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The in vivo Activity of *Fraxinus angustifolia* in Pain and Inflammation- Examination of the Biological Activities of the Sub-extracts in Mice

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ABSTRACT: *Fraxinus species (Oleaceae)* are commonly used in the treatment of several inflammatory conditions with pain, such as rheumatism, arthritis, and gout in folk medicine. We aimed to evaluate this ethnobotanical claim through in vivo experiments. The n-hexane, dichloromethane, ethyl acetate and water subextracts were obtained from the methanolic extract, which was prepared from the bark of *Fraxinus angustifolia* Vahl. The antinociceptive and anti-inflammatory activities of extracts were evaluated in mice by hot plate and formalin-induced edema assays, respectively. Mice were treated with 5-100 mg/kg methanol crude extract and 100 mg/kg subextracts intraperitoneally. Administration of methanol extract decreased paw thickness in doses of 25 and 100 mg/kg. Water and ethyl acetate subextracts decreased paw thickness while water subextract also decreased paw volume compared with the control. Paw thickness values in the dichloromethane and n-hexane subextract groups and paw volume values in the ethyl acetate, dichloromethane, and n-hexane subextract groups were comparable to those in the control group. The latency values were higher in the groups treated with the dichloromethane and n-hexane subextracts than in the control group while the other subextracts did not change the latency values compared to the control group in the hot plate test. In conclusion, the cortex of *Fraxinus angustifolia* exhibited a significant in vivo anti-inflammatory and antinociceptive activity.

KEYWORDS: Antinociceptive; hot-plate; formaline; fraxinus; ash

1. INTRODUCTION

Fraxinus angustifolia Vahl (FxA), which belongs to the Oleaceae family and is also known as narrow-leaved ash, is a deciduous ash that has approximately 40-45 m height and 1.5 m diameter. FxA has light green, glabrous leaves that are arranged in groups. The bark of the tree has gray-colored, deep, meshed furrows. FxA is most common in central and southern Europe, northwestern Africa, and southwestern Asia. The growth of FxA requires a mild climate with an annual precipitation of 400-800 mm^3 and moist soils with a pH between 5-8 [1].

Various *Fraxinus* species are medicinal plants, with an ethnobotanical importance. FxA is used in folk medicine as an analgesic, anti-inflammatory, antioxidant, and tonic [1]. FxA is widely used in Algerian folk medicine with anti-inflammatory and antioxidant properties but is also used as a diuretic, astringent, and digestive [2, 3]. Decoction and infusion of leaves and fruits have been reported to be used against rheumatism and hemorrhoids and as antipyretic agents [4]. The leaves are used to prevent and allay diarrhea, intestinal parasites, and gallstones [4], and the bark is commonly used as an antipyretic to combat passive bleeding, gout, gallstones, and especially fever [3, 5]. In addition, the stem bark of FxA is boiled and comsumed orally to treat hepatitis in Trabzon, a region of Türkiye [6]. The extract obtained from the bark of FxA has been shown to contain high amounts of polyphenolic compounds [7]. It has been reported that the ethanolic extract of leaves and bark of FxA possesses antioxidant and wound healing activity [2, 8].

There are also some studies demonstrating that various *Fraxinus* species have antinociceptive and anti-inflammatory effects. It was reported that the methanolic extract of *Fraxinus floribunda* Wallich leaves (100-400

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mg/kg; p.o.) showed anti-inflammatory effects in the carrageenan-induced paw edema test [9]. In addition, a methanolic extract of *F. floribunda* diminished inflammation in experimental arthritis induced with Freund's adjuvant [9]. The results of acetic acid-induced writhing and the tail immersion test in mice indicated that the methanolic extract of *F. floribunda* had an antinociceptive effect [9]. It was also reported that the methanolic extract of *Fraxinus xanthoxyloides* Wall. showed an antinociceptive effect in the acetic acid-induced writhing test, carrageenan-induced paw edema test, hot plate test, and subcutaneous air pouch test. These effects were suggested to be via methanolic extract induced inhibition of TNF-induced NF-κB synthesis [10].

However, we have not encountered any study reporting the antinociceptive and anti-inflammatory effects of FxA. The aim of this study was to investigate the antinociceptive and anti-inflammatory effects of extracts obtained from FxA.

2. RESULTS

To investigate the antinociceptive effects of the methanolic extract of *Fraxinus Angustifolia* (FAME), latencies were measured for 1 hour at 15-minute intervals in a hot-plate test. The results of the hot-plate test showed that FAME increased latencies only at a dose of 100 mg/kg at 30-60 min. Also the latency values were found higher in FAME administered group at the dose of 25 mg/kg at 15 min, whereas no change in latencies was detected at 5 mg/kg dose when compared with the control (Table 1 and Figure 1).

In addition, mice treated with morphine (10 mg/kg) as a positive control showed greater latencies at 15-60 min than the control group. No significant difference in latency values was observed between the morphine and FAME (100 mg/kg) groups. On the other hand, naloxone, the opioid receptor antagonist, inhibited the nociceptive effect of FAME while preventing the increase in latency values attributed to FAME in the hot plate test (Table 1 and Figure 1).

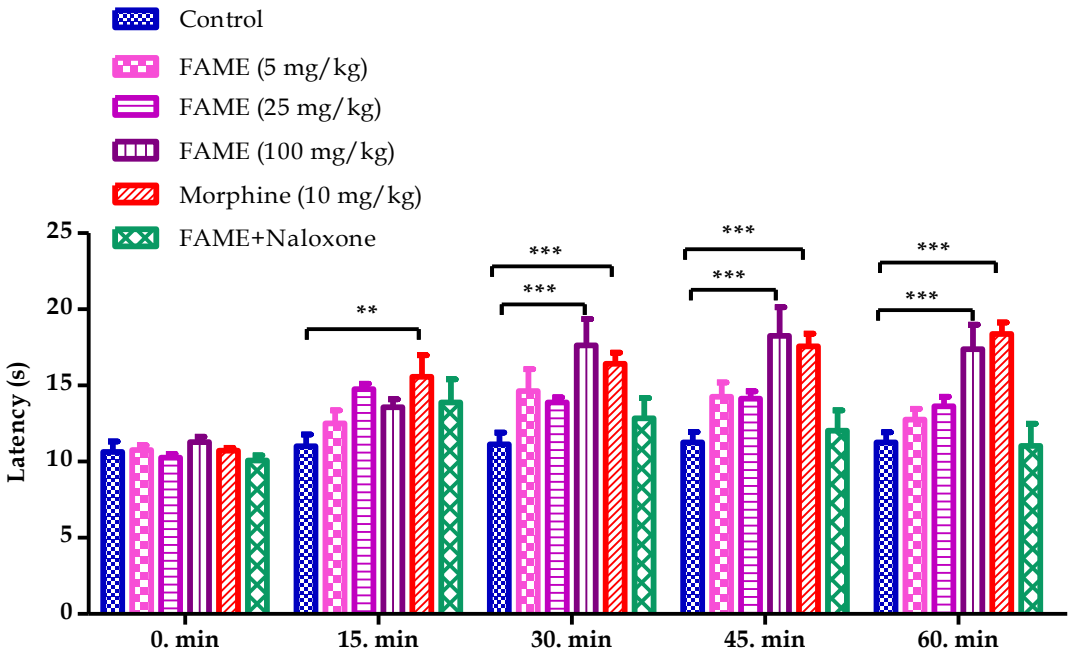

Figure 1. Effect of control (n=8), FAME (5 mg/kg; n=8), FAME (25 mg/kg; n=8), FAME (100 mg/kg); n=7), and morphine (10 mg/kg; n=7) on latency. Latency time is expressed in 's' and given as the mean ±SEM *p<0,05, **p<0,01, ***p<0,001

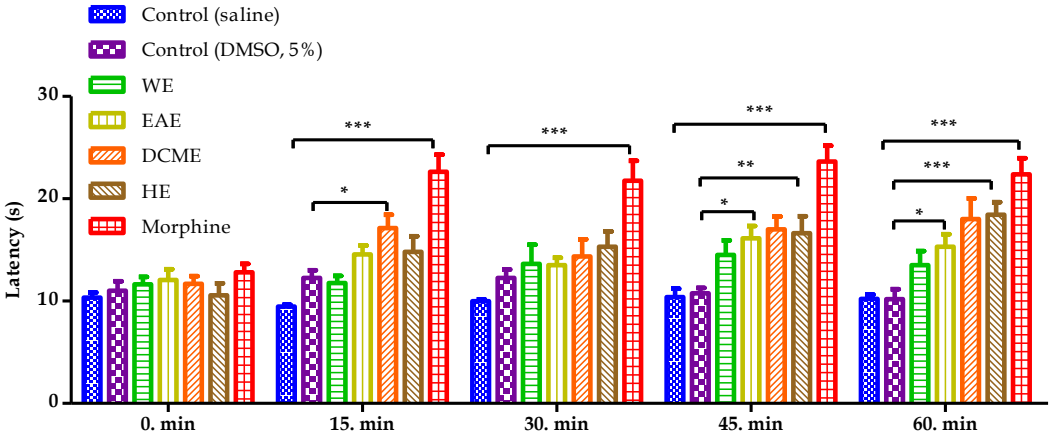

Figure 2. Effect of control (n=8), subextracts of FAME (100 mg/kg; n=8) and morphine (10 mg/kg; n=7) on latency. Latency time is expressed in 's' and given as the mean ± SEM. WE: water subextract; EAE: ethyl acetate subextract; DCME: dichloromethane subextract; HE: n-hexane subextract.
*p<0,05, **p<0,01, ***p<0,001

Further studies were performed with the subextracts obtained with n-hexane, dichloromethane, ethyl acetate, and water from FAME. The latency values were higher in the groups treated with the n-hexane, dichloromethane and ethyl acetate subextracts than in the control groups in the hot plate test (Figure 2). Subsequently, there was a marked increase in MPE values in the groups administered the n-hexane and dichloromethane subextracts while the other subextracts did not change the maximum potential effect (MPE) values compared to the control (Table 2).

Table 1. MPE values obtained from the control **(**n=8), FAME (5 mg/kg; n=8), FAME (25 mg/kg; n=8), FAME (100 mg/kg; n=7), and morphine (10 mg/kg; n=7) groups of mice. Data are presented in '%' and given as the mean±SEM.

MPE% values	15. min	30. min	45. min	60. min
Control (saline)	1.60 ± 3.55	2.21 ± 3.79	2.82 ± 3.47	3.15 ± 1.40
FAME (5 mg/kg)	9.33 ± 3.51	19.68 ± 7.97	17.77 ± 5.61	10.13 ± 4.34
FAME (25 mg/kg)	22.59 ± 2.62*	18.21 ± 2.33	19.52 ± 2.71	17.01 ± 3.27
FAME (100 mg/kg)	12.13 ± 2.71	28.89 ± 9.21**	33.58 ± 11.02***	32.94 ± 9.82***
FAME + Naloxone	19.55 ± 7.67	16.53 ± 4.65	14.02 ± 4.44	9.58 ± 4.43
Morphine	25.34 ± 7.04**	29.55 ± 3.96***	35.38 ± 4.74***	39.85 ± 4.82***

*p<0,05, **p<0,01, ***p<0,001, significantly different from control.

Table 2. MPE values obtained from control (n=8), subextracts of FAME (100 mg/kg; n=8) and morphine (10 mg/kg; n=7) groups of mice. Data are presented in '%' and given as the mean±SEM

MPE% values	15. min	30. min	45. min	60. min
Control (saline)	0.31±0.31	1.14±0.63	0.35±0.35	1.99±0.73
Control (%5 DMSO-saline)	6.24±2.02	7.23±4.09	3.34±1.68	0.68±0.68
Water subextract	4.67±3.94	17.90±6.66	17.36±5.54	13.83±5.38
Ethylacetate subextract	13.50±2.66	11.68±4.76	23.40±6.30	17.44±5.94
Dichloromethane subextract	30.48±5.18*	22.94±5.27	30.70±6.58*	37.60±10.13**
n-hexane subextract	23.35±7.07	24.74±6.04	32.58±7.34**	39.26±7.32***
Morphine	58.59±9.03#	54.10±10.67#	62.73±10.11#	55.26±9.46#

*P<0.05, **P<0.01, #P<0.001, significantly different from control.

We performed a formalin-induced paw edema test to investigate the anti-inflammatory effect of FAME. The conclusion was made that treatment with FAME inhibited formalin-induced edema in the paw edema test. FAME at lower doses (5-25 mg/kg) did not change the volume of the paws compared to that of control group. In addition, the differences in paw thickness between the control and FAME (25 and 100

mg/kg) groups were found statistically significant. No significant differences were observed between the FAME (100 mg/kg) and diclofenac groups (positive control) in the measurement of paw edema thickness and volume (Figure 3).

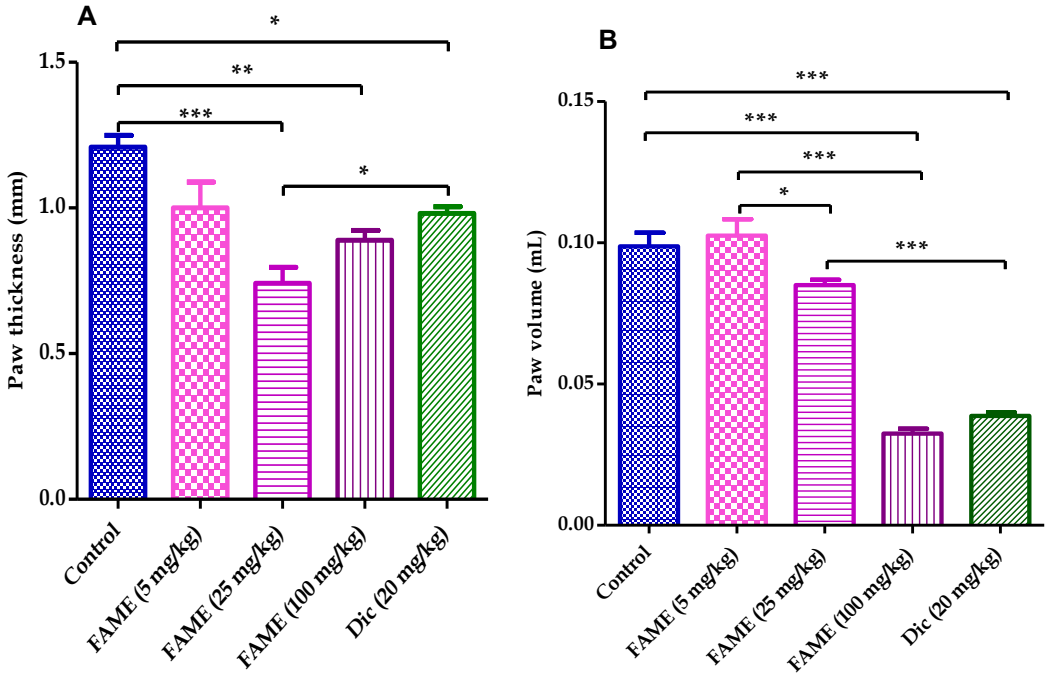

Figure 3. Paw thickness (A) and volume (B) values obtained from the control (n=8), FAME (5 mg/kg; n=8), FAME (25 mg/kg; n=8), FAME (100 mg/kg; n=8), and diclofenac (20 mg/kg; n=8) groups of mice. Data are presented in 'mm' and 'mL', given as the mean±SEM. **p*<0,05, ***p*<0,01, ****p*<0,001

Further extractions were performed with FAME to clarify the inhibitory effect of FAME on formalin-induced paw edema. The thickness and volume of paw edema were decreased in the water subextract group compared to the control group while the application of dichloromethane, and n-hexane subextracts did not cause a significant change in paw edema comparable to the control group. However, the ethyl acetate subextract reduced the paw thickness whereas it did not cause significant difference in paw volume compared to that of control group (Table 3).

Table 3. Paw thickness (A) and volume (B) values obtained from the control (n=8), subextracts of FAME (100 mg/kg; n=8) and diclofenac (20 mg/kg; n=8) groups of mice. Data are presented as the mean±SEM.

	Paw thickness (mm)	Paw volume (ml)
Control (saline)	0.99±0.03	0.07±0.007
Control (5%DMSO-saline)	0.74±0.05	0.04±0.003
Water subextract	0.45±0.03***	0.03±0.002**
Ethylacetate subextract	0.51±0.03#	0.03±0.003
Dichloromethane subextract	0.67±0.02	0.03±0.002
n-hexane subextract	0.83±0.05	0.04±0.004
Diclofenac	0.68±0.03***	0.04±0.006*

**p*<0,05, ***p*<0,01, ****p*<0,001 significantly different from control (saline)
#*p*<0.05 significantly different from control (5% DMSO-saline).

3. DISCUSSION

FxA has been considered a medicinal plant that proffers ethnobotanical significance. It has been used in folk medicine since ancient times as a plant-based analgesic, anti-inflammatory, antioxidant, and astringent. A considerable number of studies in the literature have focused on the biological activities of *Fraxinus* species finding that various *Fraxinus* species have antinociceptive, anti-inflammatory, antioxidant, antihypertensive, anticancer, and neuroprotective activities. The aim of this study was to investigate the antinociceptive and

anti-inflammatory activities of FxA and gain understanding of the underlying mechanisms that produce these properties. The effect of FAME on latency was similar to that of morphine in the hot-plate test suggesting that the antinociceptive effect of FAME is similar to that of morphine. On the other hand, the nociceptive effect of FAME occurred later than that of morphine. Based on the results, naloxone, the opioid receptor antagonist, blocks the antinociceptive effect of FxA in the hot plate test, and it can be concluded that opioid receptors mediate the antinociceptive effect of FxA. Furthermore, the results of the formalin-induced paw edema test showed that FAME prevented the development of edema and that this effect was not dose dependent. It can be concluded that FAME has an anti-inflammatory effect at the highest dose.

Further extractions were performed using FAME to identify the subextract responsible for the antinociceptive and anti-inflammatory activities of FAME. Subextracts of FAME were prepared using n-hexane, dichloromethane, ethyl acetate, and water solvents. Dichloromethane and n-hexane subextracts were found to have antinociceptive activity close to that of morphine. Due to pharmacokinetic parameters, such as delayed absorption or bioactive metabolites, these effects might have occurred later than those of morphine. On the other hand, the subextracts of water and ethyl acetate decreased formalin-induced paw edema suggesting that the subextracts have an anti-inflammatory effect. Measurements of paw thickness and paw volume showed a similar pattern in the formalin-induced edema test. Conversely, however, some differences in the compass measurements of edema volume with the plethysmometer were not found to be statistically significant. This may be due to the variations in edema thickness measurements and the higher sensitivity of the plethysmometer.

Our results from this study are consistent with previous studies. It has been reported that the extract of FxA bark contains high concentrations of phenylethanoids and hydroxycoumarins [11]. Bioactive compounds such as tannic acid, catechin, quercetin, and rutin identified in the phytochemical characterization of the barks and leaves of FxA are associated with anti-inflammatory, antioxidant, and wound healing properties [7]. Moreover, in the phytochemical characterization study, the extract of *Fraxinus chinensis* was found to contain secoiridoid glucosides such as oleuropein, (8E)-4''-O-methylligstroside, (8E)-4''-O-methyldemethylligstroside, and 3'',4''-di-O-methyl-demethyloleuropein. In addition, bioactive compounds, such as (8E)-4''-O-methylligstroside, aesculetin, and fraxetine, were found to have anti-inflammatory effects by decreasing the levels of NO, TNF-α and IL-6 via inhibition of mitogen-activated protein kinase in lipopolysaccharide-induced macrophage cultures [12]. In addition, the bioactive compounds esculin and calcelarioside isolated from the bark of FxA were reported to be responsible for the antioxidant activity of FxA by inhibiting NADH oxidase in vivo and in vitro [13]. Moreover, it was found that the hydroalcoholic extract of manna obtained from FxA exhibited antioxidant and anti-inflammatory activity in vitro. Moreover, this extract of manna was found to contain elenolic acid, tyrosol, hydroxytyrosol, catechin, fraxetin, verbascoside, gallic acid, procyanidin-B1, and luteolin 3,7 glucoside in HPLC-DAD analysis [14].

Extracts of FxA and *Fraxinus floribunda* were found to exhibit antidiabetic activity in a streptozotocin-induced diabetes model in rats by inhibiting the alpha-glucosidase enzyme in vivo [15, 16]. The ethanolic extract of FxA was shown to prevent hepatotoxicity induced by high doses of paracetamol [15].

Ethyl acetate extract of *Fraxinus xanthoxyloides*, containing rutin, caffeic acid, catechin, and gallic acid, showed antioxidant and cardioprotective effects by restoring the levels of antioxidant tissue enzymes that act against CCI4-induced oxidative stress in the cardiac tissue of rats [17]. Moreover, a coumarin secoiridoid diglucoside, named isofraxisecoside, was isolated from the bark extract of *F. Xanthoxyloides* [18]. Moreover, in vitro and in vivo, neuroprotective effects of FxA bark extract acted against Aβ aggregation and aluminum-induced neurotoxicity in mice [11].

In addition, oral administration of esculetin, isolated from *Fraxinus rhynchophylla*, was shown to decrease ear swelling, the number of scratch attacks, and infiltration of inflammatory cells. Oral administration also demonstrated increased levels of immunoglobulin E, immunoglobulin G2a, and histamine in serum and in an atopic skin inflammation model induced through the application of the house dust mite (Dermatophagoides farinae extract, DFE) and 2,4-dinitrochlorobenzene (DNCB) to the ears of mice. Esculetin is also reported to reduce the levels of Th1-, Th2-, and Th17-related cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-4, IL-13, IL-31, and IL-17, and nuclear factor-κB activation in the ear tissues of mice [19].

Novel compounds with a coumarin-related chemical structure, isolated from the stem bark of *F. rhynchophylla*, were reported to inhibit the elastase of human neutrophils in vitro. In addition, these compounds were found to inhibit the production of NO and lipopolysaccharide-induced expression of iNOS and COX-2 in RAW 264.7 cells [20].

It was found that the ethanol extracts of both the leaves and stem bark of FxA exhibited antimutagenic and antigenotoxic effects in vitro. Moreover, the ethanolic leaf extract and the aqueous/chloroform extracts of the leaves and stem bark of FxA were found to have selective cytotoxic effects on cancer cells. Phytochemical

analysis of the extracts of FxA identified phenylethanoids (calceolariosides and verbascosides) and secoiridoids (oleuropein and ligstrosides) that have been attributed to anticancer activity [21]. The bioactive compounds attributed to the biological actions of FxA showed antioxidant, anti-inflammatory, through interaction with enzymes, such as MAPK, NF-κB, PKB, JNK, Nrf-2, and ERK [22].

4. CONCLUSION

There is increasing evidence that extracts derived from *Fraxinus* species contain novel bioactive compounds that proffer therapeutic potential in clinical situations, such as inflammation, infection, and cancer. Based on the obtained results, it could be suggested that FAME displayed opioid receptor mediating antinociceptive effect. Also, in the present study it was demonstrated that the polar sub-extracts of FAME have anti-inflammatory activity. Future work should perform phytochemical characterization of these bioactive compounds to clarify their biological effects and the mechanisms that produce these effects; furthermore, these studies should assess the compounds' toxicological effects on the liver and kidneys.

5. MATERIALS AND METHODS

5.1. Plant material and extraction

The bark of FxA was collected from Ortahisar, Trabzon, in September 2016. The plant material was identified according to the Flora of Turkey and the Aegean Islands [23]. The dried and powdered bark (350 g) was extracted at 40 °C with methanol (2x1200 mL). The extraction process was repeated 3 times, and the filtrates were combined. After filtration of the solid matrix through layers of qualitative filter paper, the solvents were evaporated to dryness under vacuum, and 40.15 g (FAME) was obtained. Then, 3.31 g of the extract was separated into a vial for biological activity studies, and 36.84 g was dissolved in a mixture of methanol:water (1:9) and then extracted with n-hexane, dichloromethane, and ethyl acetate to obtain subextracts. Each extract was evaporated to dryness under reduced pressure at 40 °C to give n-hexane subextract (1.48 g), dichloromethane subextract (2.14 g), ethyl acetate subextract (8.12 g) and water subextract (24.60 g).

5.2. Animals

The antinociceptive and anti-inflammatory effects of FAME were investigated using the hot plate test and the formalin-induced edema test. The study protocol was approved by the local animal ethics committee of Karadeniz Technical University (2016/47).

Male Balb/c mice weighing 20-30 g were used for this study. Animals were housed in a temperature- and humidity-controlled environment with a 12:12 light/dark cycle and brought to the laboratory 24 h before the experiment. Mice were divided into 6 groups (n=7-8/group): vehicle, FAME, n-hexane subextract, dichloromethane subextract, ethyl acetate subextract, water subextract, and positive control (morphine for hot plate test or diclofenac for paw edema test).

5.2.1. Hot plate test

Mice were placed on a heater with a temperature of 55±0.1 °C. The latency time until they licked their hind legs or engaged in jumping behavior was measured and recorded to measure baseline response. Mice were administered vehicle (saline for FAME and water subextract or %5 DMSO in saline for n-hexane, dichloromethane, ethyl acetate subextracts), FAME (5, 25 and 100 mg/kg), subextracts (100 mg/kg), or morphine (10 mg/kg) intraperitoneally 30 minutes after baseline measurement. Latency was measured by placing mice on a hot plate every 15 minutes within 60 minutes of administration (Figure 4A). Mice were kept on the plate for a maximum of 30 seconds (cutoff time) to avoid tissue damage. The maximum potential effect (MPE%) was calculated as [(latency-baseline)/(cutoff time(30)-baseline)] * 100 [24]. The antinociceptive effect of FAME was also investigated in naloxone (opioid receptor antagonist) treated mice to evaluate the putative role of opioidergic system in the effects of FAME on pain.

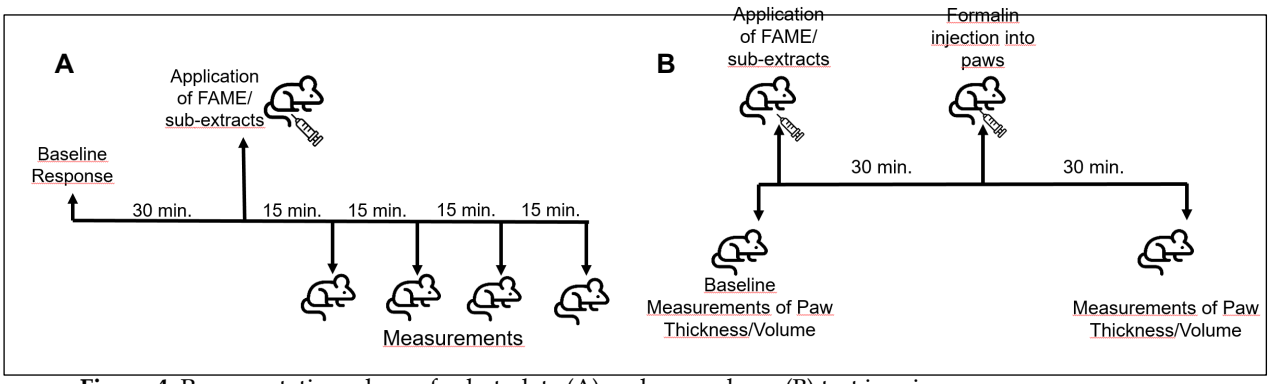

Figure 4. Representative scheme for hot plate (A) and paw-edema (B) test in mice

5.2.2. Paw-edema test

The anti-inflammatory effect of FAME (5, 25 and 100 mg/kg) and subextracts (100 mg/kg) was investigated using the formalin-induced paw edema test (25). To induce paw edema, mice were injected with a formalin solution (1%) into the subplantar surface of the right hind paws. The thickness and volume of the paws were measured with a compass and a plethysmometer, respectively. The difference between the values of paw thickness and volume before and after formalin application is considered the extent of edema. Mice were treated intraperitoneally with vehicle (0.9% NaCl or 5% DMSO), diclofenac (20 mg/kg), and FAME/subextracts 30 minutes before formalin administration (Figure 4B).

6. Statistical Analysis

Statistical analysis was performed with analysis of variances *post-hoc* Tukey test. GraphPad Prism 5.0 was used for the data analysis. If $P<0.05$ was considered as significant.

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