

Original Research

High-performance thin-layer chromatography fingerprinting and anti-inflammatory and antinociceptive activities of *Pyracantha coccinea* M.Roem.: A laboratory-based study

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Abstract: This study aimed to investigate the high-performance thin-layer chromatography (HPTLC) fingerprinting and *in-vivo* anti-inflammatory and antinociceptive activities of different leaf extracts (ethanolic extract, n-hexane, chloroform, and ethyl acetate) of *Pyracantha coccinea* M.Roem. plant. A total of one hundred and twenty-four Wistar rats for anti-inflammatory and antinociceptive tests (carrageenan and formalin tests, respectively) were treated with two doses of the ethanolic extract (100 and 300 mg/kg), two doses of other plant fractions (30 and 100 mg/kg), Diclofenac (25 mg/kg) as the positive control, and normal saline as the negative control group, by oral gavage route. HPTLC fingerprinting is used for assay of terpenoids, flavonoids, alkaloids, and antioxidant activity. Treatment of the animal with the ethanolic extract at doses of 100 and 300 mg/kg, both ethyl acetate and chloroform fractions at the dose of 30 mg/kg and 100 mg/kg decreased the pain score in the formalin test and paw edema caused by carrageenan relative to control group significantly. Moreover, these extracts reported the highest amounts of flavonoid contents. In conclusion, phytochemicals present in *Pyracantha coccinea* M.Roem. leaves have anti-inflammatory and antinociceptive activities. Future studies are needed to identify the compounds with the anti-inflammatory and antinociceptive potential present in the plant.

Key words: Flavonoid contents; Carrageenan test; Formalin test; Anti-inflammatory; Antinociceptive.

Introduction

Pain is an unpleasant sensation and is associated with actual or potential tissue damage (1). In any form, pain is a significant health problem and is one of the most common reasons for emergency room visits and in-patient and out-patient prescriptions (2). Until this day, Acetaminophen and non-steroidal anti-inflammatory agents have been the first-line treatment for pain. After that, opioids are the main drugs for pain and discomfort management. While having efficacy, these drugs can induce adverse events such as gastrointestinal upset, cardiovascular effects, renal function abnormality or dysfunction, and dependency (3, 4).

Inflammation results from many inducing factors and causes discomfort for the patients in various forms, including pain. On the other hand, inflammation can compromise the immune system, reflect on diagnosis, and delay treatment. The correlation between inflammation and nociception is obvious. This correlation is due to chemical mediators' production, which can dramatically raise the nociceptors' impulsion through the sensory afferent fibers (5).

Nature can be a rich source of compounds with pharmacological activity (6, 7). Nature and its wide pool of phytochemicals are resources for developing anti-inflammatory and antinociceptive agents (8, 9). Herbal medicine's effectiveness and safety have been tested in

in-vitro, animal models, and human (10-12).

Pyracantha genus belongs to the Rosaceae family and is geographically distributed in warm temperate such as the Mediterranean to cool and subtropical climates. *Pyracantha* spp. are native to Southwest Europe east to Southeast Asia, mainly planted and used as garden ornaments. Many species exist in the Eastern Mediterranean region and central Europe to Asia and China, and Taiwan. Almost ten species exist in the *Pyracantha* genus, including *Pyracantha coccinea* M.Roem.. *P. coccinea* M.Roem. (scarlet firethorn) is an evergreen shrub, widely distributed in Temperate Asia and Europe (Iran, Lebanon, Turkey, Armenia, Azerbaijan, Georgia, Ukraine, Albania, Bulgaria, Former Yugoslavia, Greece, France, Spain, central and southern Italy) (13). *P. coccinea* M.Roem. is used in traditional medicine for activities such as cardiac protective, anti-inflammatory, antioxidant, and wound and scar dressing. Former studies have shown antioxidant and anti-inflammatory activity. Also, there have been investigations confirming apigenin and naringenin's presence, flavonoids with anti-inflammatory and antinociceptive effects (14).

The present study aimed to determine the anti-inflammatory and antinociceptive activities of this plant and report high-performance thin-layer chromatography fingerprinting. To the best of our knowledge, this is the first report of high-performance thin-layer chromatography fingerprinting and anti-platelet-aggregation acti-

vities of different leaf extracts of *P. coccinea* M.Roem.

Materials and Methods

Plant preparation and identification

Of the 3 kg, fresh leaves of *Pyracantha coccinea* M.Roem. were collected from the Mohammad-Shahr region in Alborz, Iran, in September 2019 (Figure 1). A botanist from Shahid Beheshti University of Medical Sciences, Tehran, Iran, identified the taxonomically the plants. This specimen with the code SBMU-1150 is kept in the Phytochemistry Research Center at Shahid Beheshti University of Medical Sciences. Leaves were washed three times with distilled water and then dried at 25 ± 5 °C for one week.

Preparation of ethanolic extract and fractions

After the plant was entirely dried, it was powdered to fine particles by a mechanical grinder. With the maceration method, 1853g of the powder was extracted with 80% ethanol. The powder was macerated for 72 hours at this method, the extract was filtered, and then it was replaced with solvent for 24 more hours (15). After collecting all of the extracts, it was evaporated by a rotary evaporator (Laborota 4000, Heidolph, Germany). Three fractions were drawn up through liquid-liquid extraction with n-hexane, chloroform, and ethyl acetate solvents. Doses of 100 and 300 mg/mL from the ethanolic extract and 30 and 100 mg/mL from the fractions (n-hexane, chloroform, and ethyl acetate) were prepared following steps (Figure 2) (16).

Phytochemical screening tests

Phytochemical analysis was accomplished to confirm the presence of terpenoids, alkaloids, saponins, flavonoids, and tannins that were carried out by the methods described previously (17).

Plant fingerprinting with high-performance thin-layer chromatography (HPTLC)

HPTLC was conducted on Silica gel 60 F 254 HPTLC Plate 10*10 (Merck, Germany) as the stationary



Figure 1. *Pyracantha coccinea* M.Roem. plant used for extract analyses in this study.

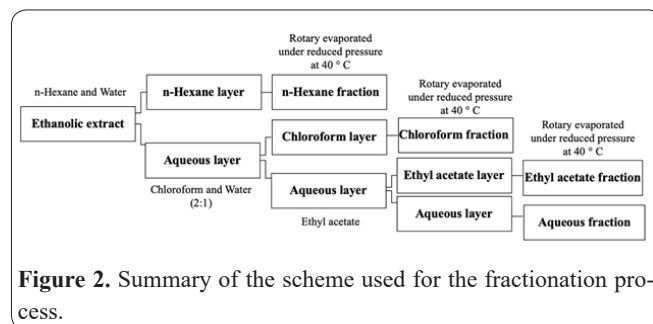


Figure 2. Summary of the scheme used for the fractionation process.

phase. A total of 20 mg each of the extracts and fractions were spotted. For terpenoid compounds identification, the mobile phase was toluene:chloroform:ethanol (4:4:1), and anisaldehyde-sulfuric acid reagent was used for derivatization. For flavonoid compounds identification, the mobile phase was chloroform:ethyl acetate:acetone:formic acid (4:3:2:1), and derivatization reagent was natural product reagent. For Alkaloids compounds identification, the mobile phase was toluene:methanol-DEA (8:1:1), and derivatization reagent was Dragendorff reagent.

For antioxidant activity by 1,1-diphenyl- 2-picrylhydrazyl (DPPH), the mobile phase was chloroform-ethyl acetate-acetone-formic acid (4:3:2:1), and derivatization reagent was DPPH(15). Chromatography bands were observed under UV, fluorescence, and visible lights.

Preparation of ethanolic extract and the fractions doses

The concentrated ethanolic extract and the fractions of *P.coccinea* M. Roem. were suspended in distilled water and tween 80, creating a heterogeneous suspension. Two doses (100 and 300 mg/mL) from the ethanolic extract and two doses (30 and 100 mg/mL) from fractions were prepared. The positive control group received Diclofenac (25 mg/kg) via intraperitoneal (IP) route. NaCl 0.9% solution (normal saline) was given to the negative control group by oral gavage. The extraction was administered by oral gavage route with a dose of 1 mL/kg.

Animal groups

A total number of one hundred and twenty-four male wistar rats weighting 100-120g were purchased from Pasteur Institute (Tehran, Iran). Test groups for paw edema test and formalin test each contained 62 rats. The rats were held in 30±10% humidity, 22±2°C temperature, 12:12 h light/dark cycle (lights on at 0800h), and free access to food and water. The research was approved by Organizational Ethics Committee, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Ethics code IR.SBMU.PHARMACY.REC.1398.337) and was conformed with guidelines (18).

Formalin test

The rats were kept in individual cages for 1 hour before the test to adapt to their environment. Thirty minutes before the test, the drugs or saline (control group) were administered by oral rout. The treatment groups received either of the treatments: the ethanolic extract (100 mg/kg and 300 mg/kg), the three fractions of extract (30 mg/kg and 100 mg/kg). The positive control group received diclofenac (25 mg/kg; i.p.). For pain

and inflammation induction, 40 μ L of 5% formalin solution in normal saline was injected subcutaneously (s.c.) into rat right hindpaw pain related behavior was assessed and scored for 60 min by method described by Dubuisson & Dennis (1977); as 0 = The rat's usual weight-bearing on the injected paw; 1 = slightly limping during its locomotion or resting the injected paw on the floor; 2 = The elevation of the paw so that at most only the nail touches the floor; and 3 = licking, grooming or biting the injected paw. The scores were recorded every 15 s and an average of each 5-min interval was calculated (19).

Carrageenan-induced paw edema test

Thirty minutes after oral administration of drugs or saline (in control group), 0.1 mL of 1% w/v carrageenan solution (in saline) was injected into sub-plantar tissues of rat's left hind paw for edema induction. The paw volume was measured by calculating the displacement of mercury in the open-top cylinder. In order to assess paw edema, paw volume was measured immediately before and 3h after carrageenan injection (20, 21).

Statistical analysis

For the analysis of pain related behavior in the formalin test, the area under the curve (AUC) of the pain score-time graph was assessed by one-way analysis of variance or ANOVA followed by Bonferroni's post-test and $P < 0.05$ was considered as significant. To investigate changes in paw volume in carrageenan test, one-way ANOVA followed by Tukey's post-test was used and $P < 0.05$ was considered as statistically significant. The statistical analysis was performed by Graphpad Prism[®] 8.0 (Graphpad Software Inc.).

Results

Phytochemical screening tests

The results of phytochemical screening tests of the ethanolic extract are shown in Table 1.

Plant fingerprinting with high-performance thin-layer chromatography (HPTLC)

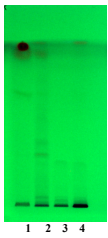
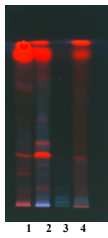
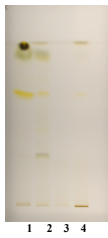

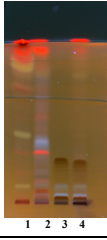
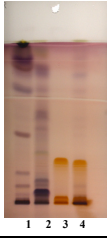
The fingerprinting with high-performance thin-layer chromatography (HPTLC) for flavonoids, terpenoids, alkaloid compounds, and antioxidant activity is shown in Table 2-5. Terpenoids appeared as purple-blue lines after spraying the plate with the reagent under the visible light. The majority of terpenoids were detected in chloroform (fraction 2).

Flavonoids appeared as sharp blue lines in fluorescent light and light-yellow spots at the visible spectrum. A significant part of flavonoids was found in chloroform (fraction 2) and ethyl acetate (fraction 3).

Table 1. Phytochemical screening test results.


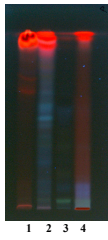


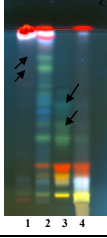
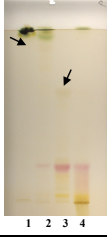
Photochemical component	Status
Terpenoids	+++
Flavanoids	+++
Alkaloids	+
Tannins	++
Saponins	++

Table 2. Identification of terpenoid compounds in *P.coccinea* M.Roem. by HPTLC. Anisaldehyde-sulfuric acid reagent was used in this assay.

	Ultraviolet	Fluorescence	Visible
Before derivatization			
After derivatization			

1: Ethanolic extract, 2: n-Hexane, 3: Chloroform, 4: Ethyl acetate.

Table 3. Identification of flavonoid compounds in *P. coccinea* M.Roem. by HPTLC. On the HPTLC plate, natural product reagent was sprayed.

	Ultraviolet	Fluorescence	Visible
Before derivatization			
After derivatization			

1: Ethanolic extract, 2: n-Hexane, 3: Chloroform, 4: Ethyl acetate.

Yellow, orange, and brown lines in the visible spectrum state the presence of alkaloids. The alkaloid compounds in this extract were insignificant.

The yellow marks on the purple background at the visible spectrum indicate certain compounds with antioxidant activity. The chloroform (fraction 3) showed high antioxidant activities among the ethanolic extract and other fractions.

Effects of *P. coccinea* M.Roem. on rat pain related behavior in formalin test

Fig. 3 shows changes in pain related behavior during 60 min of formalin test. A significant interaction between time and treatment was shown [$F(36, 336) = 5.162$; $p < 0.0001$]. Further analysis by Bonferroni's test revealed a significant decrease in pain related behavior in group received plant extract (100 mg/kg; 15, 20, 30, 35, 40, 45, 50, 55, and 60 min after formalin injection) and also group received plant extract (300 mg/kg; 15-60

Table 4. Identification of alkaloid compounds in *P. coccinea* M.Roem. by HPTLC. On the HPTLC plate, natural product reagent was sprayed.

	Ultraviolet	Fluorescence	Visible
Before derivatization			
After derivatization			

1: Ethanolic extract, 2: n-Hexane, 3: Chloroform, 4: Ethyl acetate.

Table 5. Antioxidant screening using the HPTLC. method.

	Ultraviolet	Fluorescence	Visible
Before derivatization			
After derivatization			

min after formalin injection) compared with the control group ($p < 0.01$). Also, the group received diclofenac (25 mg/kg) showed significant decrease in pain related behavior at 15-60 min after formalin injection compared with the control group ($p < 0.01$).

A significant change in AUC of pain score was shown in Fig. 4. Further analysis revealed that rats treated with *P. coccinea* M.Roem. ethanolic extract (both at 100 mg/kg and at 300 mg/kg) showed a significant decrease in AUC of pain score compared with the control group ($p < 0.001$). Also, group treated with diclofenac (25 mg/kg) showed a significant decrease in AUC of pain score compared with the control group ($p < 0.001$).

Treatment with n-hexane, chloroform, and ethyl acetate fractions of plant extract caused a significant change in AUC of pain score [$F(7, 38) = 121.1, p < 0.0001$; Fig. 5]. The most effective fractions in reducing AUC of pain score were chloroform and ethylacetate fraction ($p < 0.001$ compared with the control group at both 30 and 100 mg/kg). However, the n-hexane fraction was also effective at the dose of 30 mg/kg ($p < 0.05$) and 100 mg/kg ($p < 0.001$). The effect of n-hexane fraction was

significantly less than chloroform and ethylacetate fractions at the same administered dose ($p < 0.001$).

The effects of *P. coccinea* in carrageenan-induced paw edema test

The anti-inflammatory activity against acute paw edema in animal models of the *Pyracantha coccinea*

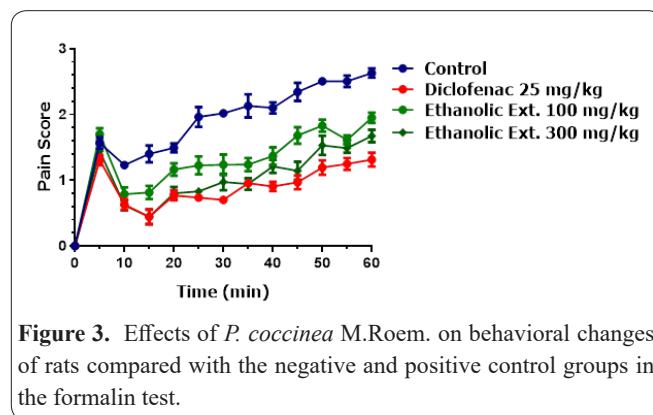


Figure 3. Effects of *P. coccinea* M.Roem. on behavioral changes of rats compared with the negative and positive control groups in the formalin test.

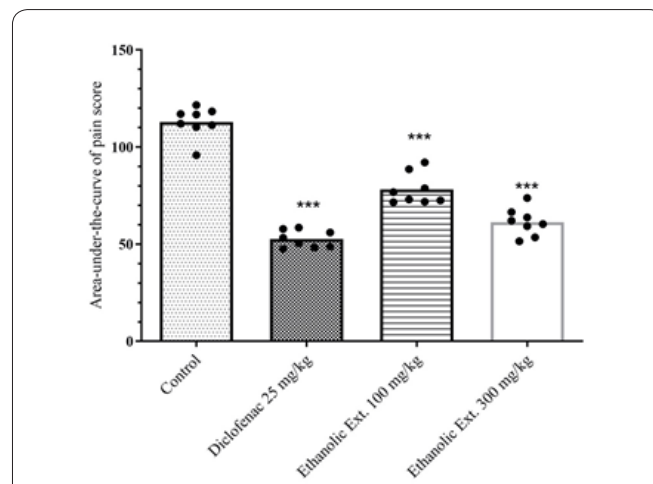


Figure 4. The area under the curve of pain score calculated from figure 3. Data were shown as Mean and the individual distribution ($n = 8$ in each group). *** $p < 0.001$ Significant difference compared with the control group (saline).

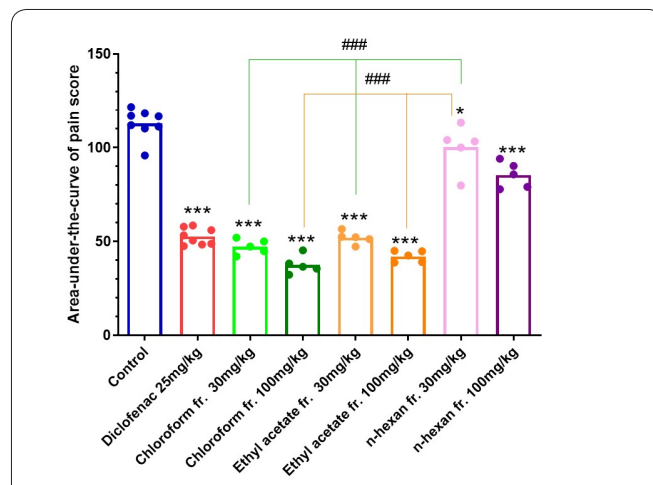


Figure 5. Effects of *Pyracantha coccinea* M.Roem. chloroform, n-hexane, and ethyl acetate fractions on rats pain scores. The AUC was calculated from pain scores obtained in 5 min intervals (data not shown). Results were shown as mean and individual distribution of data ($n = 5-8$ in each group). * $p < 0.05$, *** $p < 0.001$ significant difference compared with the control group (saline). ### $p < 0.001$ significant difference compared with the n-hexane extract at the same dose.

M.Roem. ethanolic extract at the doses of 100 and 300 mg/kg and fractions at the doses of 30 and 100 mg/kg were shown in Fig. 6 and Fig. 7, respectively.

The control (saline) group showed about 0.45 cm³ increase in paw volume 3h after carrageenan injection. Pretreatment of rats with ethanolic extract (100 or 300 mg/kg) reduced paw edema to 0.3 and 0.25, respectively. The paw edema was significantly lower compared with the control group ($p < 0.001$; Fig. 6).

Moreover, rats treated with Chloroform (30 and 100 mg/kg) and ethylacetate (30 and 100 mg/kg) fractions showed significantly lower paw edema compared with the control group ($p < 0.001$; Fig. 7) while n-hexane fraction (both at 30 and at 100 mg/kg) was ineffective.

Discussion

Medicinal plants have been a rich source for drug design and treatment of diseases for many years. Bioactive compounds extracted from medicinal herbs are a potential resource for anti-inflammatory and antinociceptive agents, and antinociceptive and anti-inflammatory activities are widely reported in plant extracts. The

ethnomedicinal references reveal the antinociceptive and anti-inflammatory effects of *P. coccinea* M.Roem.. The *Pyracantha* genus is used for different pharmacological effects. *Pyracantha fortunata* is used as a skin whitening agent in Japanese cosmetics (22). In Ayurvedic medicine, *Pyracantha* spp. is used to treat hepatic, skin, and stomach disorders (23). In traditional medicine of Europe, *Pyracantha coccinea* is taken as a heart soother. This plant is also used in diarrhea and urinary diseases (24). In one study, the pyracenic acid extracted from *Pyracantha crenulate* showed anti-inflammatory activity (25). A study by Fico *et al.*; in 2000 showed *P. coccinea* M.Roem extract contains high levels of apigenin and naringenin (14). Apigenin and naringenin both have anti-inflammatory and antinociceptive activity (26, 27). From this evidence, it can be concluded that *P. coccinea* M.Roem can show anti-inflammatory and antinociceptive activities.

The formalin test, which was a model of nociception, discriminates pain has two phases. The phases can be separated by time: first phase (0-5 minutes) generates peripherally by direct stimulation of nociceptive neurons; this phase is called the neurogenic phase. The second phase (20-25 minutes) is started through central neurons' stimulation, especially the dorsal horns neurons in the spinal cord. This phase is possibly an inflammation-induced pain because of cytokines' activity (like serotonin, bradykinin, histamine, and prostaglandins) these explanations for the antinociceptive mechanism. Our results show that the ethanolic extract and fractions of *P. coccinea* M.Roem. created antinociception against both the neurogenic phases and the inflammatory of the formalin test. Based on the paw edema test results, the ethanolic extract, chloroform, and ethyl acetate fractions have anti-inflammatory effects. Polyphenols are the critical source of the plant's antioxidant activity. The presence of polyphenols in the plant can be an indicator of the antioxidant activity of the extract. In study, the phenolic content and the HPTLC results show the significant antioxidant activity of *P. coccinea* M.Roem. ethanolic extract.

The ethanolic extract, ethyl acetate, n-hexane, and chloroform fractions of *P. coccinea* M.Roem. showed activity against nociceptive responses triggered in animal models by formalin stimulant. Also, it demonstrated anti-inflammatory activities in the carrageenan-induced paw edema test. Anti-inflammatory and antinociceptive activities were significantly increased in chloroform fractions. The flavonoids in the fractions are the compounds responsible for the effectiveness. The mechanism for antinociceptive and anti-inflammatory activities is not clear and requires further studies.

Acknowledgments

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Conflicts of interest

The authors declare no conflict of interest.

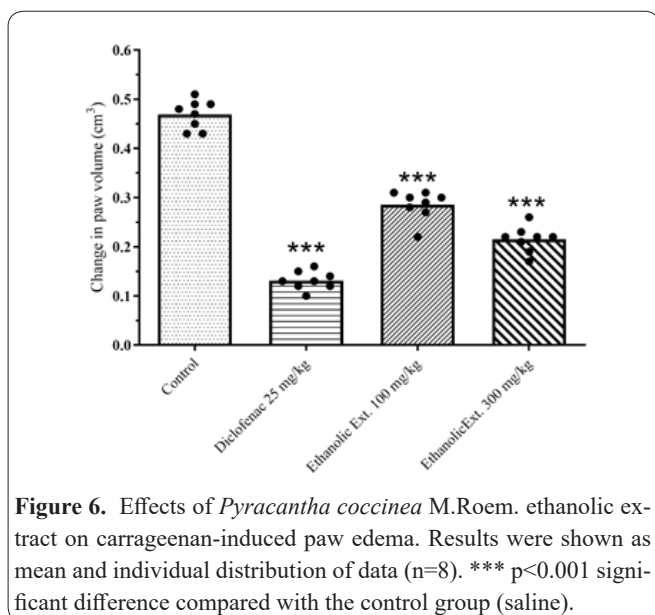


Figure 6. Effects of *Pyracantha coccinea* M.Roem. ethanolic extract on carrageenan-induced paw edema. Results were shown as mean and individual distribution of data (n=8). *** $p < 0.001$ significant difference compared with the control group (saline).

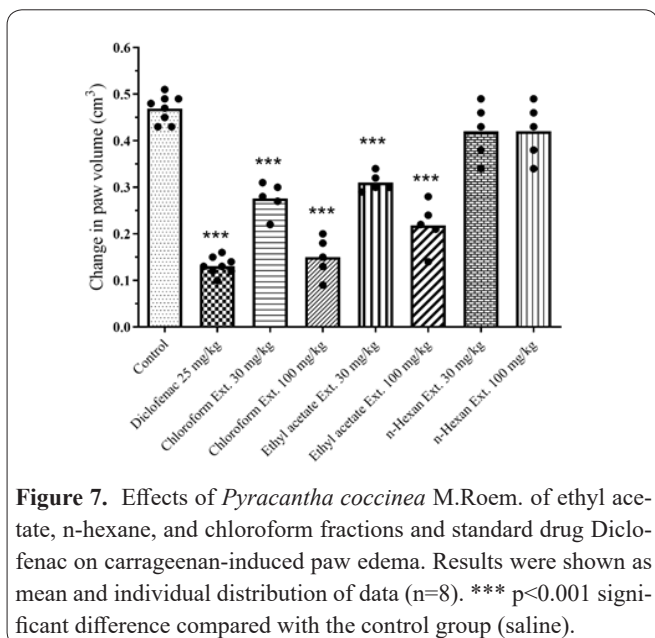


Figure 7. Effects of *Pyracantha coccinea* M.Roem. of ethyl acetate, n-hexane, and chloroform fractions and standard drug Diclofenac on carrageenan-induced paw edema. Results were shown as mean and individual distribution of data (n=8). *** $p < 0.001$ significant difference compared with the control group (saline).

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