month of July 2011 and were then dried (Figure 5). The taxonomic identification of the plant was confirmed by Mr. Ram Prasad, Department of Botanical & Environmental Sciences, GNDU, Amritsar. A voucher specimen no. (No. 29) herb has been deposited in department's herbarium.

Chemicals and Solvents

1. Solvents

Petroleum ether (60-80ºC) (Qualigens Fine Chemicals, Mumbai); Chloroform (Qualigens Fine Chemicals, Mumbai), Methanol and Ethanol (Qualigens Fine Chemicals, Mumbai) were used for the extraction of required plant material and the subsequent analysis.

2. Chemicals

Chloroform, Sulphuric acid, hydrochloric acid, Mayer's reagent, Dragendorff's reagent, magnesium turnings, hydrochloric acid, ammonia solution, benzene, pyridine, sodium nitroprusside, sodium hydroxide, ferric chloride were used for the phytochemical screening of the plant extracts.

Pharmacognostic Standardization

1. Organoleptic Features

The plant was observed with the naked eye for its varied features like color, odour, taste, size, shape and other features like touch and texture of the seeds of *E. jambolana*.

2. Microscopic Examination

2.1. Powder study

The dried seeds were powdered and was cleared in chloral hydrate solution, stained with weak iodine solution, phloroglucinol-HCl, then mounted with dilute glycerine and observed under a light microscope (Ruzin, 1999).

2.2. Photomicrograph

The microscopic descriptions of the selected tissues were supplemented with micrographs. The photomicrographs of the different magnifications were taken with Olympus Magnus Microscope and for the normal observations, a bright field was used.

Ash Values

The material remaining after incineration of the powdered drug material is called as ash content of the drug, indicating the presence

of the inorganic salts naturally occurring in the plant drug or due to the adulteration of available plant material. The ash values were determined by using total ash, water soluble ash and acid insoluble ash as per the procedure given in Indian Pharmacopeia (IP, 1996) and WHO guidelines (WHO, 1998).

1. Total Ash

This method is used to measure the total amount of the material remaining after ignition (silicates, carbonates, etc). This material obtained may include 'physiological ash' which is derived from the plant tissue itself plus 'non-physiologiacl ash' which is the residue of the powder extraneous matter as sand, soil, etc. adhering to the surface of the plant. Absolutely air-dried powdered drug (approx. 2-4 g) was weighed in a silicon crucible and was incinerated at a temperature not exceeding 400ºC in a muffle furnace (Narang scientific works, New Delhi) until it is devoid of carbon or when it appears white in colour, cooled and then weighed. The percentage of ash with reference to the air-dried drug was calculated (WHO guidelines, 1998).

2. Acid Insoluble Ash

The acid insoluble ash is the residue left after boiling the total ash content obtained above with 25 ml of 2 M hydrochloric acid and subsequently igniting the insoluble matter for 5 minutes. The resultant matter was collected in silicon crucible or most probably on an ash-less filter paper washed with hot water, ignited, cooled in a dessicator and accurately weighed. The percentage of acid insoluble ash with reference to air-dried drug was calculated (WHO guidelines, 1998).

3. Water Soluble Ash

The total ash content was boiled in a china dish containing 25 ml of distilled water for 5 minutes. The resultant insoluble matter left was collected in a sintered crucible or on an ash-less filter paper, washed with hot water and ignited in a crucible (Galaxo, Mumbai) for 15 minutes not exceeding 400ºC. Finally, calculate the difference between the weight of the residue (in mg) from the weight of total ash. The percentage of water-soluble ash with reference to air-dried drug was calculated (IP, 1996).

Loss on Drying (LOD)

Oven-dried glass-stoppered weighing bottle was weighed. Subsequently, the bottle and the powdered sample material were accurately weighed. The stopper was removed and the bottle was placed in an oven until the sample was dried to a constant weight. When absolutely dried, immediately the bottle was stoppered and was allowed to cool to room temperature while kept in a dessicator. Finally, the bottle and its contents were weighed. Loss on drying was evaluated by calculating the differences of the two (before and after drying) weighings (WHO guidelines, 1998).

Determination of Extractive Value

1. Alcohol Soluble Extractive Value

Alcohol is an ideal solvent for extraction of various phyto-chemicals as tannins, resins, etc. thus, this method is used to determine the approximate resin content of drug. The powdered drug material (approx.4-5 g) was macerated with 25 ml of ethanol in a closed conical flask for 24 hours. For first 6 hours. Shake the contents of the flask frequently and for the next 16 hours, the flask was allowed to stand still, without any disturbance. The resultant extract was filtered. The filtrate (25 ml) obtained was evaporated to dryness and the residue obtained was dried at 105ºC and finally weighed. The percentage of ethanol-soluble extractive with reference to the air-dried drug was calculated (Ayurvedic pharmacopoeia of India, 2008).

2. Water-Soluble Extractive Value

This method can be applied to plant drugs containing water soluble constituents of crude drugs as tannins, glycosides, sugars, plant acids and mucilage, etc. 4-5 g of the

powdered drug material was added to 25 ml of water (80ºC) in a tightly closed conical flask and kept in mechanical shaker. After vigorous shaking, it was allowed to stand still for 18 hours. The filtrate obtained (25 ml) was evaporated to dryness and the residue obtained was dried at 105ºC and finally weighed. The percentage of water-soluble extractive with reference to the air-dried drug was calculated (WHO guidelines, 1998). Similarly, the petroleum ether, chloroform and ethyl acetate extractive values of the powdered plant material were carried out.

Preparation of Extracts

The seeds of *E. jambolana* were dried in shade and coarsely powdered. Approx. 1.5 Kg of the powdered material was subjected to successive Soxhlet extraction using different solvents in an increasing order of their polarity viz. petroleum ether (60–80ºC), chloroform, methanol and distilled water for not less than 48 hours. After each extraction, the powdered material was dried in air at room temperature. Finally, marc was digested with distilled water for 24 hours or more to obtain the aqueous extract (Figure 6). Each extract was concentrated in-vacuo using rotatory evaporator. Extracts were weighed subsequently and the percentage yields were calculated of each extract obtained individually in terms of the air-dried weight of the plant material.

Preliminary Phytochemical Screening

The extracts of *E. jambolana* were tested for the presence of various phytochemicals such as alkaloids, glycosides, tannins, steroids, saponins, flavonoids, etc. (Kokate, 2008; Khandelwal, 2006).

Fluorescence Analysis

The powdered seed material was analyzed under visible light, short ultraviolet light, long ultraviolet light after treatment with various organic / inorganic reagents like sodium hydroxide, nitric acid, ammonia, etc (Pratt and Chase, 1949).

Animals

The experimental animals (Swiss albino mice, Laca strain) of either sex weighing 25 - 35 g were procured from the Guru Angad Dev Vetrinary and Animal Science University (GADVASU), Ludhiana, Punjab and kept at Central Animal House, Guru Nanak Dev University (GNDU), Amritsar. The animals were given standard laboratory feed and water *ad libitum*. The animals were housed in the room of controlled conditions of 24 ± 1ºC and 12 h light-12 h dark cycles. The protocol was approved as per the guidelines of Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA).

Drugs and Chemicals Used

1) Carboxy methyl cellulose (CMC) - Vehicle
2) Pentazocine - Standard
3) *E. jambolana* Petroleum ether extract
4) *E. jambolana* Chloroform extract
5) *E. jambolana* Methanol extract
6) *E. jambolana* Aqueous extract

Preparation of Doses

The various drugs used in the evaluation of biological activities such as pentazocine was dissolved in double-distilled water and administered intraperitoneally (i.p.) to the animals. The test doses (50, 100 and 150 mg/kg) of extracts of *E. jambolana* were used to test its biological activity in the form of a suspension and were prepared by triturating in 1% Carboxy methyl cellulose (CMC) and moderately vortexed for proper mixing of the extract in the suspension. All the *E. jambolana* extract formulations were administered i.p. to all the group of animals.

Evaluation of Analgesic Activity

1. Hot Plate Test

The hot plate, which is commercially available, consists of a electrically heated surface. The temperature is controlled for 55° to 56 °C. This can be a copper plate or a heated glass surface. The hot plate test has been found to be suitable for evaluation of centrally but not of peripherally acting analgesics. The paws of mice and rats are very sensitive to heat at temperature, which are not damaging the skin. The response is in the form of jumping, withdrawl of the paws or licking of the paws (Vogel and Vogel, 2002; Kulkarni, 2003). Groups of 6 mice of either sex weighing 30-40 g were used for each dose. The animals were divided into 9 groups of 5 mice each.

Group No.1: Control (1% CMC sol. i.p.)
Group No.2: Pentazocine (10 mg/kg, i.p.)
Group No.3: EJP (100 mg/kg, i.p.)
Group No.4: EJC (100 mg/kg, i.p.)
Group No.5: EJM (100 mg/kg, i.p.)
Group No.6: EJAQ (100 mg/kg, i.p.)

The bioactive extract was further evaluated at different doses: -

Group No.7: EJM (25 mg/kg, i.p.)
Group No.8: EJM (50 mg/kg, i.p.)
Group No.9: EJM (150 mg/kg, i.p.)

The control group (Group No.1) received 1% CMC suspension. Group No.2 received the reference drug Pentazocine (10 mg/kg). Group No.3, 4, 5 and 6 received the methanol (EJM), aqueous (EJAQ), chloroform (EJC) and petroleum ether (EJP) extracts at dose of 100 mg/kg and Group No.7, 8 and 9 received the methanol (EJM) extract at the doses of 25, 50 and 150 mg/kg, i.p., respectively. All the treatments were given intraperitoneally (i.p.).

2. **Tail Immersion Test**

The mice were placed into individual restraining cages leaving the tail hanging out freely. The animals were allowed to adapt to the cages for 30 min before testing. The lower 5 cm portion of the tail is marked. This part of the tail is immersed in a beaker of warm water of temperature exactly 55°C. Within a few seconds, the mice reacts by withdrawing the tail. The reaction time is recorded in seconds by a stopwatch. After each determination the tail is carefully dried. The reaction time is recorded after 15, 30, 45, 60, 90, 120 and 180 minutes following the administration of the standard and test compound. The cut off time of the immersion test is 15 seconds. The withdrawal time of untreated animals is between 1 and 4.5 seconds. A withdrawal time of more than 6 sec is regarded as a positive response. Groups of 5 mice of either sex weighing 30-40 g were used for each dose. The animals were divided in 9 groups of 6 mice each (Vogel and Vogel, 1997; Kulkarni, 1999).

Group No.1: Control (1% CMC sol. i.p.)
Group No.2: Pentazocine (10 mg/kg, i.p.)
Group No.3: EJP (100 mg/kg, i.p.)
Group No.4: EJC (100 mg/kg, i.p.)
Group No.5: EJM (100 mg/kg, i.p.)
Group No.6: EJAQ (100 mg/kg, i.p.)

The bioactive extract was further evaluated at different doses: -

Group No.7: EJM (25 mg/kg, i.p.)
Group No.8: EJM (50 mg/kg, i.p.)
Group No.9: EJM (150 mg/kg, i.p.)

After 20 minutes of treatment with each extract dose, standard (pentazocine) and control, tail of the mice was immersed in a beaker of warm water at temperature of 50 ± 5ºC for a maximum time of 15 seconds. The latency was recorded immediately when the animals withdraw their tail in seconds at an time interval of 15, 30, 45, 60, 90, 120 and 180 minutes (Vogel and Vogel, 1997; Kulkarni, 1999).

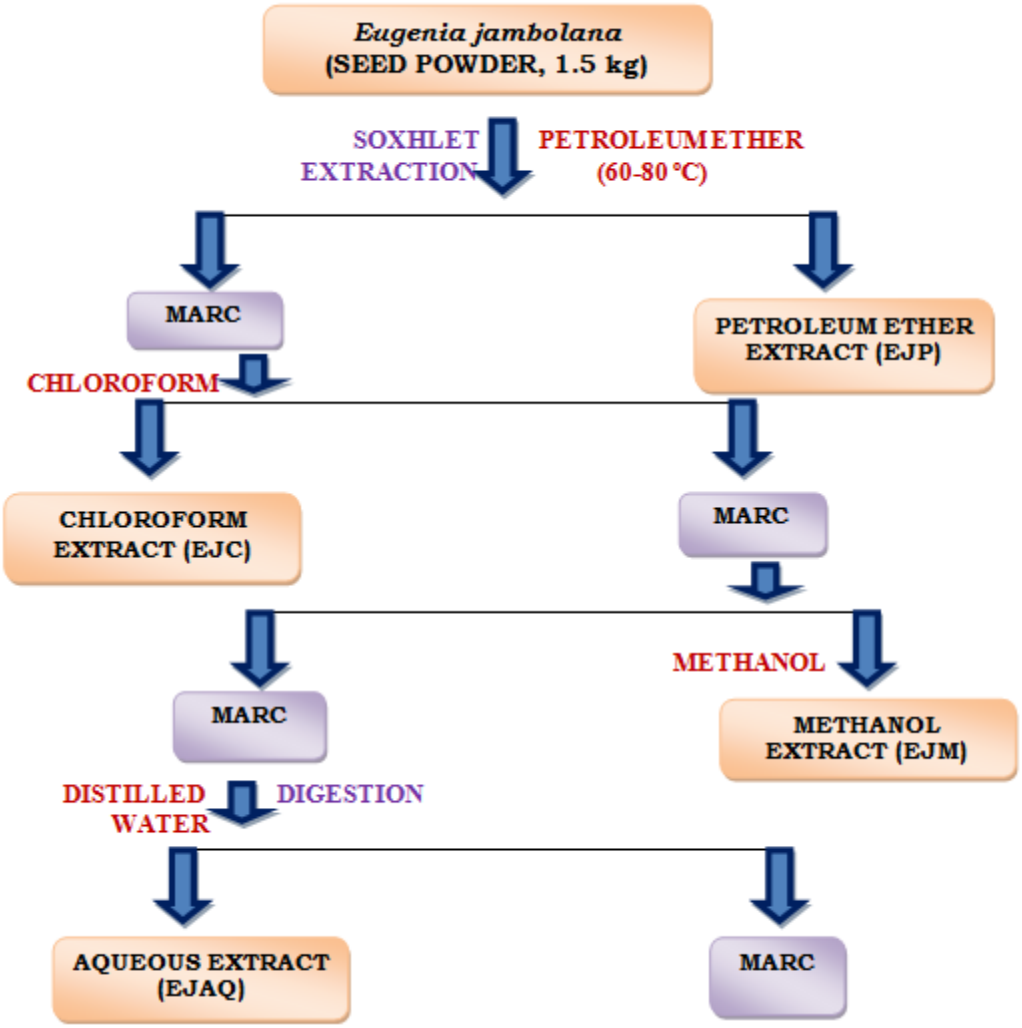


Figure 1: Preparation of different extracts from seeds of *E. Jambolana* by Soxhlet apparatus.

Column Chromatography of Bioactive Extract

The bioactive methanol extract (28 g) of *E. jambolana* seeds was loaded onto the column packed with Silica gel (60-120 mesh; Titan Biotech Ltd.) and was eluted using chloroform until 61 fractions and then the polarity was increased by using chloroform:methanol at different ratios. The obtained solution was distilled on the water bath, until 2-4 ml of the fraction is left, which is then collected in a 5 ml vial. Each fraction was simultaneously spotted on the Silica gel-G prepared TLC (Thin layer chromatogram) plates using chloroform and methanol as mobile phase at the increasing polarity. A total no. of 659 fractions was collected by the similar method.

1. Characterization of Fractions

The fractions with the similar chromatographic pattern obtained on TLC plates were characterized by using:-

i. ^{1}H- NMR (300-MHz Brukel and Jeol NMR spectrometer) and ^{13}C-NMR (75-MHz Brukel and Jeol NMR spectrometer) spectroscopic analysis;
ii. Infra-red (IR) spectroscopic analysis (FTIR Thermospectrophotometer);
iii. Mass spectroscopic (MS) analysis [HRMS (ESITOF) Mass spectrometer].

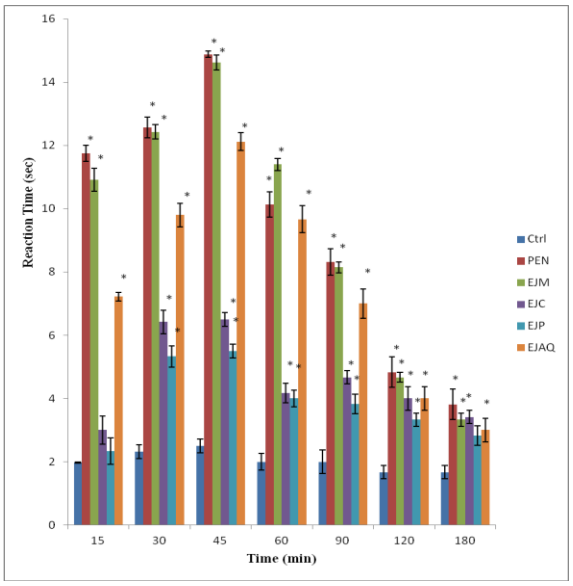
Statistical Analysis

The data is expressed as mean + S.E. (Standard error mean) and the data was analysed by one way ANOVA followed by student's t-test *p<0.001 vs control.

Results

Analgesic Activity Using Eddy's Hot Plate and Tail Immersion Method

The results showed that the reference drug, pentazocine markedly increased the pain latency in mice at all time intervals measured (15, 30, 45, 60, 90, 120 and 180 minutes) after administration. The effect of various extracts of *E. jambolana* (EJM, EJAQ, EJC and EJP) at the dose of 100 mg/kg, i.p. elicited a significant analgesic activity with the hot plate test as shown by the increase in latency time in seconds as compared with the control. The increase in nociceptive latency time was dose-dependent.

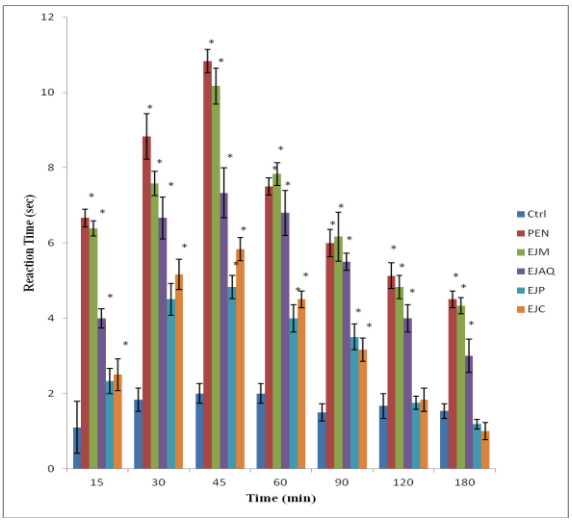


All values are expressed as the Mean ± SEM (n=5).

The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

Key: - Ctrl – Control; PEN – Pentazocine; EJM – Methanol extract of *E. jambolana*; EJAQ – Aqueous extract; EJP – Petroleum ether extract; EJC – Chloroform extract.

Figure 2: Effect of the standard and the various extracts of *E. jambolana* using hot plate method in mice.



All values are expressed as the Mean ± SEM (n=5).

The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

Key: - Ctrl – Control; PEN – Pentazocine; EJM – Methanol extract of *E. jambolana*; EJAQ – Aqueous extract; EJP – Petroleum ether extract; EJC – Chloroform extract.

Figure 3: Effect of the standard and the various extracts of *E. jambolana* using Tail immersion method in mice.

The analgesic activity of various extracts at the dose of 100 mg/kg at latency time periods 15, 30, 45, 60, 90, 120 and 180 min, after the administration of vehicle, standard and plant extracts is presented in Figure 2 and 3. The methanol extract (EJM) of *E. jambolana* showed significant increase in latency time in comparison with the reference drug in hot plate test. The other plant extracts were not as potent as the methanol extract as compared to the latency time of reference drug (Pentazocine). Because methanol extract showed the significant activity, the dose of the methanol

extract was decreased to 50 mg/kg and also increased to 150 mg/kg to observe the effect at various dose levels as shown graphically in table 1 and 2.

Discussion

The present research was focused on the seeds of *E. jambolana* extracts for the pharmacognostic studies such as fluorescence analysis, ash values, extractive values, loss on drying, etc. as per the Indian Pharmacopoeial guidelines. The total ash was 2.43%, acid insoluble ash was 1.5%, water soluble ash was 1.7% and loss on drying was 15%.

The extracts of *E. jambolana* which are best effective in analgesic activity are in conjunction with the phytochemical constituents present in the seeds of the plant.

Table 1: Effect of the most bioactive extract (EJM) of *E. jambolana* at various doses using Hot plate method in mice:

Treatment	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.	180 min.
Control	2.4 ± 0.1571	2.38 ± 0.2519	2.45± 0.2078	1.91± 0.065	1.95± 0.0391	1.9± 0.068	1.86± 0.089
Pentazocine	13.57± 0.211	14.75± 0.163	14.88± 0.098	8.5± 1.979	5.333± 0.0.422	4.833± 0.477	3.817± 0.476
EJM-50	4.827 ± 0.971	6.567± 0.206	9.483± 0.404	8.833± 0.467	7.233± 0.167	5.4± 0.201	4.7± 0.494
EJM-100	13.57 ± 0.211	14.75± 0.163	14.88± 0.098	8.5± 1.979	5.333± 0.422	4.833± 0.477	3.817± 0.476
EJM-150	13.7 ± 0.2422	14.12± 0.171	14.73± 0.2671	11.71± 0.334	9.83± 0.307	8.16± 0.482	6.98± 0.3663

All values are expressed as the Mean ± SEM (n=5) and EJM refers as Methanol extract of *E. jambolana.*

The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

Table 2: Effect of the potent extract (EJM) of *E. jambolana* at various doses using Tail immersion method in mice:

Treatment	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.	180 min.
Control	2.4 ± 0.1571	2.38 ± 0.2519	2.45± 0.2078	1.91± 0.065	1.95± 0.0391	1.9± 0.068	1.86± 0.089
Pentazocine	6.667± 0.235	8.833± 0.601	10.83± 0.307	7.5± 0.224	6± 0.365	5.917± 0.327	4.5± 0.224
EJM-50	5.75 ± 0.159	6.682± 0.241	8.3± 0.432	7.95± 0.465	6.733± 0.203	6.067± 0.067	4.66± 0.4944
EJM-100	6.383± 0.201	7.583± 0.327	10.17± 0.477	7.833± 0.307	6.167± 0.654	4.833± 0.307	4.333± 0.211
EJM-150	6.7 ± 0.2422	7.12± 0.171	11.73± 0.2671	8.71± 0.334	6.83± 0.307	6.16± 0.482	3.98± 0.3663

All values are expressed as the Mean ± SEM (n=5) and EJM refers as Methanol extract of *E. jambolana.*

The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

Thus, the phytochemical screening of *E. jambolana* extracts revealed that the petroleum ether extract contains terpenoids, chloroform extract contains alkaloids and tannins, methanol extract contains flavonoids, tannins, glycosides, saponins and alkaloids were present in aqueous extract. A tentative anthocyanin compound was isolated from bioactive methanol extract by the column chromatography and confirmed by NMR, IR and Mass spectroscopic analysis. Therefore, these phytochemicals are speculated to account for the observed pharmacological effects of the plant's extract. Many authors have reported that the phenolic compounds such as flavonoids, tannins, cyanins, triterpenoids and other phenolic compounds possess multiple biological activities such as antinociceptive and inhibitory action on arachidonic acid metabolism (Kumar *et al.*, 2008; Muruganandan, 2001). The hot plate and tail immersion tests used for the evaluation of analgesic activity have been found to be suitable for the evaluation of centrally acting analgesics. These thermal tests were selected because of several advantages including the sensitivity to strong analgesics, limited tissue damage and ability to mimic human clinical pain conditions. The present experimental study evidenced that the methanol extract of seeds of *E. jambolana* (EJM) demonstrated significantly potent analgesic activity at the different dose levels as comparable to the standard drug, pentazocine and this is due to the presence of maximum polyphenolic compounds in this fraction.

^{1}H- NMR shows the signals at aromatic region at range δ 7.61-6.81 with doublets having ortho-coupling (j = 8.1 Hz) and some resonance in proton spectrum showed at δ 5.54-4.25, due to coupling of glycosidic protons. A singlet at δ 3.55 appeared due to methoxy group and singlets of methyl group appear at δ 1.88 and 1.25 ppm, respectively. ^{13}C- NMR of this unknown compound showed various resonance at the region of aromatic δ 100-150 and aliphatic region δ 20-60. It means the unknown compound may have both aliphatic and aromatic groups. This methoxy group, already assigned by ^{1}H spectrum, further revealed a peak at the region δ 58.5 ppm by ^{13}C- NMR. Its IR spectrum shows the presence of OH group because of the stretching of OH which appeared in the IR spectrum at 34.22 cm^{-1}. It may be intermolecular H- bonding and other strong stretching at 1471 cm^{-1}, due to C-O linkage. According to the above analysis, the unknown compound may be anthocyanin. The tentative structure is finally confirmed by its base peak which appeared in the mass spectrum at 371 M^{+}.

From the spectroscopic analysis, it was observed 3-(2,2,3,3,4,5,5,6,6-nonamethylcyclohexyloxy)-3,4-dihydro-2-(4methoxyphenyl)-2H-chromene-7,8-diol be the tentative structure of the isolated compound.

The anthocyanins present in *E. Jambolana* seed may be responsible for the analgesic activity. Gonzalez and Stasi (2002) reported the analgesic activity of *Wibrandia ebracteata* due to the presence of polyphenolic compounds such as anthocyanins and flavonoids. Miladiyah *et al* (2011) reported that *Manihot esculenta* leaves possess analgesic activity, mainly because of the presence of anthocyanin compounds. Therefore, the anthocyanins are responsible for analgesic activity

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