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ANTI-INFLAMMATORY AND ANALGESIC ACTIVITY OF FRACTIONS OF *PINUS BRUTIA* AND *CEDRUS LIBANI* LEAVES ETHANOLIC EXTRACTS

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Cedrus libani and Pinus brutia are Pinaceae family members that are used in traditional medicine for wound healing and rheumatism. Various nonsteroidal anti-inflammatory drugs have been used to treat inflammation and pain but their usage is occasionally limited due to adverse effects.This study aimed to investigate the anti-inflammatory and analgesic activity of ethanolic extract fractions of both plants and explore the main phytochemical profiles of active fractions. The anti-inflammatory activity was assessed in vitro using albumin denaturation assay and in vivo with carrageenan-induced edema in rats. The analgesic effect was assessed in vivo using the formalin test and the tail flick test, by using sodium diclofenac as a reference drug. Results showed that the aqueous fraction (AQ) of cedar extract and the ethyl acetate fraction (EA) of pine extract suppressed albumin denaturation more than the other fractions ($p < 0.05$). The AQ fraction, either intraperitoneally (30 mg/kg) or topically (1% gel), could also significantly reduce edema caused by carrageenan better than the EA fraction, and both fractions have more activity than sodium diclofenac ($p < 0.001$). Furthermore, the EA fraction decreased the animals' nociceptive response in both phases of the formalin test and outperformed sodium diclofenac. Whereas, the AQ fraction exerted the same activity as diclofenac ($p > 0.05$). AQ and EA fractions also demonstrated analgesic effects by increasing pain latency in the tail flick test in a way that was better or comparable to diclofenac. These effects may be related to polyphenols in fractions, including flavonoids and tannins. In conclusion, the AQ and EA fractions possessed significant anti-inflammatory and analgesic activity compared to sodium diclofenac, making them a novel therapeutic drug. The tannins of cedar showed superior anti-inflammatory activity, while the flavonoids of pine exerted more analgesic activity. However, further toxicological studies are needed to find these new agents their clinical application.

Keywords: *Cedrus libani; Pinus brutia; anti-inflammatory; analgesic; TLC; Total tannin content; flavonoids; phenolics.*

INTRODUCTION

Inflammation and pain are involved in most human diseases. They are generally caused by biological, pharmacological, and physical stimuli [1]. NSAIDs (nonsteroidal anti-inflammatory drugs) and glucocorticoids manage inflammation, while opioids, and NSAIDs are used for pain relief. However, NSAIDs can cause gastrointestinal problems and cardiovascular complications (e.g. edema and hypertension), while glucocorticoids have long-term side effects, including adrenal suppression, osteoporosis, and ocular complications like glaucoma and cataract [1]. On the other hand, opioids induce side effects like sedation, nausea, respiratory suppression, constipation, vomiting, addiction, and bladder spasm [1]. Therefore, the search for safer anti-inflammatory and analgesic drugs from natural sources is crucial [2].

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Plants have long been used to enhance health and for their medicinal benefits [3], their medicinal properties are attributed to a variety of phytochemicals such as flavonoids, alkaloids, saponins, volatile oils, coumarins, and terpenoids [1]. Plants have several advantages, including low cost, minimal side effects profiles and a high level of acceptability [4]. Numerous plants have been found to exhibit anti-inflammatory and analgesic properties including *Bosewellia serrata* (Burseraceae), *Cannabis sativa* (Cannabinaceae), *Curcuma longa* (Zingiberaceae), *Harpagophytum procumbens* (Pedaliaceae), *Salix alba* (Salicaceae), *Zingiber officinale* (Zingiberaceae), and *Lippia dulcis* (Verbenaceae) [5,6].

Cedrus libani and *Pinus brutia* **(Fig. 1)** have long been utilized in ethnomedicine as fatigue relievers, anti-aging, anti-inflammatory, antibacterial, and antifungal agents [7,8]. *Cedrus libani* (cedar) is a tree of great medicinal and historical importance, and it was utilized in the preservation of corpses in Egypt (mummification) [9]. Cedar is commonly used in traditional medicine in the treatment of catarrhal respiratory tract disorders [10], and in Lebanon, it has been used to alleviate toothache and to treat a variety of infectious diseases [3,8]. Some studies have shown that it has anti-inflammatory, antioxidant, antiviral, antimicrobial and larvicidal effects [7,10,11].

Pinus brutia (pine) is an important tree in the Mediterranean region, it has considerable folk medicinal applications, and some research has revealed anti-inflammatory, antioxidant, and antibacterial properties [7]. Cones, tar, resin, and pine honey were all used medicinally to treat skin disorders, the common cold, cough, wounds, bronchitis, and asthma. Turpentine from *Pinus spp.* possesses potent analgesic and antioxidant effects [12].

Previous research investigated the anti-inflammatory potential of *C. libani* cone essential oil, 2-himachelen 7-ol, and *P. brutia* bark [9,11,13]. Moreover, in our previous work the anti-inflammatory and analgesic activities of an ethanolic extract of *C. libani* and *P. brutia* leaves, as well as the leaves essential oil, were investigated [14,15]. Interestingly, the ethanolic extract demonstrated superior activities over the essential oil and diclofenac sodium [15]. Therefore, the aim of this work was to enhance the anti-inflammatory and analgesic activities of ethanolic extracts from both plants by fractionating the extract with different polarity solvents and testing the activity of these fractions *in vitro* and *in vivo*, as well as to preliminary determine the major active constituents.

a **b**

Fig. 1: Leaves of *Cedrus libani* (a) and leaves and cones of *Pinus brutia* (b).

MATERIALS AND METHODS

Materials

Chemicals

Ethanol, methanol, Folin-Ciocalteu reagent (Scharlau, Spain), Distilled deionized water, Hexane, Chloroform, Butanol, HCl, Sodium chloride, toluene and formaldehyde (SCP, England), Ethyl acetate (Sham lab/Fischer, Syria), Egg Albumin powder, Sodium phosphate monobasic dehydrate (Acros organics, United States), Sodium phosphate dibasic dehydrate, (sigma Aldrich, Germany), Sodium diclofenac (Amoli Organics Pvt., India), tween 80, carrageenan, glacial acetic acid, formic acid, Aluminum chloride, ferric chloride, vanillin and TLC Silica gel 60 F254 (Merck, Germany), Na_2CO_3 (Panreac Quimica Sau, Spain), gallic acid (Prolabo, Spain), rutin (Extrasynthese, France).

Equipments

The following devices were used in this study: Electronic balance (Sartoruis AG, Germany), rotary evaporator (Heidolph Instruments, Germany), UV-1800 spectrophotometer (shimadzu, Japan), Ultra sonic (Hawashin, Korea), water bath (J.P. Selecta, Spain), STE Analgesic Meter Tail Flick (tinateb, USA), and Digital Caliper (Gilbert, China).

Plant material

Leaves of *C. libani* and *P. brutia* were collected from the campus of Aleppo University during July, 2021. The harvested leaves were shade-dried and kept in a tightly sealed container until use.

METHODS

Preparation of Plant Extracts

Cedar and pine leaves were used to prepare an ethanolic extract. Briefly, 30 g of powdered samples were extracted with 70% ethanol for 24 hours and the extraction was repeated 3 times. The extract was then evaporated using a rotary evaporator at 40 °C under low pressure, and the yield of each extract was calculated [14].

Fractionation

The ethanolic crude extract from each plant (2 g) was suspended in distilled water (100 ml), then extracted with different organic solvents such as hexane, chloroform, ethyl acetate, and butanol. All extracts were filtered separately to remove particles and evaporated completely using a rotary evaporator under reduced pressure at 40° C [16].

Evaluation the anti-inflammatory effect in vitro

In vitro inhibition of egg albumin denaturation

In a test tube, 3 ml of 1% egg albumin solution (in phosphate buffered saline pH = 7.4) was added, along with 2ml of each concentration (25, 50, 100 µg/ml) of pine and cedar ethanolic extracts and their fractions. The positive control was diclofenac Na (120 µg/ml), while the negative control was distilled water. The mixture was then incubated at 37°C ± 2 in a water bath for 30 minutes, followed by 10 minutes at 60 °C. After cooling the absorbance was measured at 660 nm using the vehicle as a blank [17].

The percentage inhibition of albumin denaturation was calculated by the formula (I):

$$(\%)\text{Inhibition} = 100\times[1 - Vt/Vc] \quad \text{.........(I)}$$

Where Vt is the absorbance of tested sample and Vc is the absorbance of control.

Evaluation the anti-inflammatory and analgesic effect in vivo

Animals

Males and females Wister albino rats weighing 120 −150 g were housed in well-ventilated plastic cages in animal house at Aleppo University's pharmacy faculty. Rats were kept under controlled conditions with a 12-hour light/dark cycle, a regulated temperature of 25 ± 3ºC, and a relative humidity of 55 ± 5. They had free access to drinking water and food. The experiments began after the animals had been acclimated for a week. The protocol of this study was approved by the Ethics Committee of Faculty of Pharmacy, Aleppo University, Syria (registration number 2/I, 2022).

Gel preparation

A base Gel was prepared using carpobol (1 g), methylparaben (0.2 g), propylparaben (0.1 g), propylene glycol 400 (5 ml), tri-ethanolamine (QS), and distilled water (until 100ml). Additionally, gels containing 1% of either the ethanolic extract of cedar or pine, or the AQ fraction, or the EA fraction were also prepared [14].

Acute skin irritation test

Six rats, three males and three females, were tested by shaving their dorsal region and marking a circle with a 1 cm radius to apply cedar ethanolic extract gel twice a day. These areas were monitored for 7 days to evaluate the presence of irritation. The previous approach was applied for pine extract, AQ fraction and EA fraction gels[14].

Assessment of the anti-inflammatory activity using carrageenan-induced edema test

Ethanloic extract of cedar and pine, as well as the aqueous and ethyl acetate fractions (the most active in vitro fractions) were tested for their anti-inflammatory effects using carrageenan test following intraperitoneal and topical application.

I.Intraperitoneal application

Rats were randomly divided into 6 groups, each with 5 rats. The first group was the negative control group, which received saline solution 0.9 % (i.p.). The second group received the positive control (diclofenac Na 30 mg/kg i.p.). The third group got a cedar ethanol extract (30 mg/kg i.p.) and the fourth group got cedar aqueous fraction (30 mg/kg i.p.). In the fifth group, pine ethanol extract (30 mg/kg i.p.) was used. Pine ethyl acetate fraction (30 mg/kg i.p.) was assigned to the sixth group.

After 30 minutes of treatment, rats were injected with 100 µl of 1% carrageenan solution in 0.9% saline in the right hind paw of rats and paw volume was measured using a Digital Calliper before and 1, 2, 3, and 4 hours after carrageenan injection [1].

The percentage inhibition of edema was calculated as follows:

$$(\%)\text{Inhibition} = 100\times[1 - Vt/Vc] \quad \text{……….(II)}$$

Vt: Edema volume in group treated with extracts or fractions.

Vc: Edema volume in negative control group.

II.Topical application

In this experiment rats were divided into 6 groups of 5 rats:

- First group: The negative control (placebo, base gel).
- Second group: The positive control (diclofenac Na gel 1%).
- Third group: *C. libani* ethanolic extract gel 1%.
- Fourth group: Aqueous fraction of *C. libani* gel 1%.
- Fifth group: *P. brutia* ethanolic extract gel 1%.
- Sixth group: Ethyl acetate fraction of *P. brutia* gel 1%.

After applying the gel for one hour, we injected 100 µl of a 1% carrageenan solution in 0.9% saline into the right hind paw of rats. We measured the paw volume using a Digital Caliper before the injection and at 1, 2, 3, and 4 hours after the carrageenan injection [18].

The percentage inhibition of edema was calculated as follows:

$$(\%)\text{Inhibition} = 100\times[1 - Vt/Vc] \quad \text{…….(III)}$$

Vt: Edema volume in treated group.

Vc: Edema volume in control group.

In vivo evaluation of analgesic activity

The analgesic effects of ethanolic extracts and fractions were evaluated using chemical-induced (formalin test) and heat-induced (tail flick test) nociception models in rats.

I.Formalin test

Rats were randomly divided into 6 groups, each with five rats and were treated with extracts as mentioned in section (3.3.2.4. I). However, after 30 minutes of treatment, all groups were injected with 50 µl of formalin solution (2.5% in 0.9% saline) into the right hind paw of rats, and the acute analgesic impact was assessed in the first five minutes (first phase), and the chronic analgesic effect was studied in the minutes 20 − 40 (second phase) [1,19]. The pain inhibition (%) was calculated as follows:

$$(\%)\text{Inhibition} = 100\times[1- LTt/LTc] \quad \text{…….(IV)}$$

LTt: Licking/biting time of treated group
LTc: Licking/biting time of control group

II.Tail flick test

Rats were treated as in section (3.3.2.4. II). The analgesic effect was evaluated using infrared light where light is focused on the animal's tail and a timer starts, when animal flicks its tail, the timer stops and the latency time, which is a measure of the pain threshold, is recorded. The maximum allowable latency time (cut-off time) was 20s to avoid tissue injury, whereas the pretreatment control latency time was 8.5 s. All rats were evaluated before any treatment and after 10, 20, 30, 40, 50, 60, 70, 80, and 90 minutes of applying the gel [20]. The mximum possible effect (MPE) was calculated as follows:

%MPE = 100 × ((post treatment latency time − pretreatment latency time) / (cut-off latency time − pretreatment latency time)) …..(V)

Phytochemical analysis
Using TLC

The phytochemical analysis of ethyl acetate fraction of *P. brutia* and the aqueous fraction of *C. libani* was performed on 20×20 cm (0.25 mm thick) TLC silica gel 60 F254 plates. 50 µl of each extract solution at a concentration of 10 mg/ml was applied as spots onto TLC plates with micro capillary tubes. Sheets were developed in previously saturated chamber with mobile phase [21]. Flavonoids, phenolic acids, tannins, and catechins were tested in each extract by using mobile phases and detection reagents shown in **Table 6**[22,23,24,25]. Saponins were absent in both plants ethanolic extract as indicated in our previous work [14].

Determination of total phenolic, flavonoids and tannin contents
I.Determination of total phenolic content (TPC)

The total phenolic content of cedar ethanolic extract and its AQ fraction as well as pine ethanolic extract and its EA fraction was determined by employing the Folin-Ciocaltaeu assay [26]. 100 µl of sample (1 mg/ml) was mixed with 500 µl of Folin Ciocalteu's phenol reagent (10%). After 1 minute of well mixing, 500 µl of Na_2CO_3 solution (7.5% w/v) was added to the mixture. After incubation for 45 minutes in a water bath (45ºC), the absorbance of blue color was read at 765 nm using a UV spectrophotometer. TPC was calculated from a calibration curve using gallic acid (GA: 0.01 − 0.1 mg/ml) as a standard and expressed as milligrams of gallic acid equivalents (GAE)/g of dried sample [26]. Dtermination was carried out in triplicate.

II.Determination of total flavonoid content (TFC)

The aluminium chloride colorimetric test was used to assess the total flavonoid content of pine ethanolic extract and its EA fraction. The reaction mixture consists of 500 µl of diluted extract and 500 µl of 2% methanolic aluminum chloride. After 60 min incubation at room temperature, the absorbance was read at 415 nm versus blank with an UV/Visible spectrophotometer. A calbration curve of rutin (0.01, 0.02, 0.03, 0.04, 0.05 and 0.06 mg/ml) was drawn and the TFC was expressed as mg of rutin equivalents /g dry weight [27]. Determination was done in triplicate.

III.Determination of total tannin content (TTC)

The total tannin content of cedar ethanolic extract and its AQ fraction was determined by Folin - Ciocalteu method as described by Kavitha Chandran CI et al. (2016) with slight modification [28]. 0.1 ml of diluted extract was added to 50 µl of Folin-Ciocalteu phenol reagent (10%). After mixing, 750 µl of distilled water and 100 µl of 35% Na_2CO_3 solution were added. The mixture was well mixed and kept at room temperature for 30 min. Absorbance of test solution was measured against a blank at 745 nm with an UV/Visible spectrophotometer. Standard curve of tannic acid (0.025 − 0.3 mg/ml) was prepared and the results were expressed as mg of tannic acid equivalents/g of dried sample. The estimation was carried out in triplicate.

Statistical analysis

Results were expressed as mean ± SD. Values were analyzed by one-way ANOVA and Student's t-test for unpaired comparison. The p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Results

Yield of ethanolic extract and its fractions

The yield of ethanolic leaves extracts of *Cedrus libani* and *Pinus brutia* were 35.6% and 38.5%, respectively. Upon fractionation the aqueous fraction of both plants had the highest yield **(Table 1)**. Whereas, the ethyl acetate fraction of *C. libani* and the hexane fraction of *P. brutia* had the lowest yield **(Table 1)**.

Evaluation of the anti-inflammatory effect *in vitro* and *in vivo*

Inhibition of egg albumin denaturation

Egg albumin denaturation test findings are summarized in **Tables 2** and 3. Results showed that ethanolic extracts of both plants and their fractions were more active than diclofenac Na (its protein denaturation inhibition was 21% at 160 µg/100ml).

At all tested concentrations the aqueous fraction of the cedar ethanolic extract demonstrated a significant activity in comparison to other fractions ($p<0.05$) **(Table 2)**. Whereas, the ethyl acetate fraction of pine ethanolic extract at all tested concentrations demonstrated statistically a significant greater activity ($p<0.05$) in comparison to other fractions **(Table 3)**, although there were no statistically significant differences between the aqueous fraction and ethyl acetate fraction at the 25 µg/ml concentration.

Table 1: Yield of ethanolic extract fractions (g) of *C. libani* and *P.brutia*.

Fraction	*C. libani* fractions yield (g)	*P. brutia* fractions yield (g)
Hexane	0.14g	0.04g
Chloroform	0.15g	0.05g
Ethyl acetate	0.10g	0.19g
Butanol	0.19g	0.08g
Aqueous	1.23g	1.50g

Table 2: Percentage inhibition of albumin denaturation of *Cedrus libani* ethanolic extract and its fractions.

Tested material	25 µg/ml	50 µg/ml	100 µg/ml
Cedrus libani	% Inhibition		
Ethanolic extract	29.90±0.04$	35.40±0.04*	41.96±0.01$
Ethyl acetate (fraction)	21.91± 0.01*	34.51± 0.05*	35.82± 0.005*
Aqueous (fraction)	26.15± 0.09	38.19± 0.03	41.71± 0.01
Chloroform (fraction)	19.97± 0.11*	29.71± 0.08*	36.82± 0.09*
Butanol (fraction)	13.83± 0.02*	15.32± 0.03*	16.35± 0.02*
Hexan(fraction)	20.74± 0.04*	21.97± 0.02*	23.45± 0.01*

Data are expressed as Mean ± SD of 3 experiments with *indicating a significant difference when compared to the Aqueous fraction ($p<0.05$) and $^{\$}$ indicating a significant difference between fractions and the ethanolic extract.

Table 3: Percentage inhibition of albumin denaturation of *Pinus brutia* ethanolic extract and its fractions.

Tested material	25 µg/ml	50 µg/ml	100 µg/ml
Pinus brutia	% Inhibition		
Ethanolic extract	31.02± 0.05$^{\$}$	40 ± 0.04$^{\$}$	46.53± 0.14$^{\$}$
Ethyl acetate (fraction)	35.14 ±0.004	38.41± 0.01	46.86±0.01
Aqueous (fraction)	31.19 ±0.01$^{\#}$	32.73± 0.02*	39.17± 0.05*
Chloroform (fraction)	23.61 ± 0.15*	29.88± 0.03*	31.14 ± 0.04*
Butanol (fraction)	13.05 ± 0.06*	17.87± 0.02*	18.18 ± 0.08*

Data are expressed as Mean ± SD of 3 experiments where * indicates a significant difference as compared with the ethyl acetate fraction ($p<0.05$), $^{\#}$ indicates a significant difference within fractions ($p<0.05$) and $^{\$}$ indicates a significant difference between fractions and the total extract.

Inhibition of carrageenan induced edema

I. Intraperitoneal administration

All tested extracts, that were administered intraperitoneally, exhibited a significant reduction in paw volume ($p<0.001$) when compared to the negative control, and the inhibition of edema by all extracts was better than diclofenac Na **(Fig. 2)**.

The inhibition of paw edema by the aqueous fraction (AQ) of cedar was time-dependent and more important than cedar ethanolic extract ($p<0.001$), except for the first hour, when the activity of the ethanolic extract was superior to the AQ fraction. When compared to the negative control, the maximum edema inhibition percentage was 99.82 ± 0.01 after 4 hours. On the other hand, the *P. brutia* ethyl acetate (EA) fraction was more effective than pine main ethanolic extract. The EA fraction inhibited paw edema by 97.14% ± 0.05 in the fourth hour ($p<0.001$). With the exception of the first hour, the AQ fraction of cedar performed better than the EA fraction of pine ($p<0.001$).

II. Topical application

The acute skin irritation test was carried out prior to topical application, as indicated in section 5.6.3. After applying *C. libani* or *P. brutia* extract, or AQ fraction, or EA fraction gels, the rats did not exhibit any indicators of irritation, such as redness or edema.

As shown in **Fig. 3** topical application of ethanolic extracts and the fractions reduced paw edema in a time-dependent manner. The cedar ethanolic extract had the best results in the first hour when compared to the AQ fraction ($p<0.001$). Following that, the AQ fraction was the most effective, with a fourth-hour inhibition ratio of 90.01 ± 0.17 ($p<0.001$). Furthermore, the AQ fraction outperformed diclofenac (p<0.001).

Pine ethanolic extract gel was less effective than diclofenac gel in the first hour, however there was no statistically significant difference between the two gels ($p>0.05$). EA fraction was more efficacious than diclofenac in the second hour ($p<0.001$), while the ethanol extract was less effective. In the third and fourth hours, all extracts and fractions outperformed diclofenac gel ($p<0.001$).

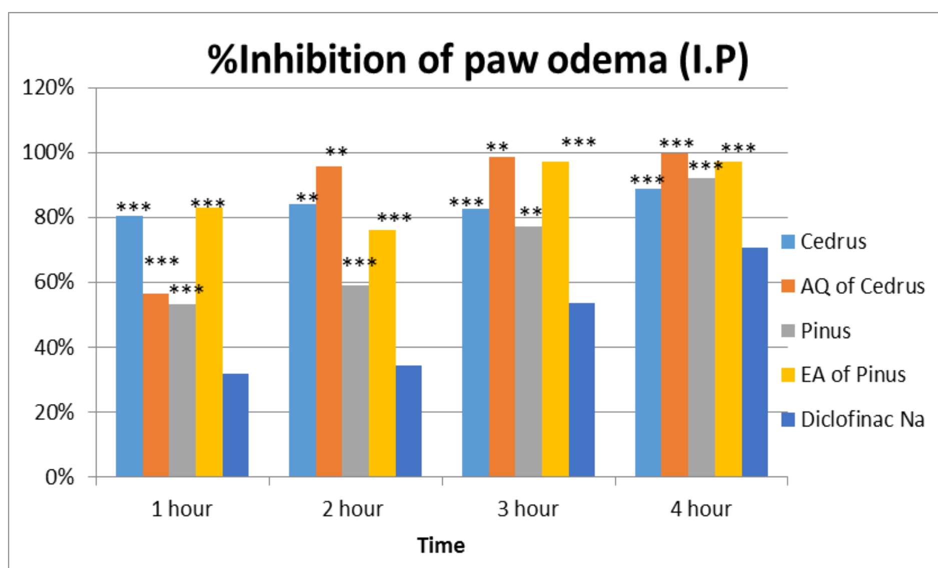


Fig. 2: Inhibition % on carrageenan-induced paw edema in rats. Data are expressed as the mean of 5 rats per group. ** $p<0.01$, *** $p<0.001$ as compared to diclofenac Na group.

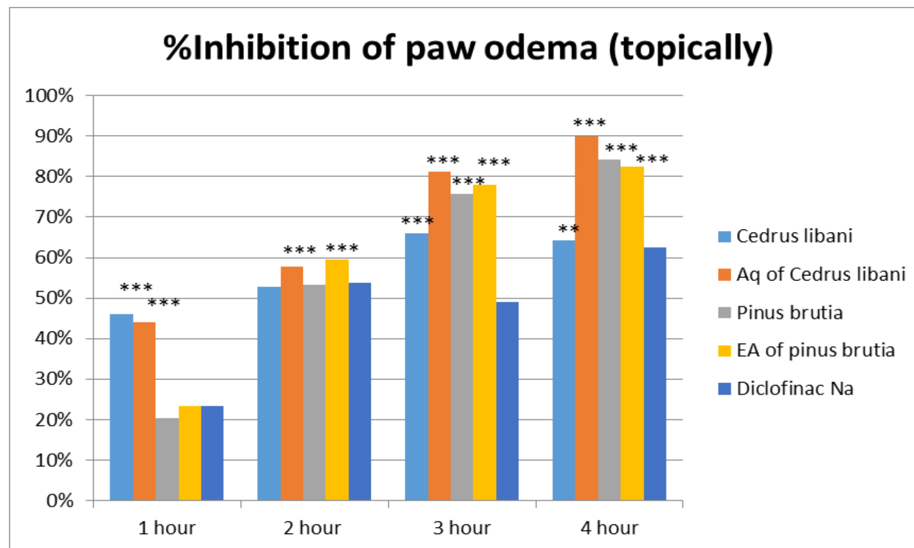


Fig. 3: Percentage inhibition of gels of *C. libani* ethanolic extract, aqueous fraction, *P. brutia* ethanolic extract and ethyl acetate fraction on carrageenan induced paw edema in rats. Data are expressed as mean of 5 animals per group where ***p<0.001 and ** p<0.01 indicate a significant difference as compared with the diclofenac group.

Analgesic effect Evaluation

I. Formalin test

As shown in **Fig. 4**, all studied extracts significantly decreased licking time in both the acute and chronic phases and outperformed diclofenac Na, with the exception of the AQ fraction.

In the first phase, all tested samples significantly decreased neurogenic pain when compared to diclofenac Na, with the exception of cedar AQ fraction, which had the same effectiveness as diclofenac (p>0.05). The highest analgesic effect was seen in the EA fraction, with an inhibition ratio of 73.67% ± 0.06 (p<0.001). The cedar AQ fraction was less effective than the cedar ethanolic extract with an inhibition ratio of 56.86% ± 0.22 (p<0.001).

In the second phase, results indicated that ethanolic extracts of both plants, as well as the pine EA fraction, reduced inflammatory pain by 43.77%, 44.88%, and 59.99%, respectively, and were more effective than diclofenac Na. The activity of the cedar AQ fraction was comparable to that of diclofenac Na (p>0.05). It's worth mentioning that the EA fraction was the most active in both phases.

II. Tail flick test

Topical Application of extracts and fractions on the rats' tail showed a significant analgesic effect in all groups as compared to the negative control group **(Fig. 5)**. The best analgesic effect was for pine ethanolic extract (p<0.001) in all time periods except in the minute 10, where the activity of cedar AQ fraction was better than that of pine ethanolic extract with %MPE of 94.78 ± 0.98. However, the analgesic effect of pine ethanolic extract decreased with time but persisted till the minute 70. EA fraction was less effective than pine ethanolic extract (p<0.001), but its analgesic effect lasted for 90 min. On the other hand, cedar AQ fraction was more effective than that of cedar ethanolic extract (%MPE of 81.38 ± 0.46 in the min10), except after 20 min where the ethanolic extract exerted better activity than that of AQ fraction (p<0.001). The activity of AQ fraction gradually decreased till the min 60.

In all times, pine ethanolic extract was more active than diclofenac gel (P<0.001). The EA fraction had more activity than diclofenac (p<0.001), except in the minute 20, when diclofenac had higher activity. Only in minutes 10, 20, and 60 did the cedar ethanolic extract's activity surpass that of diclofenac (p<0.001). On the other hand, the AQ fraction was better than diclofenac (P<0.001) in all time periods except minute 40.

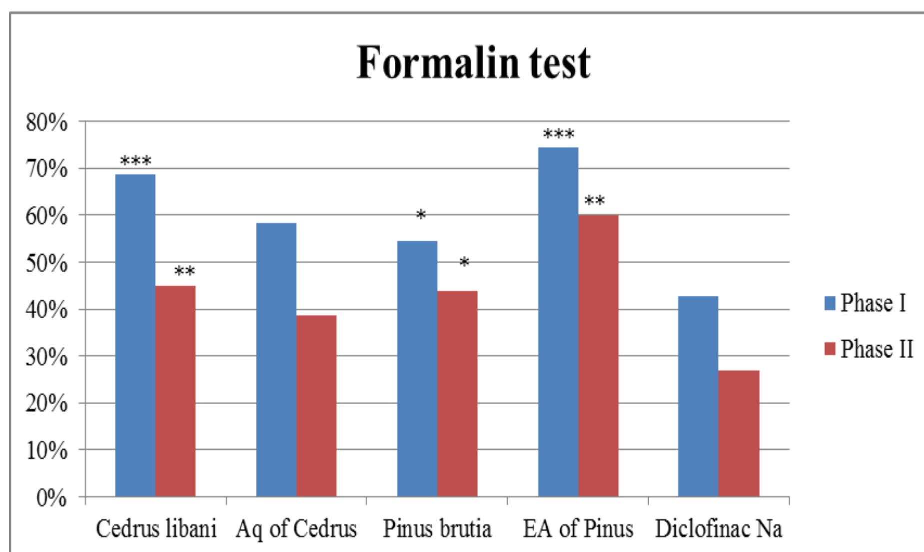


Fig. 4: Pain inhibition % of tested extracts and fractions on the phase I and phase II in formalin test in rats. Data are expressed as Mean for 5 animals per group. ****p*<0.001, ** *p*<0.01 and * *p*<0.05 indicates a significant difference when compared to the diclofenac Na group.

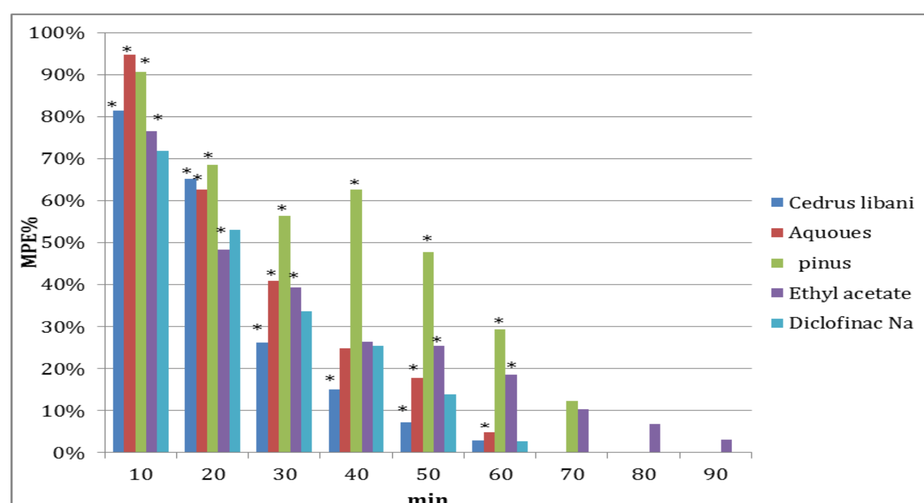


Fig. 5: %MPE of extracts, fractions and diclofenac Na. *indicates a significant difference to diclofenac group (*p*<0.001).

TLC analysis

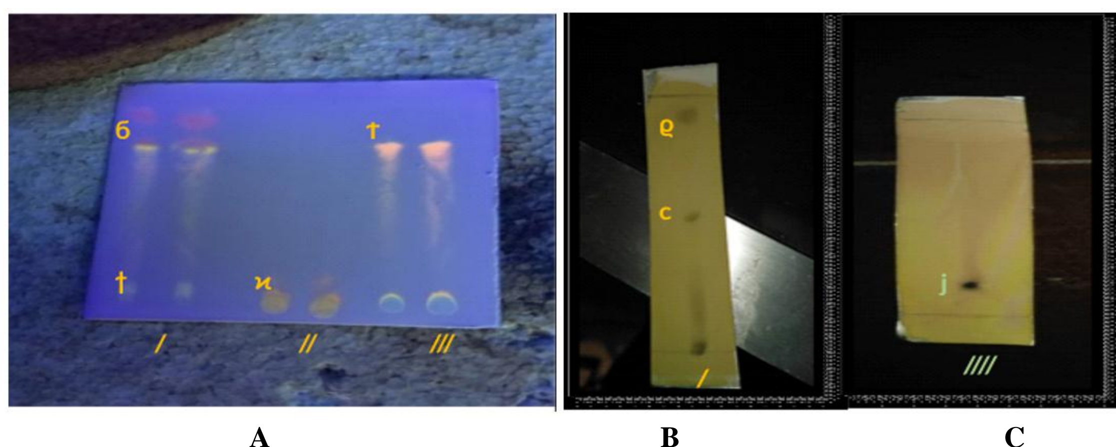
The results of the TLC analysis are presented in **Table 4**. It is clear that the EA fraction of Pine is rich in flavonoids and in comparison with Quercitin reference one spots had the same R_f of Quercitin and phenolic acids, whereas the AQ fraction is rich in polyphenols (tannins and catechins) as showed in **Fig. 6**. Saponins were not detected as they were absent in the ethanolic extract, as indicated in our previous work [14].

However, this doesn't confirm the identity of the constituents, it requires more advanced technologies.

Table 4: Results of TLC detection of Tannins, catechins, flavonoids and phenoic acids.

Secondary metabolites	Ethyl acetate fraction of *Pinus* extract R_f / color of spots	Aqueous fraction of *Cedrus* extract R_f / color of spots
Flavonoids	+ R_f= 0.8/yellow spots (MP1, detection reagent 1) as quercetin	-
	+ R_{f1}= 0.8/ orange spots (MP 2, detection reagent 2) same as quercetin R_{f2} = 0.08/ orange spots (MP 2, detection reagent 2)	-
Phenolic acid	+ R_f= 0.89/ green spots (MP1)	-
	+ R_{f1}= 0.89/ green spots (MP2) R_{f2} = 0.53/ green spots (MP2)	-
Tannins	-	+ R_f= 0.23/ brown spots
Catechins	-	+ R_f= 0.23/ pink spots

-: absence, +: presence, MP: mobile phase presented in Table 6.



A B C

Fig. 6: Results of TLC: A: Detection of Flavonoids (I: Ethyl acetate fraction of pine, II: Rutin, III: Quercitin, (MP2 as in table 1: 1)R_f = 0.8 for EA,2)R_f= 0.8 for Quercitin, 3)R_f =0.08 for EA, 4)R_f =0.1 for Rutin).
B: Phenolic acids in ethyl acetate fraction of pine (MP2 in table 1: 5)R_f =0.89, R_f =0.53 for EA).
C: Detection of Tannins in aqueous fraction of cedar(IIII: AQ fraction of cedar) (MP1in table 1: R_f =0.23 for AQ) .

Determination of total phenolic, flavonoids and tannin contents

TPC, TFC, and TTC of both plant ethanolic extracts and their fractions were calculated from the regression equation of corresponding calibration curve Y = 13.988x + 0.0337 (R^2 = 0.9953), Y = 9.2856x + 0.0406 (R^2 = 0.9983), and Y = 3.6609x + 0.658 (R^2 = 0.9843), respectively.

The TPC, TFC, and TTC were expressed as mg standard equivalents per g of extract in dry weight as well as per g of leaves dry weight (mg/g).

According to the findings, cedar aqueous fraction had the greatest quantity of phenolics, but the ethanolic extract of both plants contained the least amount **(Table 5)**. On the other hand, the EA fraction was richer in

flavonoids (87 ± 0.021 mg RE/ g dry weight of extract, 2.697 ± 0.021 mg RE/ g dry weight of plant) in comparison to the pine ethanolic extract (11.3 ± 0.002 mg RE/ g dry weight of extract, 4.35 ± 0.002 mg RE/ g dry weight of plant. The AQ fraction had higher tannin content (161 ± 0.012 mg TAE/ g dry weight of extract , 33.005 ± 0.012 mg TAE/ g dry weight of plant than the cedar ethanolic extract (72 ± 0.008 mg TAE/ g dry weight of extract 25.63 ± 0.008 mg TAE/ g dry weight of plant.

Dıscussıon

C. libani and *P. brutia* are used in traditional medicine for the treatment of rheumatism, pile, pains, arthritis. In this study the anti-inflammatory and analgesic effects of ethanolic extract fractions of both plants were tested. *In vitro* results showed that the albumin denaturation inhibition was concentration dependent. The ethyl acetate (EA) fraction from *P. brutia* and the aqueous (AQ) fraction of *C. libani* inhibit albumin denaturation better than other fractions ($p<0.001$). This inhibition activity is due to secondary metabolites, where TLC screening **(Table 4)** confirm that EA fraction of pine contains phenolic acids and flavonoids and the AQ fraction of cedar contained basically polyphenols such as tannins and catechins. Quantitative determination of phenolics revealed that the content of the two ethanolic extracts was approximately equal; however, the phenolic content of the cedar AQ fraction was higher than that of the pine EA fraction **(Table 5)**.

On the other hand, EA fraction war rich in flavonoids and the AQ fraction was rich in tannins. Flavonoids and tannins may be responsible for the in vitro activity of the fractions as they were responsible for the anti-inflammatory activity of Sterculia setigera extract using albumin denaturation inhibition test (I: 72.23 ± 3.14% at 1000 µg/ml) [29].

Table 5: Total phenolic content (TPC) in ethanolic extract of *C. libani* and *P. brutia* and their fractions.

Extract or fraction	TPC mg GAE/ g dry weight of extract	TPC mg GAE/ g dry weight of leaves
Ethanolic extract of *Cedrus*	64.8± 0.012	23.06± 0.012
Aqueous fraction of *Cedrus*	179.2± 0.012	36.73 ± 0.012
Ethanolic extract of *Pinus*	63.4± 0.015	24.40± 0.015
Ethyl acetate fraction of *Pinus*	162.8± 0.013	5.046 ± 0.013

GAE: gallic acid equivalents

Table 6: Mobile phases and detection reagent of phytochemicals using TLC.

Chemical groups	Mobile phase (MP)	Detection reagent
Flavonoids	1. Ethyl acetate: glacial acetic acid: formic acid: water (100 : 11 : 11 : 26) [22] 2. Toluene: ethyl acetate: glacial acetic acid (30 : 40 : 5) [23]	1. Aluminum chloride (2% methanolic) + UV light (354 nm) 2. 1% methanolic diphenylboryloxyethylamine and 5% ethanolic polyethylene glycole 4000 + UV light (354nm).
Phenolic acids	1. Chloroform: methanol: water: formic acid (organic phase) (80 : 13 : 2 : 5) [22] 2. Chloroform: methanol (2 : 3) [24]	2% ethanolic ferric chloride (acidified with 2N HCl) + visible light
Tannins	Toluene: ethyl acetate: formic acid: methanol (3 : 3 : 0.8 : 0.2) [22]	2% ethanolic ferric chloride + visible light
Catechins	Ethyl acetate: methanol: water (10 : 2 : 1) [25]	Vanillin: sulfuric acid (1:1)

The anti-inflammatory activity was evaluated *in vivo* using carrageenan test, which is highly sensitive to nonsteroidal anti-inflammatory drugs and has long been accepted as a useful model to ascertain the anti-inflammatory effects of natural products[30]. This test is a reasonable model for evaluating the effects of different agents on acute inflammation[30]. In addition, it has been demonstrated that reduction of carrageenan-induced inflammation is a highly predictive measure of anti-inflammatory medication efficacy in human inflammatory illnesses, and the dose of NSAIDs in this model correlates with the effective dose that patients should receive[31].

Carrageenan injection induces inflammation of two phases. The early phase (2 h after carrageenan injection) is due to the release of serotonin and histamine, while later phase of edema is attributed to the production of prostaglandin and bradykinin. The later phase was reported to be sensitive to both non-steroidal and steroidal anti-inflammatory agents. Results of this study showed that the inhibition activity of paw edema of EA fraction was better than ethanolic extract and diclofenac Na. This effect could be due to the presence of flavonoids in higher amount than ethanolic extract. It has previously been stated that flavonoids have an anti-inflammatory effect[3,31]. Flavonoids inhibit the enzymes lipoxygenase and cyclooxygenase, which are responsible of the first step of inflammatory responses, and have also inhibitory effects on bradykinin and prostaglandins which are involved in the second phase of edema.

In general, the inflammatory response started by production tissue activators, especially prostaglandin and nitric oxide and flavonoids inhibit key enzyme which biosynthesis these activators by the inhibition of gene expression of cyclooxygenase1 (COX1) and cyclooxygenase 2 (COX2)[31]. Moreover, beside arachidonic acid metabolites, the oxygen-derived free radicals play also an important role in acute inflammation and flavonoids because of their antioxidant effects act as free radicals scavenger [3].

Results of intraperitoneally administration of AQ and EA fractions on carrageenan-induced paw edema inhibition in rats showed remarked activity at a dose of 30 mg/kg. EA fraction had good flavonoids content and this compounds were responsible for the anti-inflammatory activity of Trigonella foenum-graecum aqueous fraction injection on carrageenan- induced edema (inhibition activity was 94.80 ± 3.83% at 4th hour)[32]. Furthermore, the aqueous fraction of cedrus showed anti-inflammatory activity better than diclofenac Na and that may be due to the presence of tannins and catechins which are known to inhibite nuclear Factor-Kappa-B (transcription factor) as well as pro-inflammatory cytokines Interleukin B, Interleukin L, COX 2 and nitric oxide synthase and cause reduction in prostaglandin E2 and nitric oxide. However, the effect of AQ fraction was better than EA fraction because of the presence of high amount of tannin in this fraction. Tannins were also responsible for the anti-inflammatory effect of Alchornea cordifolia in albumin induced edema test (inhibition activity 0.65% ± 0.10 in first hour) and of Ricinus communis [4,5].

Topical application of fraction gels showed also significant inhibition activity better than diclofenac, except for the first hour for EA fractions, where its activity was as that of diclofenac. The activity may be due to polyphenols that possess antioxidant activity which involved in cellular protection. The polyphenols have the ability to scavenging the reactive oxygen and radical species, that are created during inflammatory process and also act as inhibitors of lipid peroxidation, which influence in the formation of prostaglandin and other mediators of inflammation, that decrease erythema and edema [18].

In formalin test the central analgesic agents can inhibit both phases of formalin-induced pain while peripherally effective ones inhibit the late phase of pain. The first phase is inhibited by the opioid receptor blockers [33]. In this study, EA fraction and pine ethanolic extract inhibit the pain in both phases. However, the activity of EA fraction was better than the ethanolic extract and this may be due to the enrichment of this fraction in flavonoids (as showed in the TLC screening and the determination of flavonoids). Flavonoids have analgesic effect through the activation of Nrf 2/HO-1 pathway, and activation of PKG/cGMP/ ATP-sensitive potassium

channels pathway like opioids and cannabinoids which means that activation HO-1 pathway inhibition pain, This is a relevant mechanism because it has been confirmed that morphine stimulated PI3Kγ/AKT pathway that, in turn, activates NO production and NO also indirectly activation of cGMP/PKG and causes the up-regulation of ATP-sensitive potassium channels to the hyperpolarization of primary nociceptive neurons. In addition to the inhibition of the aforementioned pro-inflammatory signaling pathways [33]. The analgesic effect of pine EA fraction in pain both phases aligns with the effect of EA fraction of Ilex dipyrena in both phases [34] and the analgesic effect is due to the involvement of GABA receptors in the test animals because of the presence of phenolics and flavonoid [34]. On the other hand, the AQ fraction of cedar showed important analgesic effect comparable to diclofenac Na ($p>0.05$) but was less active than the ethanolic extract. This means that not only tannins are involved in the analgesic activity but the synergism between different phytochemicals in cedar ethanolic extract such as phenolics and flavonoids. Apart from the AQ fraction, all tested extracts showed better activity than that of diclofenac Na.

The tail flick test revealed a significant difference between the treatment groups, the positive and negative control groups. The analgesic effect of the 1% gel of the EA fraction was shown to be more than diclofenac gel ($p<0.001$), although it was less effective than the ethanolic extract over a longer time span. Thus, the ethanolic extract's synergistic effects among phytochemicals are more active than those of flavonoids in EA fraction. The AQ fraction demonstrated a considerable analgesic effect superior to the cedar ethanolic extract, because of the presence of polyphenols, which play a vital role in suppressing prostaglandin formation and exerting analgesic and anti-inflammatory effects. In Jahromi MAF *et al*. (2020), flavonoids and phenols were responsible for the analgesic effect of ethyl acetate fraction of Solenanthus circinatus because they inhibited certain enzymes like COX or blocked the activity of various mediators like histamine and serotonin[32].

Conclusıon

AQ and EA fractions showed significant anti-inflammatory effects as well as analgesic effects using chemically induced (formalin test) and heat-induced (tail flick test) nociception models in rats. These characteristics can be linked to active phytochemicals found in the fractions, such as polyphenolics. The results showed that interaperitoneal and topical application of AQ and EA fractions produced significant anti-inflammatory activity, outperforming diclofenac Na. In formalin test, the EA fraction and both ethanolic extracts were better than diclofenac Na. Whereas, the AQ fraction exhibited an activity equivalent to that of diclofenac Na, indicating the role of flavonoids in this activity. Nevertheless, in the tail flick test, pine ethanolic extract outperformed EA fraction, albeit for a shorter duration (until minute 70). Conversely, cedar ethanolic extract was underperformed by AQ fraction (except in minute 20). With the exception of minute 40, AQ fraction performed better than diclofenac. Moreover, the EA fraction outperformed diclofenac with the exception of minute 20. The tannins of cedar showed superior anti-inflammatory activity, while the flavonoids of pine exerted more analgesic activity. Novel topical anti-inflammatory and analgesics medicines may be derived from cedar AQ fraction and pine EA fraction. Further toxicological research is, nonetheless, required.

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نشرة العلوم الصيدليـــة
جامعة أسيوط

الفعالية المسكنة للألم والمضادة للالتهاب لأجزاء الخلاصة الإيتانولية لأوراق الصنوبر البروتي والأرز اللبناني

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يستخدم نباتي الصنوبر البروتي والأرز اللبناني من الفصيلة الصنوبرية في الطب التقليدي لشفاء الجروح والروماتيزم. وتستخدم الأدوية المضادة للالتهاب غير الستيروئيدية (NSAIDs) لعلاج الالتهاب والألم كعلاج موضعي أو عن طريق الفم، ولكن استخدامها محدود في بعض الأحيان بسبب آثارها الضارة. ولذلك، فمن الضروري اكتشاف مواد كيميائية جديدة مشتقة من النباتات الطبية. كان الهدف من هذا العمل هو دراسة النشاط المضاد للالتهاب والمسكن للألم لأجزاء الخلاصة الإيتانولية لكلا النباتين بالإضافة إلى تحديد المواد الكيميائية النباتية الرئيسية المسؤولة عن هذه الأنشطة باستخدام كروماتوغرافيا الطبقة الرقيقة والتحديد الكمي لهذه المواد الفعالة في الخلاصة الأساسية والجزء الفعال. تم تقييم النشاط المضاد للالتهاب في المختبر باستخدام اختبار تمسخ الألبومين وعلى حيوانات التجربة بإحداث الوذمة الناجمة عن الكاراجينان، وتم تقييم التأثير المسكن باستخدام اختبار الفورمالين واختبار Tail Flick وتم استخدام ديكلوفيناك الصوديوم كشاهد إيجابي.

أظهرت النتائج أن جزء خلات الايتيل من خلاصة الصنوبر والجزء المائي من خلاصة الأرز يثبط تمسخ الألبومين أكثر من الأجزاء الأخرى. كان للجزء المائي لخلاصة الارز بالطريق الحقني أو موضعيًا على شكل هلام أيضًا فعالية مثبطة للوذمة الناتجة عن الكاراجينان بشكل أفضل من جزء خلات الايتيل حتى الساعة الرابعة، وكلا الجزأين لهما نشاط أكثر من ديكلوفيناك الصوديوم وأكثر من الخلاصة الأساسية لكلا النباتين.

كما قلل جزء خلات الايتيل من استجابة الحيوانات المسببة للألم في كلا مرحلتي اختبار الفورمالين وكان أداؤه أفضل من ديكلوفيناك، لكن الجزء المائي كان له نفس النشاط مثل ديكلوفيناك، وأظهرت الأجزاء أيضًا تأثيرًا مسكنًا عن طريق زيادة كمون الألم في اختبار Tail Flick بطريقة أفضل أو مماثلة للديكلوفيناك. ومع ذلك، كان الجزء المائي أكثر نشاطًا من جزء خلات الايتيل حتى الدقيقة ٣٠. لكن التأثير المسكن لجزء خلات الايتيل استمر لمدة ٩٠ دقيقة. وكانت هذه التأثيرات مرتبطة باحتواء الخلاصات على عديدات الفينول كما ظهر في نتائج كروماتوغرافيا الطبقة الرقيقة فقد تبين احتواء جزء خلات الايتيل على الفلافونوئيدات والحموض الفينولية بشكل رئيسي بينما كان الجزء المائي غني بالتانينات الكاتشية.

أخيراً تم تحديد المحتوى من الفينولات والفلافونوئيدات والتانينات في الخلاصة الأساسية والجزء الفعال لكلا النباتين وقد كان المحتوى أعلى في الجزء الفعال.