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LC-MS-Based Chemical Profiling and *In-Vivo* Evaluation of The Anti-Inflammatory and Anti-Nociceptive Activities of The Defatted Methanolic Extract of *Crataegus Sinaica* (Rosaceae) Fruits

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Abstract

In this study, the anti-inflammatory and anti-nociceptive properties of the defatted methanolic extract (DME) of *Crataegus sinaica* fruits were evaluated *in vivo*. The anti-inflammatory effect of DME was evaluated in a model of carrageenan-induced rat paw edema against indomethacin (20 mg/kg, *p.o.*), and the anti-nociceptive effect was tested against acetylsalicylic acid (150 mg/kg, *p.o.*) using acetic acid-induced writhing method to evaluate the peripheral analgesic effect, and hot plate method to evaluate the central analgesic effect. DME (at the three dose levels; 50, 100 and 200 mg/kg, *p.o.*) significantly ($P \leq 0.05$) suppressed carrageenan-induced inflammation after 1, 2 and 3 hr. In addition, treatment with DME at 200 mg/kg significantly reduced the level of prostaglandin E_2 (PGE_2, 0.8±0.04 ng/paw), tumor necrosis factor-alpha (TNF-α, 246.33±9.38 pg/ml) and myeloperoxidase (MPO, 2.09±0.37 ng/ml) in paw exudate close to that shown by indomethacin (0.72±0.04, 242.56±5.39 and 1.59±0.37, respectively). Furthermore, DME at 100 and 200 mg/kg as well as acetylsalicylic acid significantly ($P \leq 0.05$) decreased the number of writhes (in acetic acid-induced writhing test), and increased latency time (in the hot plate test) compared to control. The chemical profile of DME was identified *via* UPLC/PDA/ESI-MS. As a result, a total of 40 compounds were tentatively identified, including phenolic acids, procyanidins (many as oligomers of epi-catechin and their gallate esters), flavonoids and their glycosides. These findings confirm that DME has anti-inflammatory as well as central and peripheral analgesic effects, and support the traditional use of *C. sinaica* fruits against several inflammatory ailments.

Keywords: Crataegus sinaica; defatted methanolic extract; chemical profile; anti-inflammatory; anti-nociceptive effect.

1. Introduction

Hawthorn, *Crataegus* species (family Rosaceae) are widely used in folk medicine as venotonic for various cardiovascular diseases, and number of *Crataegus* preparation from leaves, flowers and fruits are used to treat high blood pressure and heart disorders [1] *Crataegus monogyna* Jacq., the common hawthorn in north-eastern Portugal, is one of the species that is highly recommended in folk medicine and fruits are used as healthy and nutritious foodstuff for the

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management and prevention of age-related diseases [2].

Crataegus species are rich source of phenolic constituents such as simple phenolic acids, chlorogenic acids, proanthocyanidins, flavonoids and its glycosides. Karar and Kuhnert [3] have identified 113 compounds in *C. monogyna and C. laevigata*, leaves, fruits and their herbal derived drops (Crataegutt Tropfen) using UPLC-ESI-qTOF-MS/MS and HPLC-ESI-MS^n. Constituents identified were mainly phenolic compounds and their derivatives. Hawthorn phenolics were reported to decrease blood lipid levels, have antioxidant activities, lower blood pressure, and reduce the risk of cardiovascular diseases [4].

Crataegus sinaica BOISS. (also known as Hawthorn Azarola) is a hybrid of two other hawthorn species, C. azarolus and C. monogyna [5,6] . In Egypt, the plant grows in the mountains of Saint Catherine area where it is known as Za'rour or Za'rour Alawdiyah [7].

Refaat et al. [8] reported that the acetone extract of *C. sinaica* (leaves with flowers and the young stems), as well as its successive fractions, have potential cardiovascular and hepatoprotective activities in animal models. Phenolics such as hyperoside, vitexine-2"-O-rhamnoside, epicatechin, procyanidin B2 and procyanidin C1 are of the main active constituents isolated from the young stems were found to demonstrate anti-complementary and antiviral activities against HIV-1 and herpes simplex virus type 1 (HSV-1) [9,10,11]. The ethanol extract of *C. orientalis* leaves displayed remarkable anti-inflammatory and anti-nociceptive activities [12], and the methanolic extract of *C. sinica* root as well as its methylene chloride fraction exhibited anti-inflammatory activity in rats [13].

In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [14]. Studies show that chronic inflammation is linked to a wide range of progressive diseases, including cancer, metabolic disorder and cardiovascular diseases [15].

Meanwhile, non-steroidal anti-inflammatory drugs are widely used for the treatment of inflammation and pain disorders. However, their use may be associated with common side effects such as bleeding and peptic ulcers. Accordingly, there is an urgent need to find new and safe anti-inflammatory compounds. Epidemiologic studies indicate that the incidence of chronic disease and cancer is inversely correlated with the consumption of fruits and vegetables rich in phenolics, and this is attributed to their possible anti-inflammatory activities [16,17]. Herein, we report the anti-inflammatory and anti-nociceptive effects of the defatted methanolic extract (DME) of the fruits of *C. sinica* growing in Egypt. Furthermore, the effect of DME on the number of pro-inflammatory markers was discussed and the chemical profile of DME was tentatively identified using UPLC/PDA/ESI-MS.

2. Materials and Methods

2.1. Plant material

The fruits of *C. sinaica* BOISS (family *Rosaceae*) were collected from the mountains of Saint Catherine in South Sinai, Egypt in September 2017. The plant material was identified by Mrs. Trease Labib, Consultant of Plant Taxonomy at Ministry of Agriculture and the former director of El-Orman botanic garden. A voucher specimen (# 2017.10.05) was deposited at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The collected fruits were air-dried, ground and milled to a powder of mesh No. 36. The powder was kept in tightly closed containers till use.

2.2. Extraction

The powdered fruits (3 kg) were extracted by cold maceration in methanol (5 Lx3) by sonication for 20 minutes every time. After filtration, the solvent was evaporated under reduced pressure at 40ºC to obtain a gummy residue (250 g). Part of the residue obtained (50 g) was suspended in water (1L) and was further defatted by liquid-liquid fractionation with CH_2CL_2(1L X 5 times) to obtain a defatted methanolic extract (DME; 45 g). The dried extract was kept at 4°C in a tightly closed container.

2.3. *UPLC/PDA/ESI-MS analysis*

Mass spectrometric analysis was achieved using Waters ACQUITY Xevo TQD system, consisting of an ACQUITY UPLC H-Class system and Xevo^{TM}TQD triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA). Acquity BEH C18 100 mm × 2.1 mm column (particle size, 1.7 µm) was used to separate analytes (Waters, Ireland). The solvent system composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) by applying the following mobile phase gradient: 0-4 min, 30% B; 4-8 min, 35% B; 8-25 min, 70% B; 25-30 min, 30% B. The flow rate was 200 µl/min, and the injection volume was 10 µl. The samples were dissolved in methanol at a concentration of 1 mg/ml then filtered through a filter of pore size 0.22 µm. Mass ranges from 100 to 1000 *m/z*. The MS scan was applied at the following conditions: capillary voltage, 3.5 kV; detection at

cone voltages, (20V - 95V); radio frequency (RF) lens voltage, 2.5V; source temperature,150 °C; desolvation gas temperature, 500°C. Nitrogen was selected as desolvation and cone gas at a flow rate of 1000 and 20 L/h, respectively. System operation and data acquisition were controlled using Mass Lynx 4.1 software (Waters).

2.4. Animals

Fifty male Wistar rats weighing 150-200 g and 25 mature albino mice weighing 20-25 g were purchased from the animal unit at the National Research Centre. Standard conditions 12:12 light-dark cycle and well-ventilated rooms have been established for animals' housing. Animals were kept in hygienic cages and given free access to clean standard pellet diet food and water. One week before experiments, all the animals were shifted to be adapted to the laboratory environment. All animal procedures were performed after approval of the Medical Research Ethics Committee (MREC), National Research Centre, Egypt (#MREC-16-156) and following the recommendations of proper care and use of laboratory animals of the National Institutes of Health [18].

2.5. Chemicals and kits

Carrageenan was obtained from Sigma Aldrich, Germany. PGE_2 and MPO assay ELISA kits were purchased from Kamiya Biomedical Company, USA. Assay kit for TNF-α was obtained from Abcam Company, USA.

2.6. Carrageenan-induced inflammation in rats

Carrageenan was dissolved in distilled water 1% (w/v) and 0.1 ml was injected subcutaneously in the sub-plantar region of the rats' left hind paw [19]. Rats were randomly allocated into five groups each of 5 rats. Group1 was given distilled water (DW) daily and kept as control. Groups 2- 4 were treated orally (*p.o*) with DME in doses of 50, 100 and 200 mg/kg, *p.o,* respectively. Group 5 was given indomethacin (20 mg/kg, *p.o*) and was assigned as a standard group. After 1, 2, 3 and 4 h of injection of carrageenan, the paw volumes were measured using Plethysmometer, (UGO-Basile Italy).

Edema rate and inhibition rate were calculated at the mentioned intervals using the following equations [20]: Edema rate (%) = Vt - Vo/ Vo)x 100, and the Inhibition rate (%) = (Ec - Et/ Ec) x 100. Where, Vo is the volume before carrageenan injection (ml), Vt is volume at t h after carrageenan injection (ml), Ec is the edema rate of control group and Et is the edema rate of treated group.

Three h after carrageenan injection, all animals were sacrificed and the left hind paws were separated, lacerated and the inflammatory exudates were drained from the inflamed lacerated paws and centrifuged at 1800*g* for 15 min [21]. Exudates were then used for determination of PGE_2, TNF-α and MPO levels using commercial kits.

The total amount of PGE_2 expressed in the edematous fluid was calculated using the following equation:

PGE_2 (ng/paw) = [ng PGE_2 in sample x paw edema vol. (mL)] /sample vol. (= 0.1 mL).

2.7. Writhing test in mice

Peripheral analgesic activity was tested in mice [22]. Animals were divided randomly into five groups each of 5 animals. Group1 was kept as a negative control. Groups 2- 4 were given DME (50, 100 and 200 mg/kg *p.o*, respectively. Group 5 was given acetylsalicylic acid (150 mg/kg, *p.o*) and was kept as a standard group. Thirty minutes before the treatment, all animals were injected intraperitoneally (*i.p*) with acetic acid (0.7% aqueous solution) in a dose of 10 mL/kg b.wt. Pain is indicated with abdominal writhes. The number of writhes was counted for 20 min after injection. The protection percent was calculated using the following equation [23]:

Protection% = (Control mean - Treated mean)/Control mean ×100

2.8. Hot plate test in rats

Central analgesic activity was examined using hot plate apparatus (UGO Basile, Italy). Rats were divided randomly into 5 groups (each of 5 animals). Group1 was kept as negative control. Groups 2, 3 and 4 were given DME (50, 100 and 200 mg/kg *p.o*, respectively). Group 5 was given acetylsalicylic acid (150 mg/kg, *p.o*) as a standard group. Pain was induced thermally by exposing rats to a hot plate at 53±0.5 ºC [24]. The reaction time was calculated for each rat, which is the interval between placing the animal in the hot plate and the first response (jumping or paw licking). Exposure to thermal stimulus over 1 min was avoided to prevent tissue damage. Percentage of maximum possible effect (% MPE) was calculated according to the equation:

% MPE= (Post drug latency- pre drug latency/cut-off latency- pre drug latency) × 100 [25].

2.9. Statistical analysis

Results were compared to untreated and standard groups and analyzed using one-way ANOVA followed by Dunnett's multiple comparisons using SPSS statistics 17.0 (Chicago, IL, USA), and expressed as means ± standard error (SEM).

3. Results

3.1. Metabolic profiling using UPLC/PDA/ESI-MS

UPLC/PDA/ESI-MS was used to characterize metabolites (Table 1) in DME (Figure 1). Identification of metabolites was based on their retention time, molecular weight and UV-Vis absorption maxima.

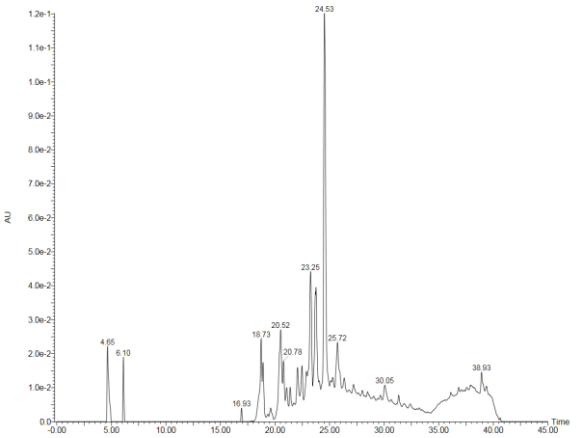

Figure 1
UPLC/PDA/ESI/MS profile of metabolites detected in DME.

Forty compounds including 17 phenolic acids and their derivatives, and 23 flavonoids and their derivatives were tentatively identified in DME by comparing their UV-Vis spectrum and fragmentation pattern with those reported in the literature (Table 1). Among them, 4 of the phenolic acids; three caffeoylshikimic acid isomers (**4**, **5** and **28**) and caffeoylquinic acid isomer (**18**), and 3 of the flavonoid derivatives; procyanidin dimer (**22**), quercetin hexoside/isomer (**29**) and myriceticin hexoside glucuronide (**34**) were found to be major constituents in DME (Figure 1).

Compounds **4** (Rt 4.70 min), **5** (Rt 6.10 min), and **28** (Rt 24.43 min) in their mass spectra had a $[M-H]^-$ ion at *m/z* 335 and the MS/MS of the three compounds gave ions at *m/z* 191, 179, and 135 (or 133) corresponding, respectively, to diagnostic fragments derived from a caffeoyl moiety in the caffeoylshikimic acid derivatives. The deprotonated molecular ions at *m/z* 335 indicated that compounds **4**, **5**, and **28** were one H_2O less than caffeoylquinic acid. We assume that these compounds are esters of caffeic acid and dehydrated quinic acid (shikimic acid). Therefore, compounds **4**, **5**, and **28** were identified as caffeoylshikimic acid isomers previously reported [28,40]. While the peak number **18** (eluted at Rt 20.83 min) revealed a deprotonated molecular ion at *m/z* 353 $[M-H]^-$ and a fragment ion at *m/z* 190 is indicative of quinic acid moiety in its structure and suggest that compound **18** is a caffeoylquinic acid isomer previously reported in the genus [3]. The peak eluted at Rt 23.23 min (#**22**) gave a deprotonated molecular ion at *m/z* 577 $[M-H]^-$ characteristic for a procyanidin dimer. The RDA fragmentation gave major ions supporting the structure information are *m/z* 425 $[M-H-152]^-$ and 289 $[M-H-288]^-$ (resulted after cleavage of the interflavan bond between the top and base units [38]. The presence of UV absorption bands at 220, 330 nm, together with molecular ion peak at m/z 463 [M-H]- and MS/MS at 301 [M-H-162]-, 179 and 153 nm are indicative of quercetin hexoside isomer for compound # **29** (Rt 24.58 min). Peak # 34 (Rt 25.74 min) with a molecular ion peak at *m/z* 655 and MS/MS fragment ion at *m/z* 317 [M-H-(162+176)] for the aglycone myricetin after the loss of hexosyl and hexuronyl moieties and suggested that **34** is a myricetin hexosyl hexuronide isomer [45].

3.2. Anti-inflammatory effect of DME against carrageenan induced paw edema in rat

As shown in (Table 2), administration of the DME at dose levels 50, 100 and 200 mg/kg showed significant ($P \leq 0.05$) decrease in edema rate compared to the control group after 1, 2 and 3 h of carrageenan injection and their potencies were calculated as % in comparison to standard group (indomethacin). DME at a dose of 50 mg/kg showed inhibition rates of 43.5, 39.3 and 35.3% at 1, 2, 3 h post carrageenan, respectively; with respective potency % of 103.8, 64.7 and 58.1. Whereas, DME at 100 mg/kg showed inhibition rates of 55.8, 47.8 and 34.8% at 1, 2, 3 h post carrageenan, respectively; and exerting potency % of 133.2, 78.7 and 57.2% at 1, 2, 3 h post carrageenan, respectively. Likewise, DME at 200 mg/kg showed inhibition rates of 60.3, 54.0 and 46.9% at 1, 2, 3 hours post carrageenan, respectively; and exerting potency % of 143.9, 88.9 and 77.1. Notably, the anti-inflammatory inhibition rates and potencies were dose dependent and reached the highest values for all doses after the 1st hour of carrageenan injection.

3.3. Effect of DME on PGE2, TNF-a and MPO levels in paw exudate

Carrageenan injection elevates the level of PGE_2 which contributes to the nociceptive and hyperalgesia at the site of the peripheral inflammation and the control group value was 13.3±0.43 ng/paw. Treatment with DME at 50, 100 and 200 mg/kg, showed significant ($P \leq 0.05$) reduction in PGE_2 levels; 1.87±0.17, 1.79±0.25 and 0.8±0.04ng/paw, respectively. However, treatment with indomethacin significantly ($P \leq 0.05$) reduce PGE_2 level to 0.72±0.04 ng/paw. Meanwhile, there was no significant difference between the inhibitory effect of

all DME doses and indomethacin on PGE_2 synthesis inhibition (Figure 2A).

TNF-α is a cell-signaling protein involved in systemic inflammation and is induced by carrageenan injection in paw exudate. Administration of indomethacin (20 mg/kg) showed a significant ($P\leq0.05$) reduction in the level of TNF-α (242.56±5.39 pg/ml). Similarly, administration of DME at 50, 100 and 200 mg/kg showed a significant ($P\leq0.05$) reduction in the level of TNF-α; 308.93±8.31, 276.16±17.64 and 246.33±9.38pg/ml, respectively, as compared to the control group (448.67±4.35 pg/ml). Moreover, the doses of 100 and 200 mg/kg of DME showed no significant difference from indomethacin group (Figure 2B).

Carrageenan-induced paw edema displays high tissue levels of myeloperoxidase (MPO) which is a standardized marker of neutrophil infiltration and have been shown to correlate with severity of inflammation. All doses of DME; 50, 100 and 200 mg/kg showed significant ($P\leq0.05$) reduction in MPO activity in paw exudate; 2.92±0.55, 2.26±0.39 and 2.09±0.37 ng/ml, respectively, when compared to the control group (11.68±0.59 ng/ml). The treatment with indomethacin (20 mg/kg) showed a significant ($P\leq0.05$) reduction in MPO activity; 1.59±0.37 ng/ml. It was noteworthy that, there was no significant difference between the inhibitory effect of all DME doses and indomethacin on MPO activity (Figure 2C).

3.4. Peripheral analgesic activity of DME

After thirty minutes of intraperitoneal injection of animals with acetic acid (0.7% aqueous solution) in a dose of 10 mL/kg b.wt. DME at examined doses was orally administrated and the number of writhes was counted for 20 minutes. Animals treated with DME at 100 and 200 mg/kg as well as acetyl salicylic acid (150 mg/kg) showed a significant ($P\leq0.05$) decrease in number of writhes 40.6±1.81, 35.6±1.44 and 22.4±1.08 writhes/20min, respectively, as compared to control group 50.4±1.99 writhes/20 min, showing protection % of 19.44, 29.37 and 55.56%, respectively (Table 3).

3.5. Central analgesic activity of DME

After 30 min. animal groups given oral dose of DME (50 mg/kg) and acetyl salicylic acid showed no significant ($P\leq0.05$) difference as compared to control group. After one hour of the administration of DME and acetylsalicylic acid, the reaction time for all animal groups was significantly ($P\leq0.05$) prolonged by 13.9±0.9, 17.2±0.85, 17.6±0.6 and 16.1±0.98 sec. when compared to control group (10.7±0.11 sec.). After 90 minutes from administration of DME at 50, 100 and 200 mg/kg, all animal groups significantly ($P\leq0.05$) prolonged the reaction time by 15.8±0.75, 17.7±1.12 and 18.2±0.87 sec. when compared to control group (11.3±0.29 sec.). All animals given DME at all dose levels did not show any significant changes from that of acetylsalicylic acid group starting from 1 hour of administration representing potent protective effect of DME against central pain induced by the thermal stimulus (Table 4).

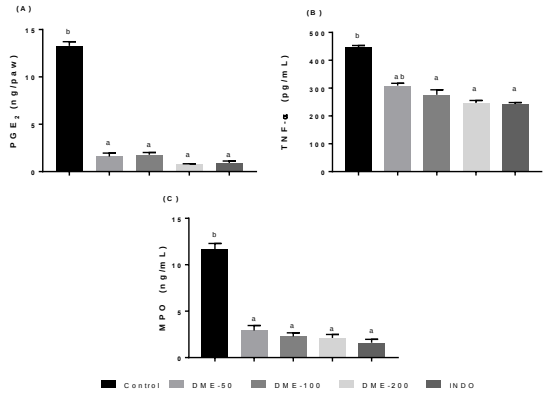

Figure 2
Effect of DME on PGE_2 and TNF-α levels and MPO activity in paw exudate of rats (n=5). Values are mean ± SEM (n=5). $^aP<0.05$, $^bP<0.05$ in comparison to control and indomethacin groups, respectively (LSD followed by Dunnett's test).
DME-50, *C. sinaica* defatted extract (50 mg/kg, *p.o*); DME-100, *C. sinaica* defatted extract (100 mg/kg, *p.o*); DME-200, *C. sinaica* defatted extract (200 mg/kg, *p.o*); INDO, Indomethacin (20 mg/kg, *p.o*); PGE_2, Prostaglandin E_2; TNF-α, Tumor necrosis factor-α; MPO, Myeloperoxidase.

Table 1

Characterization of metabolites in DME using UPLC/PDA/ESI-MS.

No.	Rt/min	Compound	UV-Vis	$[M-H]^-$	$[M+H]^+$	Fragment/s	Reference
1	4.26	Quinic acid**	198	191		179	[26]
2	4.32	Catechin*	278	289		179, 151	[27]
3	4.65	Quinic acid derivative	196	215		191	[26]
4	4.70	Caffeoylshikimic acid/isomer**	244, 326	335		191, 179, 173,133	[28]
5	6.10	Caffeoylshikimic acid/isomer**	245, 336	335		191, 179, 173,133	[28]
6	6.40	Coumaric acid-O-hexoside/isomer**	275, 325	325		163	[26]

7	8.84	Acyl-feruloylcaffeoylquinic acid	298	571		530, 355	[27]
8	8.79	Quercetin acetyldihexoside	254, 354	667		503, 463, 301	[29]
9	8.85	Naringenin deoxyhexoside	230, 352	417		273, 271	[3]
10	8.93	Gallagic acid	258, 378	601		575, 549	[30]
11	9.40	Rutin	254, 354	609		463, 301	[31]
12	9.73	Gallocatechin gallate/isomer	276	457		169, 331, 305	[32]
13	9.98	Caffeoylquinic acid/isomer**	220, 330	353		190	[3]
14	12.64	Syringic acid-O-hexoside/isomer**	276	359		197, 175	[3]
15	12.80	Coumaroylquinic acid/isomer	229, 336	337		191,163	[33]
16	13.70	Caffeic acid derivative	246, 328	635		179	[34]
17	17.60	(Diacetoxy-methoxy phenyl) Acroyl-O-p-Coumaroyl-O-caffeoylquinic acid	242, 336	777		717, 499, 245, 217	[27, 35]
18	20.83	Caffeoylquinic acid/isomer**	220, 330	353		190	[3]
19	21.83	Syringic acid-O-hexoside/isomer	197	359		198	[36]
20	22.35	Methylluteolin hexuronide (Chrysoeriol hexuronide)	269, 343	475		299, 285	[37]
21	23.08	Coumaric acid-O-hexoside/isomer**	275, 325	325		163	[26]
22	23.23	Procyanidin dimer* (Epi-catechin-epi-catechin) (isomer)	278	577		425, 289	[38]
23	23.31	Procyanidin derivative	234, 278	675		610, 577	[39]
24	23.80	Methylgallocatechin gallate/isomer	279	471		441, 407	[40]
25	23.91	phlorizin**	220, 336	435		227	[41]
26	24.02	Vitexin*	222, 332	431		341, 311	[3]
27	24.13	Ellagic acid pentoside	262, 363	433		301	[42]
28	24.43	Caffeoylshikimic acid/isomer**	244, 326	335		179, 135	[28]
29	24.58	Quercetin hexoside/isomer	220, 330	463		301,179, 153	[31]
30	25.22	Procyanidin dimer derivative	234, 278	785		577	[38]
31	25.32	Luteolin dihexoside/isomer**	223, 335	609		285,151	[43]
32	25.37	Cyanidin glucoside**	230		449	287, 259	[44]
33	25.42	Cratenacin/isomer**	232	619		413, 293	[3]
34	25.74	Myricetin hexosyl hexuronide	266, 355	655		317	[45]
35	26.50	Quercetin-di-*O*-deoxyhexoside)**	221, 335	755		301	[3]
36	27.16	Pelargonidin hexoside	278, 504		433	271	[38]
37	28.29	Catechin C-hexoside/isomer**	289	451		272	[3]
38	37.22	Quercetin pentoside**	254, 330	433		301,179	[46]
39	38.29	Dimethoxyluteolin	252, 318	313		298, 283	[33]
40	40.95	Pelargonidin-3-*O*-dihexoside	278, 504		595	433, 271	[38]

* reported in the same species
** reported in the same genus

Table 2
Anti-inflammatory effect of *DME* against carrageenan-induced paw edema in rats.

Group	1 hour		2 hours		3 hours	
	Edema rate (%)	Potency (%)	Edema rate (%)	Potency (%)	Edema rate (%)	Potency (%)
Control *	25.7±2.19 [b]	----	50.7±4.58 [b]	----	51.8±4.70 [b]	----
DME-50	14.5±0.99 [a] (43.5)	103.8	30.8±1.27 [ab] (39.3)	64.7	33.5±1.40 [ab] (35.3)	58.1
DME-100	11.4±0.97 [a] (55.8)	133.2	26.5±2.45 [a] (47.8)	78.7	33.8±3.14 [ab] (34.8)	57.2
DME-200	10.2±0.55 [a] (60.3)	143.9	23.3±2.36 [a] (54.0)	88.9	27.5±2.21 [a] (46.9)	77.1
INDO	15.0±1.39 [a] (41.9)	100	19.9±1.71 [a] (60.7)	100	20.3±1.01 [a] (60.8)	100

* Control group, sub-plantar injected with carrageenan.

Values represent the mean ± SEM of five rats for each group.
Each value in parenthesis indicates the percentage inhibition rate.
[a]*P*<0.05: Statistically significant from control (LSD followed by Dunnett's test).
[b]*P*<0.05: Statistically significant from indomethacin (LSD followed by Dunnett's test).
The potency was calculated compared to the reference drug indomethacin.
DME-50, *C. sinaica* defatted extract (50 mg/kg, *p.o*); DME-100, *C. sinaica* defatted extract (100 mg/kg, *p.o*); DME-200, *C. sinaica* defatted extract (200 mg/kg, *p.o*); INDO, Indomethacin (20 mg/kg, *p.o*).

Table 3
Peripheral analgesic activity of *DME* against acetic acid-induced writhing in mice.

Group	No. of writhes /20 min.	Protection (%)#
Control*	50.4±1.99 [b]	-----
DME-50	44.2±1.88 [b]	12.30
DME-100	40.6±1.81 [ab]	19.44
DME-200	35.6±1.44 [ab]	29.37
ASA	22.4±1.08 [a]	55.56

* Control group, *ip* injected with acetic acid (10 mL/kg).
Values represent the mean ± SEM of five mice for each group.
[a]*P*< 0.05: Statistically significant from control (LSD followed by Dunnett's test).
[b]*P*< 0.05: Statistically significant from acetylsalicylic acid (LSD followed by Dunnett's test).
Protection % = (Control mean-Treated mean)/Control mean ×100
DME-50, *C. sinaica* defatted extract (50 mg/kg, *p.o*); DME-100, *C. sinaica* defatted extract (100 mg/kg, *p.o*); DME-200, *C. sinaica* defatted extract (200 mg/kg, *p.o*); ASA, Acetylsalicylic acid (150 mg/kg, *p.o*).

Table 4
Central analgesic activity of *DME* against the thermal stimulus in mice.

Group	Reaction time (sec.) post-treatment			
	0 min	30 min	60 min	90 min
Control*	10.2±0.64	10.5±0.39	10.7±0.11 [b]	11.3±0.29 [b]
DME-50	10.7±0.73	13.1±1.10	13.9±0.90 [a]	15.8±0.75 [a]
DME-100	10.9±0.28	14.7±1.29 [a]	17.2±0.85 [a]	17.7±1.12 [a]
DME-200	10.7±0.58	15.0±1.37 [a]	17.6±0.60 [a]	18.2±0.87 [a]
ASA	10.0±0.51	13.3±0.32	16.1±0.98 [a]	16.1±0.52 [a]

* Untreated control group
Values represent the mean ± SEM of five rats for each group.
[a] *P*< 0.05: Statistically significant from control (LSD followed by Dunnett's test).
[b]*P*< 0.05: Statistically significant from acetylsalicylic acid (LSD followed by Dunnett's test).
DME-50, *C. sinaica* defatted extract (50 mg/kg, *p.o*); DME-100, *C. sinaica* defatted extract (100 mg/kg, *p.o*); DME-200, *C. sinaica* defatted extract (200 mg/kg, *p.o*); ASA, Acetylsalicylic acid (150 mg/kg, *p.o*).

4. Discussion

In previous studies, the flavonoids 3,4-di-O-acetyl-2-O-α-rhamnosyl-vitexin and 2*R*,3*R*-dihydroquercetin-3-*O*-xylopyranoside, (+)-catechin, (-)-epicatechin, vicenin II, 2-*O*-*α*-rhamnosyl-vitexin and 4-*O*-acetyl-2-*O*-*α*-rhamnosyl-vitexin were reported as main constituent of the methanolic extract of *C. sinaica* leaves [47]. These constituents were also found in the young stems and flowers of the plant and suggested to exert cardiovascular and hepatoprotective effects of its extract [8,9]. Phenolic compounds, flavonoids and vitamin C were also identified in the fruits of 20 selected genotypes of *Crataegus* species explaining its antioxidant effects [48]. The fruits of *C. sinaica* were reported to contain flavonoids which are not as much as leaves but consists primarily of oligomeric and polymeric procyanidins [10]. In this study, UPLC/PDA/ESI-MS analysis of DME of the fruits of *C. sinaica* was done and phenolic compounds were tentatively identified based on UV absorption maxima, molecular weight, mass fragmentation patterns, and by comparison with reported data (Table 1). Metabolic profiling of DME revealed the presence of several phenolic constituents. Some of them were reported previously from the same species such as catechin and its congeners including procyanidins [8,9] and flavonoid *C*-glycosides such as vitexin [47]. Some others were reported from other *Crataegus* species such as quinic acid, caffeoylquinic acid derivatives [49], caffeoylshikimic acid derivatives [50], phenolic acid glycosides such as

coumaric acid-*O*-hexosides [51], syringic acid-*O*-hexosides [52], anthocyanins such as cyanidin glucoside [51], flavonoids such as luteolin dihexoside [53], cratenacin, quercitin-*O*-pentoside [49], quercetin-di-*O*-deoxyhexoside, (epi)catechin-*C*-hexoside [50], and the dihydrochalcone glycoside phlorizin [51]. In general, the structures of 40 phenolic compounds were tentatively identified (Table 1).

Moreover, DME was investigated for possible anti-inflammatory effects as well as peripheral and central analgesic effects. Results revealed that DME (at all doses tested) demonstrated high potencies to inhibit the carrageenan-induced inflammation. This effect was accompanied by a significant decrease in the proinflammatory mediators PGE_2 and TNF-α induced by carrageenan injection. *Crataegus* species is known to be rich in phenolics and flavonoids. The high contents of phenolic compounds proved to exert anti-inflammatory activities through the modulation of NF-κB, which regulate the synthesis of inflammatory proteins, such as IL-6 and TNF-α [54,55]. Likewise, the ability of DME (at all dose levels) to significantly decrease myeloperoxidase enzyme activity (MPO) in the paw exudates after its dramatic increase as a response to carrageenan injection indicated that the extract has also a powerful antioxidant effect. Accordingly, the inhibition of neutrophil infiltration and free radical production could be the mechanisms of the anti-inflammatory activity of DME. Previous study demonstrated that *Crataegus microphylla* has antioxidant capacity due to its content of total phenolics and flavonoids [54]. These findings suggest that Crataegus species are valuable source of natural antioxidants with potential anti-inflammatory effects of DME.

Additionally, Crataegus species content of some flavonoids, procyanidins, organic acids, tannins and triterpene derivatives were detected and have been reported to have anxiolytic, sedative and analgesic/anti-nociceptive activities [56, 57].

Significant, dose-dependent increase in the reaction time against thermal stimulus in hot-plate test, as well as the decrease in the number of writhes and stretching behavior in writhing test (chemical stimulus) designating the analgesic actions of *C. sinaica* extract.

The peripheral analgesic effect of the methanolic extract was investigated by induction of pain via intraperitoneal injection of acetic acid as a chemical noxious stimulus in mice. The result showed significant decrease in number of writhes produced by mice in response to pain induction, indicating a mild-to moderate peripheral analgesic activity of the plant extract. This finding is in line with the use of Crataegus fruits in traditional Chinese medicine, mainly to improve circulation, and to treat indigestion, diarrhea, and abdominal pain [58].

It was also worth investigating the central analgesic effect of DME using hot plate method; our results showed a potential analgesic effect of all doses of the plant extract after 1 h of administration, indicating a pain-relieving property of the extract and suggesting that the analgesic effect of DME may be mediated *via* the activation of delta and/or kappa opioid receptor subtypes [57].

5. Conclusions

It could be concluded that the defatted methanolic extract prepared from the fruits of C. *sinaica* growing in Egypt has significant anti-inflammatory and analgesic effects. These activities could be attributed to its high content of phenolics and flavonoids that were found responsible for most of the biological activities of Hawthorn. Though, we recommend more in-depth research to be carried out to clarify safety and efficacy of the bioactive fraction before use.

6. Conflict of interest

Authors declare that there is no conflict of interest.

7. References

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