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EVALUATION OF ANTINOCICEPTIVE EFFECT OF SEED EXTRACTS OF *EUGENIA JAMBOLANA* LINN

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Abstract

Eugenia jambolana belonging to family Myrtaceae is a large evergreen tree of about 50 ft height and possesses a large crown. The present study was designed to evaluate the analgesic activity of the petroleum ether, chloroform, methanol and aqueous extract of seeds of *Eugenia jambolana* Linn. and isolation of bioactive compound. The shade dried, coarsely powdered dried seeds of *E. jambolana* were successively extracted with petroleum ether, chloroform and methanol for 48 hours each using soxhlet apparatus and finally boiled with distilled water for 4 hours. The extracts were filtered, concentrated in *vacuo*. The crude dried extracts were evaluated for analgesic activity using hot plate test and tail immersion test. Maximum bioactive extract was subjected to column chromatography. The effect of various extracts of *E. jambolana* (*intraperitonially*) elicited a significant analgesic activity in hot plate test and tail immersion 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. The methanol extract of *E. jambolana* showed significant increase in latency time. The other plant extracts were not as potent as the methanol extract as compared to the latency time of the standard drug (Pentazocine). As the methanol extract showed maximum analgesic activity therefore, it was subjected to column chromatography and an anthocyanin glycoside was isolated.

The results clearly indicate that the seeds of *E. jambolana* showed good analgesic activity in both the experimental models which could be due to the presence of anthocyanin compound and exert its effect predominantly through μ-opioid receptors.

Key words : *Eugenia jambolana,* Analgesic activity, Anthocyanin glycoside.

Introduction

Eugenia jambolana belonging to family Myrtaceae is a large evergreen tree. It is about 50 ft (8-15 m) in height and possesses a large crown. The tree was introduced from India and tropical Asia to southern Africa for its edible and attractive purple-red fruits. Now it is grown throughout the tropics and subtropics, the best forms are frequently cultivated in Java and Florida. The completely ripe fruit has a combination of sweet, mildly sour and astringent flavor (Warrier *et al.,* 1996).

Stem bark contains friedelin, friedelan-3-α-ol, betulinic acid, β-sitosterol, kaempferol, β-sitosterol-Dglucoside, gallic acid, ellagic acid, gallotannin and ellagitannin and myricetine (Rastogi *et al.,* 1990; Sagrawat, 2006).

Leaves contain β-sitosterol, betulinic acid, mycaminose, crategolic (maslinic) acid, n-hepatcosane, n-nonacosane, n-hentriacontane, noctacosanol, n-triacontanol, n-dotricontanol, quercetin, myricetin, myricitrin and the flavonol glycosides myricetin 3-O-(4"-acetyl)-α-L rhamnopyranosides (Rastogi *et al.,* 1990; Sagrawat, 2006). Flowers contain oleanolic acid, ellagic acid, isoquercetin, quercetin, kampferol and myricetin (Sagrawat, 2006). Fruit pulp contains anthocyanins, delphinidin, petunidin,malvidin-diglucosides (Sagrawat, 2006; Veigas *et al.,* 2007).

The seeds of *E. jambolana* contain various chemical constituents such as jambosine, gallic acid, ellagic acid, corilagin, 3, 6-hexahydroxy diphenoylglucose, 4, 6-hexahydroxydiphenoylglucose, 1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol, etc (Rastogi *et al.,* 1990; Sagrawat, 2006).

Traditionally, *E. jambolana* has been used as a medicine for diabetes, gingivitis, hypertension, polyuria, diarrhoea, dysetry, piles, astringent, liver disorders, stomachic, female sterility, etc. Its theraputic usage has

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been long standing history in traditional system of medicine as in Ayurveda, Unnani, Chinese medicine and in Homeopathy (Warrier *et al.,* 1996).

The several parts of *E. jambolana* possess multifarious pharmacological activities such as antiviral (Bhanuprakash *et al.,* 2007), anti-inflammatory (Kumar *et al.,* 2008), gastroprotective (Ramirez and Roa Jr, 2003), hepato-protective (Das and Sarma, 2009), antioxidant (Sultana *et al.,* 2007), hypolipidaemic (Sharma *et al.,* 2003), cardioprotective (Mastan *et al.,* 2009), antidiarrhoeal (Mukherjee *et al.,* 1998), anti-fertility (Rajasekaran *et al.,* 1988), anti-allergic (Brito *et al.* 2007), anti-pyretic (Chaudhuri *et al.,* 1990), anti-neoplastic (Barh and Viswanathan, 2008), radio-protective and chemo-protective (Li *et al.,* 2009; Zhang and Lin, 2009), CNS depressant (Chakraborty *et al.,* 1986; Kumar *et al.,* 2007), anti-bacterial (De Oliveira *et al.,* 2007), anti-fungal (Jabeen and Javaid, 2010), etc.

Despite a long history of use of *E. jambolana* as a traditional medicine for the treatment of various ailments, the plant has not been subjected to analgesic activity study. Keeping in view the traditional/alternative and complementary medicinal uses, sporadic phytochemical and pharmacological reports, *E. jambolana* seems to hold a great potential for in depth investigation for analgesic activity.

The present study was designed to demonstrate the analgesic activity of petroleum ether (PE), chloroform (CE), ethanol (EE) and water (WE) extract of *E. jambolana* seeds and efforts were made to isolate some compound(s) of most bioactive extract using column chromatography.

Materials and Methods

Plant Material

The seeds of *E. jambolana* were collected from the Botanical garden of Guru Nanak Dev University, Amritsar in the month of July and were dried in shade. The taxonomic identification of the plant was confirmed by Mr. Ram Prasad, Department of Botanical & Environmental Sciences, Guru Nanak Dev University, Amritsar. A voucher specimen number (No. 29) herb has been deposited in herbarium of the same department.

Preparation of extracts and doses

Coarsely powdered dried seeds of *E. jambolana* were successively extracted with petroleum ether, chloroform and methanol for 48 hours each using soxhlet apparatus and finally boiled with distilled water for 4 hours. The extracts were filtered, concentrated in *vacuo* and dried in an oven at 40-50ºC. After removal of solvents under *vacuo* from various extracts, the dried extractives were preserved in vacuum dessicator. Phytochemical screening of various extracts *viz* petroleum ether (EJP), chloroform (EJC), ethanol (EJM) and water (EJAQ) was carried out using standard procedures [Evans, 1996[a], Evans, 1996[b], Evans, 1996[c], Farnsworth, 1996, Khandelwal, 2004).

The test doses 50, 100 and 150 mg/kg of extracts of *E. jambolana* 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* extracts were administered intraperitoneally (*i.p.*) to all the groups of animals.

Animals

The experimental animals Swiss albino mice (Laca strain, 20-25 g) of either sex were procured from Guru Angad Dev Veterinary and Animal Sciences, University, Ludhiana, Punjab, India. The animals received a standard pelleted diet and water *ad libitum,* were maintained under standard environmental conditions (25±5^{0}C with 12hr of light/dark cycle). The experimental protocol was approved by Institutional animal ethical committee and experiments were conducted according to CPCSEA, India guidelines on the use and care of experimental animals.

Evaluation of analgesic activity

Hot plate test

The hot plate, which is commercially available, consists of an electrically heated surface. The temperature is maintained at 55° to 56°C. 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, withdrawal of the paws or licking of the paws (Vogel and Vogel, 2002; Kulkarni, 2003).

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 maintained at exactly 55°C. Within a few seconds, the mice react 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 (Kulkarni, 2003).

Statistical analysis

Each group consisted of five animals. Results were expressed as mean ± S.E.M and all the extracts were compared with control using one way analysis of variance (ANOVA) followed by student's t-test. Differences were considered significant at *p<0.001 vs. control.

Column chromatography of most 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 with chloroform and chloroform: methanol at different ratios (Table 1). The obtained solution was distilled off on the water bath until 2-4 ml of the fraction was left, which was then collected. Each fraction was simultaneously spotted on the silica gel-G coated TLC (Thin layer chromatogram) and plates were developed using chloroform and methanol as mobile phase at different polarities. A total 659 fractions were obtained and the fractions which have similar TLC pattern were pooled together (Table 1).

Table 1: Table showing fractions with similar TLC pattern.

Fraction No.	Pooled fraction	Elutant	Polarity
F1	28-31	Chloroform	-
F2	55-61	Chloroform	-
F3	72-85	$CHCl_3$: MeOH	99:1
F4	103-113	$CHCl_3$: MeOH	98:2
F5	149-166	$CHCl_3$: MeOH	95:5
F6	202-238	$CHCl_3$: MeOH	9:1
F7	296-302	$CHCl_3$: MeOH	9:1
F8	315-327	$CHCl_3$: MeOH	85:15
F9	366-419	$CHCl_3$: MeOH	8:2
F10	439-451	$CHCl_3$: MeOH	3:1
F11	455-472	$CHCl_3$: MeOH	7:3
F12	481-497	$CHCl_3$: MeOH	65:35
F13	501-515	$CHCl_3$: MeOH	3:2
F14	525-550	$CHCl_3$: MeOH	55:45
F15	550-659	$CHCl_3$: MeOH	1:1

Characterization of fractions

The fractions with the similar chromatographic pattern obtained were characterized by using ^{1}H- NMR (300-MHz Brukel and Jeol NMR spectrometer), ^{13}C- NMR (75-MHz Brukel and Jeol NMR spectrometer), Infra-red (IR) (FTIR Thermospectrophotometer) and Mass spectroscopic (MS) analysis [HRMS (ESITOF) Mass spectrometer].

Results and Discussion

The phytochemical screening of *E. jambolana* extracts revealed the presence of terpenoids in petroleum ether extract, alkaloids and tannins in chloroform extract, flavonoids, tannins, glycosides, saponins and alkaloids in methanol and aqueous extract.

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* (EJP, EJC, EJM, and EJAQ) at the dose of 100 mg/kg, *i.p.* elicited a significant analgesic activity in hot plate test and tail immersion 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. 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 Fig. 1 and 2.

Tail immersion test

Hot Plate test

The methanol extract (EJM) of *E. jambolana* showed significant increase in latency time in comparison with the reference drug. The other plant extracts were not as potent as the methanol extract as compared to the latency time of the reference drug (Pentazocine). As the methanol extract showed maximum analgesic activity as compared to other extracts therefore, 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 in table 1 and 2.

Acute pain is a protective response to injury and most often it is nociceptive (*i.e.* resulting from injury or inflammation of somatic or visceral tissue) (Morris and Bill, 2005). The recent literature on pain study shows that pain threshold is relatively constant for an individual, but pain tolerance is influenced by psychological state. For example, the patients with acute pain show normal personality profile, but the degree of pain experienced is related to the degree of anxiety present (Sternbach, 1975). Studies indicate that specialized neural pathways are involved in transmission of pain and these pathways are sensitive to changes in stimulus features, such as intensity, quality and duration which are modulated by opioid peptides, serotonin and norepinephrine (Dubner and Hargreaves, 1989). Transmission of painful stimulus

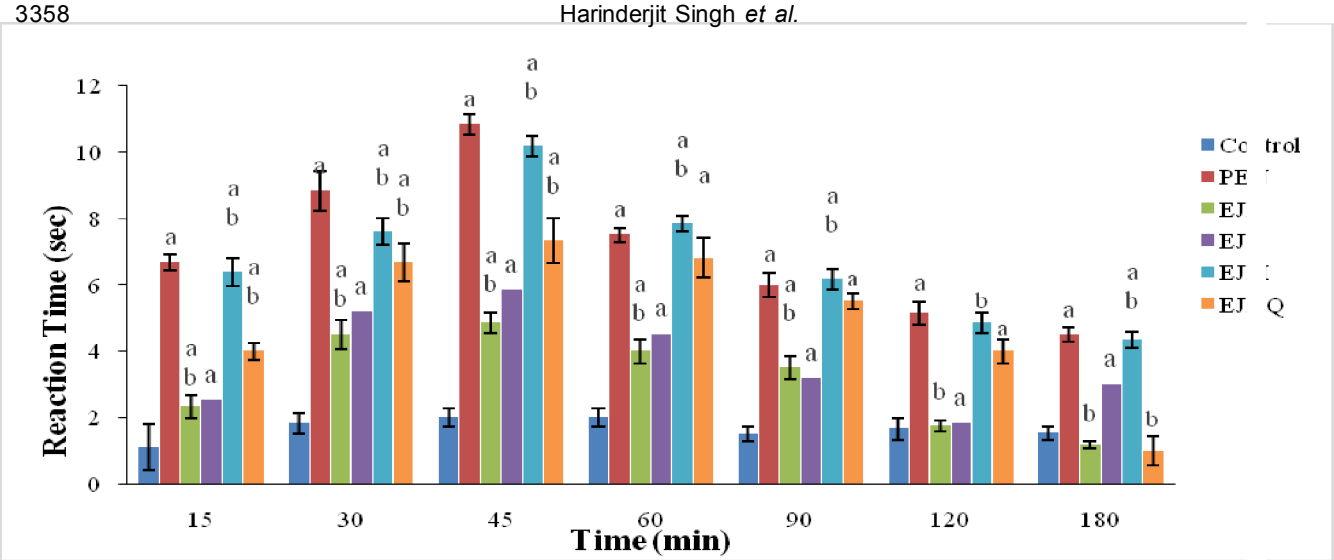

Fig. 1: Effect of the standard and the various extracts of *E. jambolana* using hot plate method in mice. All values are expressed as the Mean ± SEM (n=5). The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

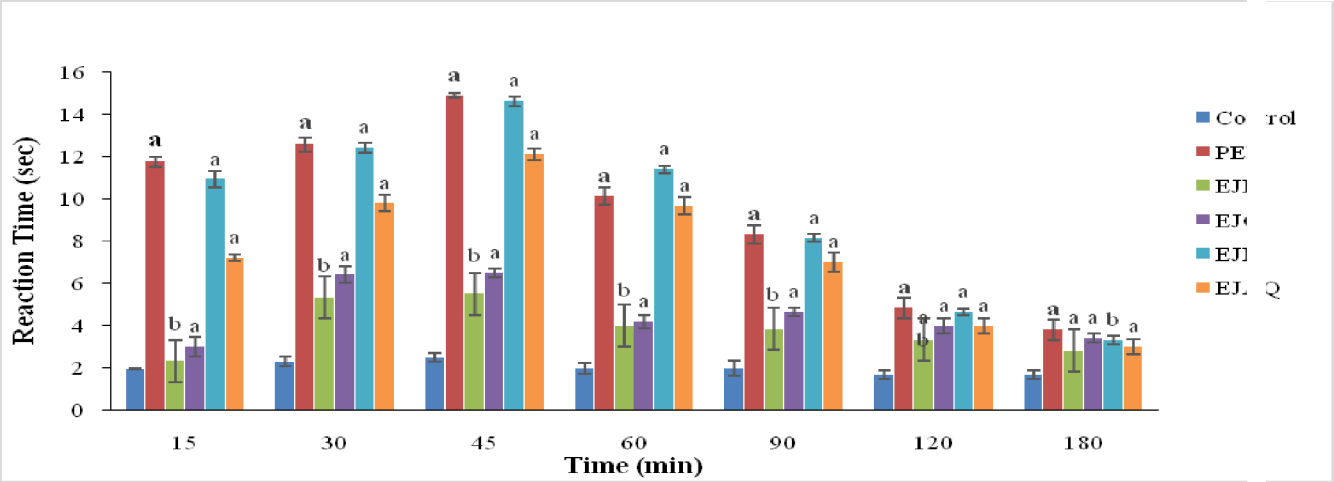

Fig. 2: Effect of the standard and the various extracts of *E. jambolana* using Tail immersion method in mice. All values are expressed as the Mean ± SEM (n=5). The data was analysed by one way ANOVA followed by Student's t-test * p< 0.001 vs. control.

Table 2: Effect of the most bioactive methanol extract (EJM) of *E. jambolana* at various doses using Hot plate method.

Treatment	15 min	30 min	45 min	60 min	90 min	120 min	180 min
Control	2.4±0.15	2.38±0.25	2.0±0.19	2.0±0.08	2.1±0.03*	2.2±0.06	1.86±0.08
Pentazocine	13.57±0.21*	14.75±0.16*	14.88±0.09*	8.5±1.97*	5.33±0.42*	4.83±0.47*	3.8±0.40*
EJM-50	4.82±0.97*	6.56±0.20*	9.48±0.40*	8.83±0.46*	7.23±0.16*	5.4±0.20*	4.7±0.49*
EJM-100	13.57±0.21*	14.75±0.16*	14.88±0.09*	8.5±1.97*	5.33±0.42*	4.83±0.47*	3.81±0.47*
EJM-150	13.7±0.24*	14.12±0.17*	14.73±0.26*	11.71±0.33*	9.83±0.30*	8.16±0.48*	6.98±0.36*

All values are expressed as the Mean ± SEM (n=5). The data was analyzed by one way ANOVA followed by Student's t-test. * p< 0.001 vs. control. EJM refers as methanol extract of *E. jambolana*.

through spinal column and CNS is modulated by excitatory and inhibitory neurotransmitters, as well as action at sodium and potassium channels; norepinephrine and serotonin may be excitatory or inhibitory but they are inhibitory on pain transmission (Morris and Bill, 2005). Nociceptive pain is usually treated with anti-inflammatory or analgesic agents (Morris and Bill, 2005). Non-steroidal anti-inflammatory drugs suppress noxious signals by reducing the sensitivity of peripheral nociceptors and opioid drugs are administered to provide long-lasting pain relief (Dubner and Hargreaves, 1989). Continued and prolonged use of narcotics in patients with pain is not recommended because of serious behavioral consequences, the development of tolerance, and dependence liability. Long-term use of analgesics usually produces behavioral complications that are more difficult

Table 3: Effect of the most bioactive methanol extract (EJM) of *E. jambolana* at various doses using Tail immersion method.

Treatment	15 min	30 min	45 min	60 min	90 min	120 min	180 min
Control	2.35 ± 0.10	2.4 ± 0.20	2.45 ± 0.20	1.91 ± 0.06	1.95 ± 0.05	2.1 ± 0.06	1.9 ± 0.07
Pentazocine	6.66 ± 0.23*	8.83 ± 0.60*	10.83 ± 0.30*	7.5 ± 0.22*	6.0 ± 0.36*	5.91 ± 0.32*	4.5 ± 0.22*
EJM-50	5.75 ± 0.15*	6.68 ± 0.24*	8.3 ± 0.43*	7.95 ± 0.46*	6.73 ± 0.20*	6.06 ± 0.06*	4.66 ± 0.49*
EJM-100	6.38 ± 0.20*	7.58 ± 0.32*	10.17 ± 0.47*	7.83 ± 0.30*	6.16 ± 0.65*	4.83 ± 0.30*	4.33 ± 0.21*
EJM-150	6.7 ± 0.24*	7.12 ± 0.17*	11.73 ± 0.26*	8.71 ± 0.33*	6.83 ± 0.30*	6.16 ± 0.48*	3.98 ± 0.36*

All values are expressed as the Mean ± SEM (n=5). The data was analyzed by one way ANOVA followed by Student's t-test. * $p < 0.001$ vs. control. EJM refers as methanol extract of *E. jambolana*.

to manage than the pain it was desired to eliminate (Halpen, 1977).

The hot plate and tail flick are the most common tests of nociception that are based on a phasic stimulus of high intensity. The nociceptive experience is short lasting and it is well accepted that agonists of μ-opioid receptors produce analgesia in acute pain models (Shuanglin *et al.,* 2000). Therefore, it is believed that substances that are effective in tail flick exert their effects predominantly through μ-opioid receptors. The hot plate test is considered to be selective for opioid- like compounds, which are centrally acting analgesics in several animal species (Janssen *et al.,* 1963).

The extracts of the seeds of *E. jambolana* exhibited analgesic activity but maximum activity was observed with methanol extract. The phytochemical screening of methanol extract of *E. jambolana* revealed the presence alkaloids, flavonoids, glycosides, saponins and tannins.

Fernanda *et al*., (2002) observed that alkaloids have been found to be responsible for analgesic activity in some natural products (Fernanda *et al.*, 2002). The role of flavonoids in pain perception was observed by Bittar *et al*., (2000) and Santa *et al.*, (2011) (Bittar *et al.,* 2000; Santa-Cecilia *et al.,* 2011). Also, there are few reports by Starec *et al.*, (1988), Hosseinzadeh & Younesi ((2002) and Wang *et al.*, (2008) on the role of tannins and saponins in anti-nociceptive activity (Starec *et al.,* 1988; Hosseinzadeh and Younesi, 2002; Wang, *et al.,* 2008).

The methanol extract when subjected to chromatographic separation of different fractions, the clear spectral data was obtained with fraction $F_{13.}$ ^{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 resonances 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 above discussed data, the structure and the IUPAC nomenclature of the compound is 3-(2, 2, 3, 3, 4, 5, 5, 6, 6-nonamethylcyclohexyloxy)-3, 4-dihydro-2-

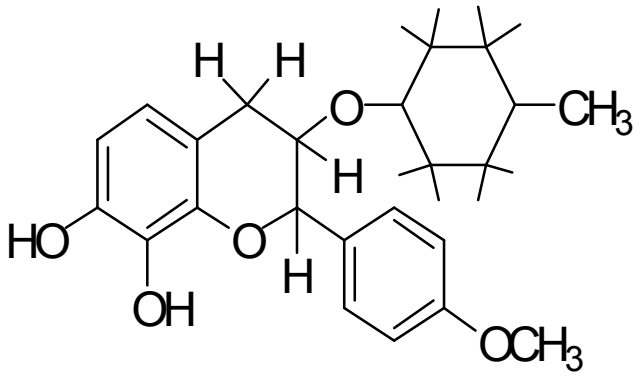

Fig. 3: 3-(2,2,3,3,4,5,5,6,6-nonamethylcyclohexyloxy)-3,4-dihydro-2-(4methoxyphenyl)-2H-chromene-7,8-diol.

(4 methoxyphenyl)-2H-chromene-7, 8-diol (Fig. 3).

An anthocyanin compound in the form of glycoside was isolated from the maximum bioactive methanol extract by the column chromatography and its structure was confirmed by NMR, IR and Mass spectroscopic analysis.

The anthocyanins present in *E. Jambolana* seed may be responsible for 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 (Gonzalez and Di Stasi, 2002). Miladiyah *et al.,* (2011) reported that *Manihot esculenta* leaves possess analgesic activity, mainly because of the presence of anthocyanin compounds (Milandiyah *et al.,* 2011).

Therefore, the anthocyanins may be responsible for the analgesic activity of *E. Jambolana* seeds.

It is concluded that the seeds of *E. jambolana* showed good analgesic activity which could be due to the presence of anthocyanin compound and exert its effect predominantly through μ-opioid receptors.

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