

Original Research Article

Phytochemical Constituents and Analgesic Activity of Ethyl Acetate Fraction of *Punicagranatum* L (Punicaceae)

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Abstract

Purpose: To investigate the active fraction of pomegranate fruit extract and screen it for analgesic activity.

Methods: The analgesic activity of pomegranate ethyl acetate fraction (EtOAc) was examined using three models of pain: writhing, hot tail flick and plantar tests. EtOAc was administered by oral gavage in doses of 100, 150 and 200 mg/kg, p.o., for all the tests and compared to aspirin (100 mg/kg, p.o.) which was used as standard drug. Phytochemical studies of EtOAc were carried out by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection and mass spectrometry (MS).

Results: In the writhing test, the index of pain inhibition (IPI) was 41 % for EtOAc (200 mg/kg, p.o.) and 56 % for aspirin. In the hot tail flick test, EtOAc (200 mg/kg, p.o.) showed analgesia reaching its peak at 60 min with maximum possible analgesia (MPA) of 30.5 %, compared with 43.8 % for aspirin. Plantar test showed that pain was reduced by EtOAc in a dose-dependent manner and compared well with aspirin at 100 mg/kg, p.o., dose. The 200 mg/kg dose showed the highest effect, prolonging withdrawal latency in the left hind paw to 11.9 ± 0.3 compared to aspirin with 13.4 ± 0.2 ($p < 0.001$). HPLC analysis of EtOAc revealed the presence of gallic acid, ellagic acid and punicalagins A & B. Confirmation of their structures was achieved by mass spectroscopy.

Conclusion: EtOAc has a central and peripheral analgesic effect that is most likely due to the presence of gallic acid and ellagic acid.

Keywords: Analgesia, Pomegranate, Gallic acid, Ellagic acid, Punicalagins, Phytochemical constituents

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INTRODUCTION

Punica granatum belongs to the Punicaceae family and is found in over 1000 cultivars [1]. It is cultivated in Iran, Afghanistan, India, Mediterranean countries and to some extent in the USA, China, Japan and Russia. The pomegranate plant is a large shrub or small tree, reaching a height of 20 ft at maturity. It is more or less spiny and deciduous, with small, narrow oblong leaves and short stems. The pomegranate fruit is berry-like with a leathery rind enclosing many seeds surrounded by juicy

arils [2]. Pomegranate fruit is very rich in antioxidants [3] and contains bio-active compounds which aid in good health [4]. Among the antioxidants, punicalagin and ellagic acid have been identified [4]. Punicalagins possess two isomeric forms in pomegranate: α and β . Punicalagin is an ellagitannin in which the gallagic acid and ellagic acid are linked through a molecule of glucose [5]. Punicalagin could inhibit proliferation of human tumor cells (oral, colon or prostate) [6]. Punicalagins and ellagic acid are also responsible for the antioxidant activity and healthy benefits of pomegranates [7]. Ellagic acid

shows health benefits against cancer, cardiovascular diseases and other diseases [8]. Punicalagins inhibit proliferation of human tumor cells (oral, colon or prostate) [6]. Gallic acid possesses several biological activities, including antioxidant, anti-tyrosinase, antimicrobial and anti-inflammatory activities [9].

In our previous work, we have affirmed that the crude ethanol extract was able to produce significant analgesic effect [10]. The present study was undertaken to provide further pharmacological insight into the active fraction, and the mechanisms of analgesic activity.

EXPERIMENTAL

Plant material

Pomegranate fruits were collected from Tripoli, Libya in the fall of 2010. The plant material was identified and authenticated taxonomically at Tripoli-University Herbarium by Dr. Al-Sheriff (Department of Botany, Tripoli-University). A voucher specimen (01563) was deposited at the same herbarium for future reference. Fruits were transferred to 4 °C store room on the harvest day.

Animals

Albino mice (20 - 30 g) and Sprague Dawley rats (180 - 200 g) of either sex were used in this study. The animals were procured from the animal unit of Faculty of Medicine Animals were housed at a temperature of 24 ± 2 °C and relative humidity of 30–70 %. A 12:12 light: dark cycle was followed. All experiments were carried out in strict compliance with the ethical guidelines for investigation of experimental pain in conscious animals [11], after obtaining approval from the animal ethics committee of University of Malaya (Ethic number: FIS/27/01/ 2012/LAB (R)).

Extraction method

Pomegranate extract and its fractions were prepared with references to methods of [10]. The whole fruits were cleaned, freeze-dried and grounded into powder using a blender. Then the powder was dried in an oven at 40 °C for 24 h. The powder was sieved through a 24-mesh filter. The resultant powder of one kilogram was extracted with 2500 ml of 80 % ethanol in water at room temperature for 24 h in a shaking water bath and then was filtered. The ethanol was removed by using RV10 rotary evaporator (IKA, Guangzhou, China) and the resultant residue was crude ethanolic extract (EPE, yield 11 %).

Approximately 100 g of crude ethanol extract was mixed with 200 ml of n-hexane to dissolve the non-polar compounds. The mixture was filtered and the filtrate was concentrated by the rotary evaporator yielding the hexane fraction. The hexane insoluble residue was subjected to partitioning between the ethyl acetate and distilled water (immiscible solvents) using a separating funnel. The ethyl acetate was removed using rotary evaporator and the water was removed by a freeze dryer to give the ethyl acetate fraction (EtOAc) and the water fraction. The yield of the ethyl acetate fraction was 3.5 %. Both the crude ethanolic extract EPE and EtOAc were stored in air tight containers in the dark until further use.

HPLC analysis with UV detection and structure confirmation by mass spectrometry

The sample was prepared and run on Beckman System Gold HPLC with UV- detection to monitor gallic acid, ellagic acid and punicaligin elution. Standards were used to establish the retention time of the compounds of interest. The sample and standards were separated on a 15 cm x 4.6 mm, 3 µm SUPELCO, Supelcosil LC-18-T (58970-U) and HPLC column by employing the following method: Mobile Phase A: 0.1 % Phosphoric Acid in water (buffer system), Mobile Phase B: 100 % Acetonitrile with the following gradient: 0-20 min, 0 % B; 20 - 25 min, 50 % B; 25 - 45 min, 0 % B with a flow rate of 0.5 ml/min, and UV detection monitored at 220, 246, 280 nm. The identification of each compound was made by comparing the retention times, UV spectra and high resolution mass spectra with those corresponding to standards. Gallic Acid (TCI, Portland, OR; #G0011): 50 µg/µl stock made up in MeOH

Ellagic acid (Indofine, Hillsborough, NJ; #E-001): 0.604 µg/µl stock made up in MeOH. Punicalagin (Sigma, St Louis, MO; #P0023): 5 µg/µl stock made up in MeOH, Sample (9.38 mg), was dissolved in (98 µL DMSO) to give a final concentration of 100 µg/µL. 500 µg of sample was loaded and the fractions were collected. The fractions matching the retention time of the standards were checked by direct infusion into a LTQ-Velos Orbitrap Pro using an Advion Triversa nano spray robot after cleanup of a small portion using C18 zip-tips (to desalt). HPLC traces and mass spectra are attached. Mass error relative to theoretical is indicated in the spectra.

Acetic acid writhing test

Mice were divided into five groups of six mice each and pretreated with (saline), aspirin (100

mg/kg, p.o.), and EtOAc (100, 150 and 200 mg/kg, p.o.) 30 min before injection of acetic acid (0.6 v/v) i.p. The number of abdominal constrictions (full extension of both hind paws) produced in each group was counted for 30 min and compared to the response in the control group. The antinociceptive activity was expressed as percentage of inhibition of the abdominal constrictions [12].

Hot tail flick test

Mice were divided into five groups of six mice. The lower part of each tail was immersed in a beaker of water maintained at a temperature of 55 ± 0.5 °C. The reaction time was the time for the tail to be withdrawn from water with a cut-off time set at 10 s. The reaction time was measured at 15, 30, 45, 60 min after oral (by gavage) administration of EtOAc (100, 150, 200 mg/kg, p.o.) or aspirin (100 mg/kg, p.o.) [13].

Plantar test

The Hargreaves' test was performed using a standard apparatus (Ugo Basile). The test consisted of placing a rat in a transparent acrylic box and a mobile infrared heat lamp was placed underneath the hind paw to be tested. The latency of the paw withdrawal response was measured automatically with the help of a photoelectric-sensitive device after the thermal radiant stimulus was applied to the plantar surface. The latency of the withdrawal response of each hind paw was determined at 30, 60, 90, 120, 150, 180, 210, 240, 300 min post-challenge, with the left paw being stimulated with the

carrageenan and the right one stimulated with sterile saline. EtOAc (100, 150, 200 mg/kg, p.o.) and aspirin (100 mg/kg, p.o.) was administered by oral gavage 30 min before the subcutaneous injection of both rat hind paws [14].

Statistical analysis

Data were represented as mean \pm standard error of the mean (SEM). The results were analyzed using one way analysis of variance (ANOVA) for comparison between groups followed by students't-test.

RESULTS

Phytochemical profile

HPLC analysis showed the presence of gallic acid (50.3 mg/kg), ellagic acid (81.7 mg/kg) and punicalagins A & B (4.98 mg/kg) (Figure 1). The mass spectra of gallic acid and ellagic acid are shown in Figures 2 and 3.

Writhing

EtOAc at doses (100, 150, 200 mg/kg, p.o.) reduced pain response in a dose dependent manner when compared to control group aspirin (100 mg/kg, p.o.), the standard drug significantly reduced the number of writhing. The index of pain inhibition (IPI) induced by the ethanol pomegranate extract at all doses was 24, 31 and 41 %, respectively, while aspirin produced IPI of 56 % (Figure 4A)

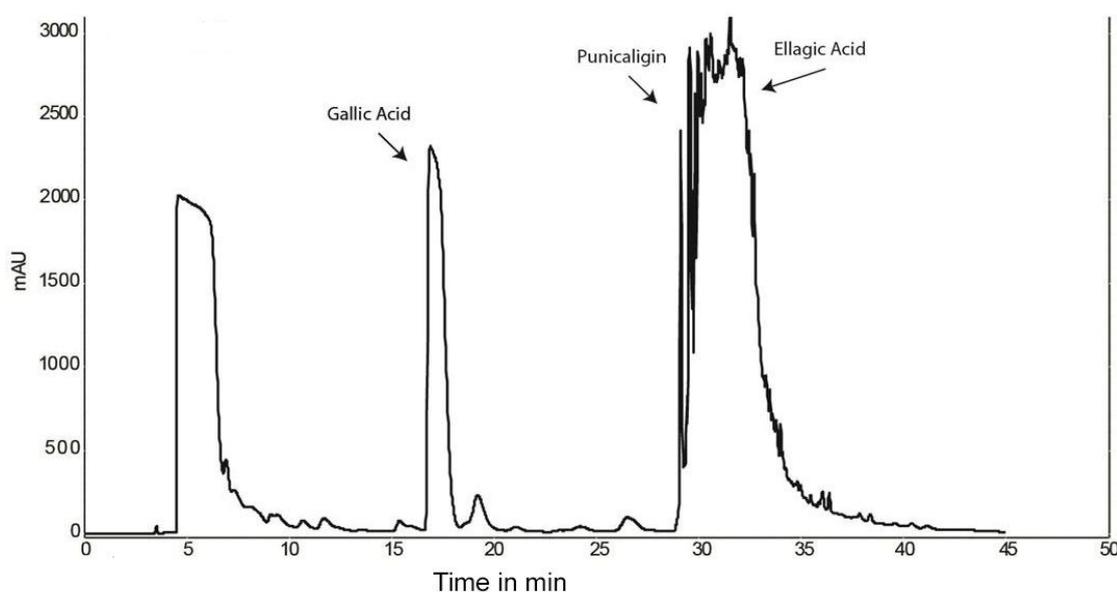


Figure 1: High performance liquid chromatogram of EtOAc. Peaks indicate gallic acid, punicaligin and ellagic acid

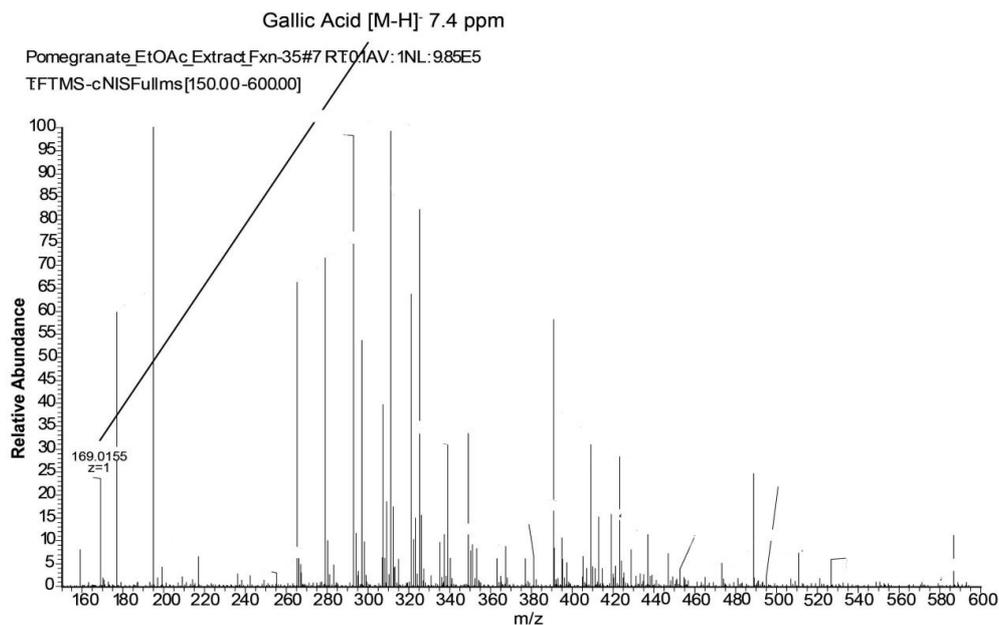


Figure 2: Mass spectrum of EtOAc showing the presence of gallic acid ([M-H]-m/z 169)

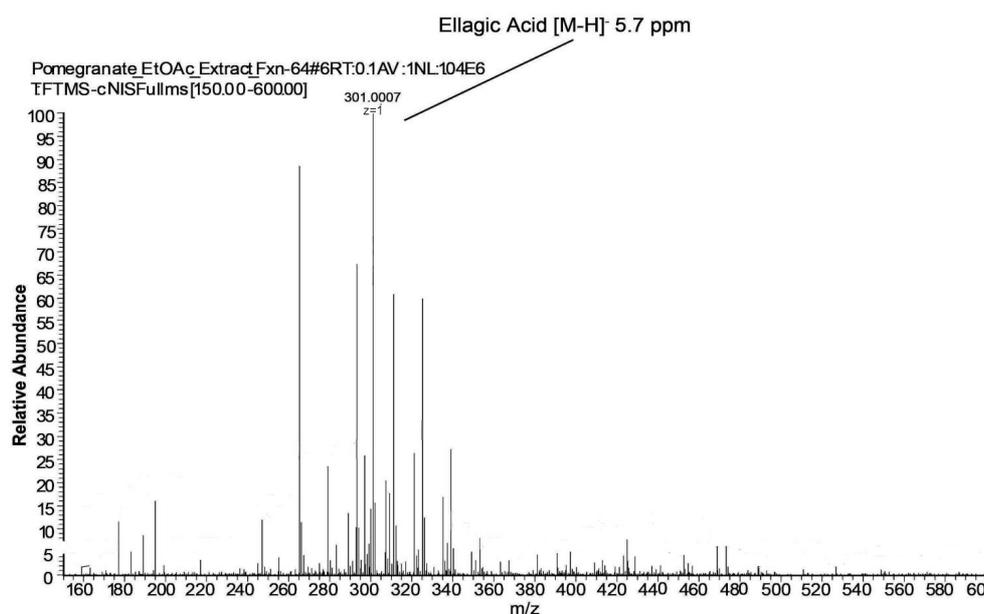


Figure 3: Mass spectrum of EtOAc showing the presence of ellagic acid ([M-H]-m/z 301)

Hot tail flick

The hot tail flick test results showed that MPA (maximum possible analgesia) increased in a dose-related manner, reaching a peak at 60 min for all doses, i.e., 12.3, 20.6 and 30.5 % at 100, 150 and 200 mg/kg, respectively, and 43.8 % for aspirin (Figure 4B).

Peripheral anti-nociceptive action on right hind paw injected with carrageenan

For the control group (carrageenan) withdrawal latencies were shortened from 10.0 ± 0.2 to $5.6 \pm$

0.2 indicating (hyperalgesia) and for the EtOAc (100,150 and 200 mg/kg), carrageenan induced hyperalgesia was reduced. The anti-nociceptive effect was exhibited in a dose dependent manner where 200 mg/kg showed significant response which was comparable to aspirin, the significant effect started at 90 min ($p < 0.05$) and remained constant throughout the observation period of 5 hours. The highest effect withdrawal latency prolonged to 13.4 ± 0.3 ($p < 0.001$) compared to aspirin 13.7 ± 0.2 ($p < 0.001$) Figure 5A.

Central anti-nociceptive action on right hind paw injected with saline

Latencies were shortened from 10.5 ± 0.2 to 5.8 ± 0.2 for the control group (saline) and for the ethyl acetate fraction of pomegranate (100, 150 and 200 mg/kg). Algesia was reduced and antinociceptive effect was exhibited in a dose

dependent manner. Significant analgesia was observed at 90 min ($p < 0.01$) and remained constant throughout observation period of 5 hours this significant effect was comparable to aspirin. At 200 mg/kg withdrawal latency prolonged to 11.9 ± 0.3 ($p < 0.001$) compared to aspirin 13.4 ± 0.2 ($p < 0.001$) Figure 5B.

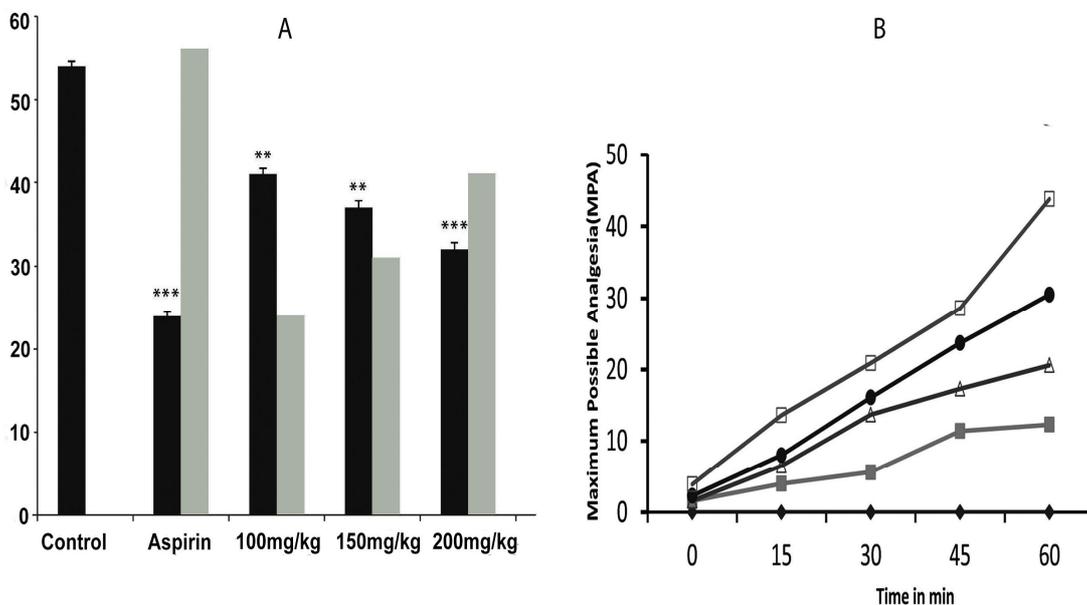


Figure 4: (A) Effect of EtOAc (100, 150 and 200 mg/kg, p.o.) and aspirin (100 mg/kg, p.o.) on writhing of mice. IPI: index of pain inhibition. Key: Number of writhes (■), IPI(■); (B) Effect of mg/kg (■), 150mg/kg(Δ), 200mg/kg(●), aspirin(□). Data are expressed as mean \pm SEM (n = 6). The differences between groups were statistically analysed by ANOVA followed by t-test; ** $p < 0.01$, *** $p < 0.001$ vs control (saline)

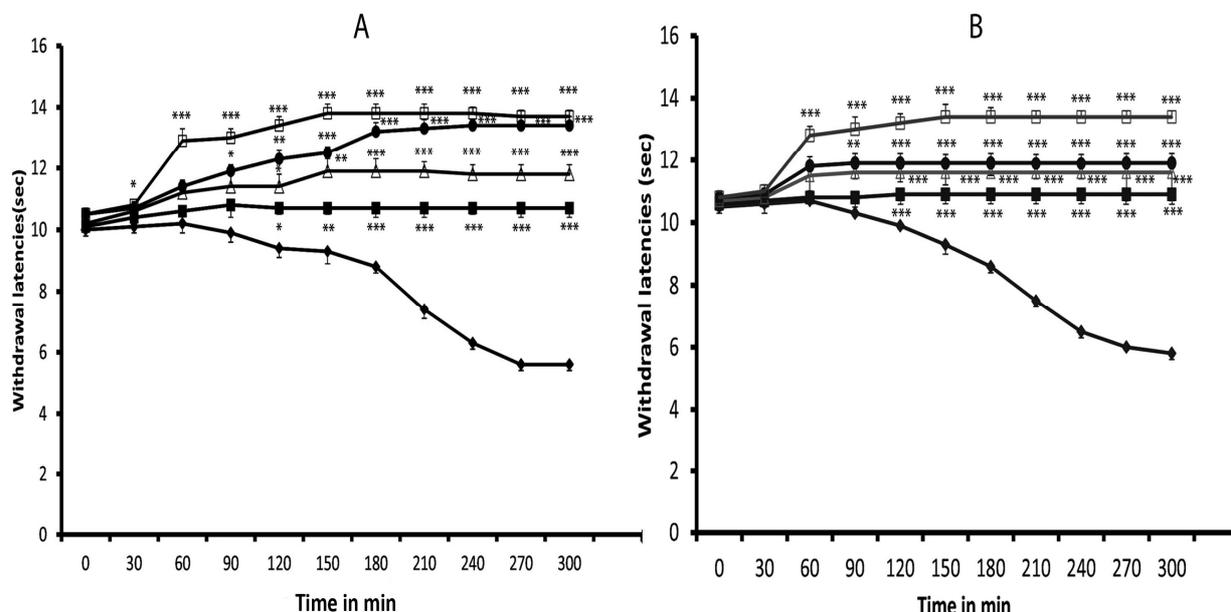


Figure 5: (A) Effect EtOAc (100, 150, 200 mg/kg, p.o.) on withdrawal latencies and aspirin (100 mg/kg, p.o.) vs control (carrageenan) on right hind paw in plantar test; (B) Effect of EtOAc (100, 150, 200 mg/kg, p.o.) on withdrawal latencies and aspirin (100mg/kg, p.o.) vs control (saline) on left hind paw in plantar test; **Key:** Saline (♦), 100 mg/kg (■), 150 mg/kg (Δ), 200 mg/kg (●), aspirin (□); data are expressed as mean \pm SEM (n = 6); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs control

DISCUSSION

In the present study, we performed a phytochemical analysis, investigated EtOAc analgesic properties and tried to elucidate EtOAc mechanism of action.

The acetic-acid-induced abdominal constriction test is a well-known model for inflammatory pain and has been used to screen for analgesic or anti-inflammatory properties of new agents [12]. In addition, it is frequently used as a model to study the peripheral anti-nociceptive effect of extracts [15]. This model of nociception is well known to represent the stimulation of peripheral mechanism since the administration of phlogogen lead to an increase in the levels of cyclooxygenase (COX) and lipoxygenase (LOX) [16]. The ability of EtOAc to attenuate acetic-acid-induced abdominal constriction suggests that anti-nociceptive mechanism may involve the inhibition of COX and LOX in the peripheral tissues leading to a decrease in PGEs synthesis and impediment of the pain transduction in primary afferent nociceptor.

The hot tail flick test is usually used in conjunction with the writhing test to distinguish central nervous system effects from peripheral ones. Our results indicated that EtOAc significantly prolonged the time that mice could endure heat stimuli in a dose dependent manner. Similar findings were reported by Parminder [17]. The Hargreaves model (1988) is actually a suitable model in determining the anti-inflammatory effect and its mechanism of action either acting on COX-1 or COX-2 and via either the peripheral or CNS response [18]. This model is also a sequel to carrageenan-induced oedema model. Thus, rat's hind paw injected with carrageenan can be used to evaluate both anti-inflammatory and anti-nociceptive effects.

EtOAc given orally produced a dose-dependent significant suppression on pain induced by the radiant heat applied to the plantar surface of the heel of the right hind paw injected with carrageenan (Figure 5A) and left hind paw injected with saline (Figure 5B).

Ellagic acid has been reported to possess both peripheral and central anti-nociceptive activities when tested by writhing test and hot tail flick test [19,20]. Some studies have confirmed the anti-nociceptive effects of gallic acid in models of acute pain such as the acetic acid writhing test and cyclophosphamide-elicited cystitis [9,21]. In addition gallic acid has also demonstrated to

possess anti-nociceptive property when studied on a neuropathic pain model [7].

According to the previous reports, ellagic acid and gallic acid exhibit analgesic potential through peripheral and central mechanisms [19-21,7]. EtOAc showed analgesic potential through both peripheral and central components. Therefore, it is suggested that ellagic acid and gallic acid present in EtOAc fruit extract may be the compounds responsible for its analgesic effect.

An important limitation of this study is that despite the successful isolation of both ellagic acid and gallic acid, they have not been tested for their relative analgesic activity. This was due to lack of funds at the time of study. However, this limitation has been expanded in our progressing studies which are examining the possible mechanism of action of pure isolates of both ellagic acid and gallic acid.

CONCLUSION

The findings of this study demonstrate that EtOAc exhibits significant analgesic activity, possibly, via central and peripheral mechanisms. Ongoing studies in our laboratory will further confirm whether ellagic acid and/or gallic acid are responsible for its analgesic actions.

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