Research article

Investigating the anti-inflammatory and analgesic properties of leaves ethanolic extracts of Cedrus libani and Pinus brutia

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ABSTRACT

This investigation aimed to assess the anti-inflammatory and analgesic effects of Cedrus libani and Pinus brutia leaves. The anti-inflammatory property was evaluated by Human Red Blood Cells (HRBC) membrane stabilization assay and Albumin denaturation assay using Sodium diclofenac as a positive control. To evaluate the analgesic property, formalin and tail flick tests were carried out using ethanolic extracts at a dose of 30 mg/kg and gel containing 2% (w/v) of ethanolic extract of each plant. Diclofenac sodium, diclofenac gel 1% and lidocaine gel 2% were used as positive controls.

Results: The effect of inhibiting hemolysis was observed at concentrations (2.5–12.5) μg/ml for P. brutia, and (2.5–25) μg/ml for C. libani. Moreover, albumin denaturation test showed protection effect for both plant extracts with IC50 of 47.74 and 81.50 μg/ml for C. libani and P. brutia extract, consecutively. In Formalin test, both extracts could significantly reduce paw licking time, and in tail flick test, each plant extract gel showed greater efficacy than diclofenac gel by calculating the maximum possible effect (MPE %) for both extracts and Diclofenac.

Conclusion: We concluded that both extracts showed in vitro anti-inflammatory activity at different concentrations when compared to standard drug of diclofenac as well as analgesic activity in formalin and tail flick tests.

1. Introduction

Inflammation is defined as a reaction that produces redness, warmth, oedema, and soreness as a result of infectious, chemical and physical agents such as microorganisms, toxins, radiations, bruises and caustic chemicals [1, 2].

The inflammatory response is a protective process aims to restrict the harmful agents. Another aim is to remove damaged cells to reach healing of the affected tissues or organs [3]. Inflammatory process starts by various chemical mediators which are released from macrophages and neutrophils which are responsible for the initiation, progression, regulation, and eventual resolution of the acute stage of inflammation. Monocytes play a main role in the clearing of cell debris. If the resolution is not occur in the acute stage, a chronic stage will develop [4]. Chronic inflammatory illnesses have been recently considered as the most cause of death worldwide, with more than half of all deaths being attributed to diseases related to inflammation such as ischemic heart disease, chronic kidney disease, cancer, diabetes and neurodegenerative and autoimmune conditions [5].

The main types of anti-inflammatory medications are the steroidal and non-steroidal drugs. Corticosteroids (steroidal drugs) are used to treat asthma and autoimmune inflammatory response. In addition, non-steroidal drugs are used for mild to moderate pain and as antipyretic through the inhibition of cyclooxygenase enzyme [6]. Nevertheless, non-steroidal anti-inflammatory drugs (NSAIDs) have many side effects, such as cardiovascular risk and gastric irritations [7]. Therefore, extensive research was conducted on different plant species and their active compounds, which could constitute a source of new compounds which have anti-inflammatory property with fewer side effects and lower cost [2, 8]. In addition, researches were not exclusively conducted to evaluate the anti-inflammatory effect. Therefore, studies varied to determine all the potential therapeutics. One of the thoroughly evaluated activities in plant species is the analgesic activity.

Pain is defined as a feeling that ranges from mild discomfort to extreme suffering. Pain may be situated in a discrete area, as in an injury, or it may be more diffuse. Analgesic medications are usually administered to treat mild to moderate pain. On the other hand, opiates and other narcotics may be used for severe pain. When pain is associated with inflammatory conditions, non-steroidal anti-inflammatory or steroidal drugs are administered [1]. According to the World Health Organization, approximately 80% of the world population still use plant-based drugs.
which include the medicinal use of plants as anti-nociceptive drugs in traditional treatment [9]. Some examples of medicinal plants that show anti-inflammatory or analgesic therapeutic properties are: Clerodendrum phlomidis L. (Lamiaceae) that could in vitro and in vivo reduce mediators responsible for synovial inflammation [10], Erycibe obtusifolia Benth (Convolvulaceae) that prevent paw swelling and articular damage index score [11], Glycyrrhiza uralensis (Fabaceae) which inhibit in vivo arthritis induced by collagen [12], Magnolia officinalis (Magnoliaceae) that reduce in vivo inflammatory arthritis [13], Lippia dulcis (Verbenaceae) that inhibit in vitro elastase activity [14], Withania somnifera (Solanaeae) and Panax ginseng C.A. Mey. (Araliaceae) both modulate in vivo COX-2 expression [15, 16], Mangifera indica Wall. (Anacardiaceae) could in vivo inhibit the production of IgE [17], and Arnica montana that used topically as counterirritant to relieve pain [18].

Cedrus libani is one of true cedar which is grown in the eastern Mediterranean, Lebanon and western Syria [19]. C. libani was mentioned 75 times in the Bible (Old Testament), and its oil was used to embalm the ancient Pharaohs of Egypt. In the recent years, antioxidants properties of wood cedar oil have been demonstrated [20]. Moreover, C. libani was used traditionally to heal wounds and to treat many diseases in humans and animals, both internally and externally [19].

Pinus brutia Ten. has several names depending on the geographical area: Turkish pine, East Mediterranean Brutia and Calabrian pine (in the southern part of Italy, where pine was first described). Pine trees are useful in many industrial processes; turpentine obtained from pine trees is used in medical, pharmaceutical and nutritional applications, and it is also used in the cosmetics and paint products [21]. In addition, the resin extracted from the P. brutia trees is traditionally used to treat coughs and peptic ulcers, and it is also used externally to heal wounds [22]. According to studies, essential oil (EO) of P. brutia showed significant anti-inflammatory activity at different concentrations (2.5–12.5) μg/ml when compared to standard drug of diclofenac sodium and this effect could be related to the active compounds of EO such as α-Terpineol, Carveol and cis-Verbenol [23]. On the other hand, P. brutia bark extract showed significant effects in inhibiting pancreatic cholesterol esterase which may reduce cholesterol absorption and slow down micro- and macro vascular complications progression [24].

Nature-based medicines having increased attention in order to have novel drugs that could have more therapeutic potential. The 2015 Nobel Prize in Physiology or Medicine was awarded to the developers of Aveurmectin and Artemisinin, which are nature-based drugs. This highlights the possible effects of naturally derived medicines in tropical illnesses treatment. This development follows the World Health Organization’s 2008 ratification of The Beijing Declaration, which promotes the efficacy and safety of folk medicines and claims greater assimilation of these into national health care systems [25].

Therefore, this study aims to evaluate the anti-inflammatory and analgesic properties of both plants, especially there are no studies conducted in the medical literature.

2. Materials and methods

2.1. Chemicals and apparatus

Sodium phosphate dibasic dehydrate (sigma Aldrich, Germany), Sodium phosphate monobasic dehydrate (Acros organics, United States), Distilled deionized water, Sodium chloride (HiMedia Laboratories, India), Sodium Diclofenac (Amoli Organics Pvt., India), Formaldehyde (Merck, Germany), Anhydride acetic acid (Acros organics, USA), Picric acid (BDH Laboratory, England), Hydrochloric acid (Chemlab, Belgium), Chloroform (Eurolab, UK), Sodium bicarbonate (HiMedia, India), Sodium hydroxide (HiMedia, India), 3,5 di-nitro Benzolic acid (Merck, Germany), Vanillin (Merck, Germany), Aluminum chloride (Merck, Germany), Ferric chloride (Merck, Germany), Bismuth nitrate (Merck, Germany), Potassium iodide (Rectapur, prolab, CE), Ethanol (Schalau SL, Spain) and Sulfuric acid (Szurechem product LTD, England). Apparatus used in this study were Electronic balance (Sartoruis AG, Germany), Ultrapure TM aqua purification system (Lotus Co., Ltd., Taipei, Taiwan), UV-1800 spectrophotometer (Shimadzu, Japan), Water bath, Rotary evaporator (Heidolph Instruments, Germany) and TSE Analgesia Meter Tail Flick (tinateb, USA).

2.2. Plant collection

The leaves of both plants were collected in the spring of 2019 from classified trees growing on the campus of Aleppo University. Leaves were dried in shade in a well-ventilated place, then stored in airtight containers.

2.3. Preparation of extracts

C. libani and P. brutia ethanol extracts were prepared using ethanol 70%, the maceration process was done at room temperature for 24 h with stirring, the leaves were re-extracted according to the previous method three times, the solvent was removed by rotary evaporator at 40 °C [26]. Yield% of each extract was calculated using Eq. (1):

\[ \text{Yield}\% = \left( \frac{\text{dry extract weightiness}}{\text{dried plant powder weightiness}} \right) \times 100 \]

2.4. Qualitative analysis of phytochemicals of studied plants

Plant powder or ethanolic extracts (1:10 ethanol 70%, 1 h at room temperature) were tested using tests tabulated in Table 1:

2.5. Evaluation of anti-inflammatory activity in vitro

The anti-inflammatory effect of plant ethanolic extracts was tested using human red blood cell (HRBC) membrane stabilization assay and the albumin denaturation assay.

2.5.1. HRBC membrane stabilization assay

The effects of the P. brutia and C. libani ethanolic extracts on heat-induced HRBC haemolysis was assessed using Shinde’s et al. method with minor modifications [34].

2.5.1.1. Preparation of RBCs suspension

3 ml of fresh whole blood were drawn from healthy nonsmokers’ volunteers, who did not take any alcoholic drinks, and did not use any chemical medicine for one week at least. All experiments were done according to the guidelines of clinical trials Ethics Committee of Aleppo university (Registration No: 866/II) and complies with the ethics of the world medical association declaration of Helsinki. Informed consent was obtained from volunteers who donated blood samples.

Blood samples were pipette into heparin tubes and centrifuged for 10 min at a speed of 3000 rpm, then red blood cells were resuspended using a volume of normal saline solution equal to that of the supernatant and reconstituted as a 40% suspension with 10 mM sodium phosphate buffer (pH 7.4). The buffer solution consists of NaH2PO4 (0.2 g), Na2HPO4 (1.15 g) and NaCl (9 g) in 1 L of distilled water.

Table 1. Qualitative Tests for Phytochemical constituents.

| Phytochemical constituents | Test/reagent |
|----------------------------|--------------|
| Alkaloids                  | Dragendorff reagent [27] |
| Coumarins                  | NaOH + UV light [28] |
| Carbohydrates              | Molisch's test, Resorcinol test and Fehling test [29] |
| Cyanogenic glycosides      | Sodium picate paper [30] |
| Tannins                    | Ferric chloride [31], and vanillin reagent [32] |
| Flavonoids                 | Aluminium chloride 1% [33] |
| Phenols                    | Lead acetate 1% [31] |
| Saponins                   | Foam test [28] |
2.5.1.2. Examining the ability of plant extracts to stabilize red blood cells membranes. The above mentioned phosphate buffer was used to prepare series of dilutions of each plant extract; 5, 10, 15, 20, 25 μg/ml for P. brutia and 5, 15, 20, 25, 50 μg/ml for C. libani.

Two groups of centrifuge tubes were prepared in such a way that each tube contained 5 ml of extract, 4.85 ml of isotonic buffer solution and 0.15 ml of RBCs suspension 40%. One of the groups was incubated 20 min in a water bath at 54 °C. The other group was placed in the refrigerator.

Afterwards, the tubes were centrifuged for 7 min at 3500 rpm and the absorbance of the supernatant was measured at 560 nm, the experiment was run in triplicate. The ability of the plant extract to stabilize the membrane was calculated in percent by the Eq. (2).

\[
\text{Protection \%} = 1 - \frac{A2 - A1}{A3 - A1} \times 100 \tag{2}
\]

Where:

\( A1 \): Absorbance of unheated test sample.
\( A2 \): Absorbance of heated test sample.
\( A3 \): Absorbance of heated control sample.

Saline solution and diclofenac sodium were used as a negative control and a standard drug, respectively.

2.5.2. Evaluation of extract effect on albumin denaturation

The following reaction mixture was prepared; 0.2 ml of hen egg albumin, 2.8 ml of isotonic phosphate buffer (10 mM sodium phosphate, pH 7.4) and 2 ml of extract solution at concentrations (25, 50, 100, 200, 300 or 400 μg/ml) for C. libani and (10, 25, 50, 100 or 200 μg/ml) for P. brutia. The mixture was incubated for 20 min at 37 °C then for 5 min at 70 °C. After cooling, the absorbance was measured at 660 nm. To calculate the ability of the extract to protect protein from denaturation in percent, formula (3) was used [35].

\[
\text{Protection \%} = 1 - \frac{\text{Absorbance of heated test sample}}{\text{Absorbance of heated control sample}} \times 100 \tag{3}
\]

Sodium diclofenac was used as a positive control, while distilled water was used as a negative control.

2.6. Evaluation of analgesic effect

The analgesic effect was evaluated by formalin and tail flick tests.

2.6.1. Animals used in experiments

Wistar Albino rats of both sexes (176 ± 14.7 g for formalin test and 150–200 g for tail flick test were obtained from the animal laboratory at Faculty of Pharmacy. They were allowed for free access to water and food. The light/dark cycle was 12/12 h, and the temperature was 22 ± 2 °C. The protocol of this study was approved by the Ethics Committee of Faculty of Pharmacy, Aleppo University, Syria (registration number (862/1a). All experiments and procedures used in this study were according to the established public health guidelines in Guide for Care and Use of Laboratory Animals (2011).

2.6.2. Formalin test

In this test experimental animals were divided in 4 groups of 5 rats in each group:

- **Group 1**: Negative control- saline solution
- **Group 2**: Positive control- diclofenac sodium (30 mg/kg, intraperitoneally)
- **Group 3**: C. libani leaves extract (30 mg/kg, intraperitoneally)
- **Group 4**: P. brutia leaves extract (30 mg/kg, intraperitoneally)

After 30 min of treatment all groups were injected subcutaneously with 50 μl formalin solution (2.5% v/v) into right hind paw of each rat. Paw licking time was measured by stopwatch for each rat for 10 min (acute pain phase) and from 11-40 min (chronic pain phase) [36, 37]. The percentage of inhibition of paw licking was calculated using formula (4) [38].

\[
\text{Inhibition \%} = \left( \frac{\text{Licking Time (control)} - \text{Licking Time (treatment)}}{\text{Licking Time (control)}} \right) \times 100 \tag{4}
\]

2.6.3. Tail flick test

2.6.3.1. Gel preparation. To prepare gel containing 2% extract of C. libani leaves, 1 g of Carbopel 934 was dispersed in 50 ml of distilled water under continuing stirring for 20 min. Methyl paraben and propyl paraben were dissolved in 10 ml distilled water on a water bath. After cooling, propylene glycol 400 was added. Then leaves extract was added. After that, the previous mixture was mixed with Carbopel 940 solution and the volume was made up to 100 ml using distilled water. Finally, for the adjustment of pH at (6–7) sufficient quantity of triethanolamine (TEA) was used under continuing stirring. The same previous method was followed to prepare a gel from the ethanolic extract of Pinus brutia leaves at concentration of 2%.

Control gel was prepared without adding extracts to check the activity of the vehicles. Materials used in preparing the control gel were weighed according to Table 2.

2.6.3.2. Acute skin irritation test. Test was conducted on 6 rats, 3 males and 3 females by shaving their dorsal area and marked a circle with 1 cm radius to apply P. brutia extract gel twice a day. These areas were checked for 7 days to determine the occurrence of irritation. The same previous method was used for C. libani extract gel [39].

2.6.3.3. Evaluation of analgesic effect. The analgesic effect of gels was evaluated by Tail Flick test. Lidocaine and Diclofenac gels were used as positive control. The experimental animals were divided in 5 groups:

| Group | Gel Description                                |
|-------|------------------------------------------------|
| 1     | Negative control gel                           |
| 2     | Positive control gel- Commercial lidocaine gel 2% |
| 3     | Positive control gel- Commercial Diclofenac gel 1% |
| 4     | P. brutia leaves extract gel 2%                |
| 5     | C. libani leaves extract gel 2%                |

The tail flick latency was measured based on Soni et al. procedure [40]. Pre-dose latency time was measured for each rat twice by applying thermal light beam to the tail of each rat. Beam intensity was adjusted to the level that give a tail-flick latency of 8 s in control animals, then the extracts gels, negative and positive control gels were applied on the distal two thirds of each rat tail and the rat was tested in response to painful stimulation every 10 min for 90 min [41].

Maximum Possible Effect (MPE) was calculated by Eq. (5) [42].

\[
\%\text{MPE} = \frac{\text{postdose latency time} - \text{predose latency time}}{\text{cut-off latency time} - \text{predose latency time}} \times 100 \tag{5}
\]

A 20 s cut-off time was used to avoid thermal injury.

### Table 2. Materials used in control gel preparation.

| Formulation | Ingredients                        | Quantity |
|-------------|-----------------------------------|----------|
| Control     | Carbopel 934                      | 1 g      |
|             | Methyl paraben                    | 0.2 g    |
|             | Propyl paraben                    | 0.1 g    |
|             | Propylene glycol 400              | 5 ml     |
|             | Triethanolamine                   | 1.2 ml   |
|             | Distilled Water                   | Q.S. 100 ml |
2.7. Statistical analysis

Values were expressed as mean ± SD. Values were analyzed by one-way ANOVA. The p-value < 0.05 was statistically significant when compared with control.

3. Results

3.1. Yield of plant extracts

The yield of ethanolic leaves extract of *P. brutia* reached 33.63% and was higher than that of *C. libani* leaves ethanolic extract (29.56%).

3.2. Qualitative analysis of phytochemicals of studied plants

The qualitative analysis showed that the ethanolic leaves extracts of *C. libani* and *P. brutia* contained carbohydrates, tannins, flavonoids and phenols. *C. libani* extract was characterized by containing coumarins, as shown in Table 3.

3.3. Assay of membrane stabilization by heat-induced hemolysis

Ethanolic extracts of both plants showed a concentration dependent activity in stabilization of HRBCs membranes. Moreover, both extracts showed higher efficiency than sodium Diclofenac. *P. brutia* extract showed the highest efficiency which reached 92.88% at 12.5 μg/ml, whereas *C. libani* extract showed an efficiency of 91.54% at 25 μg/ml as shown in Tables 4 and 5.

3.4. Evaluation the extracts effects on albumin denaturation by heat

Ethanolic extracts of both plants showed an efficiency in protecting the albumin from denaturing as shown in Tables 6 and 7. *P. brutia* extract efficiency in protection was higher than *C. libani* extract, with IC₅₀ of 47.74 and 81.50 μg/ml, respectively. Sodium diclofenac does not give any noticeable efficacy at a concentration of 40 μg/ml.

3.5. Evaluation of analgesic effect

3.5.1. Formalin test

Both extracts exerted inhibition of pain behavior. In the acute pain phase after formalin injection in right hind paw the duration of paw licking in negative control group was 207.6 ± 24.8 s. Pinus hydro alcololic extract reduced significantly (p < 0.01) the licking time to 88.6 ± 16.4 s. In this group the inhibition rate was 57.2 % (Figure 1).

| Phytochemicals | Test             | Cedrus libani extract | Pinus brutia extract |
|----------------|------------------|-----------------------|----------------------|
| Saponins       | Foam test        | -                     | -                    |
| Carbohydrates  | Molisch's Test   | +                     | +                    |
|                | Resorcinol test  | -                     | -                    |
|                | Fehling test     | +                     | +                    |
| Coumarins      | NaOH             | +                     | -                    |
| Cyanogenic glycosides | sodium picate | -                     | -                    |
| Tannins        | FeCl₃            | +                     | +                    |
|                | vanillin reagent | +                     | +                    |
| Alkaloids      | Dragendorff reagent | -                 | -                    |
| Flavonoids     | aluminum chloride | +                     | +                    |
| Phenols        | lead acetate     | +                     | +                    |

| Table 4. Effect of *Pinus brutia* extract on heat induced haemolysis of HRBCs. |
|-----------------------------------------------|
| Treatment | Concentration μg/ml | Protection% | Mean ± SD |
|-----------|---------------------|-------------|-----------|
| *Pinus brutia* extract  | 2.5 | 74.71 ± 8.36* | |
|                | 5 | 86.98 ± 1.82 | |
|                | 7.5 | 89.96 ± 1.39 | |
|                | 10 | 92.71 ± 2.98 | |
|                | 12.5 | 92.88 ± 2.73 | |
| Sodium Diclofenac | 100 | 89.21 ± 2.54 | |

*p < 0.01 as compared to positive control.

| Table 5. Effect of *Cedrus libani* extract on heat induced haemolysis of HRBCs. |
|-----------------------------------------------|
| Treatment | Concentration μg/ml | Protection% | Mean ± SD |
|-----------|---------------------|-------------|-----------|
| *Cedrus libani* extract | 2.5 | 84.53 ± 1.60* | |
|                | 7.5 | 86.20 ± 3.29 | |
|                | 10 | 89.96 ± 2.23 | |
|                | 12.5 | 91.49 ± 1.07 | |
| Sodium Diclofenac | 100 | 89.21 ± 2.54 | |

*p < 0.01 as compared to positive control.

| Table 6. Effect of *Pinus brutia* extract on Albumin denaturation. |
|-----------------------------------------------|
| Treatment | Concentration μg/ml | %Protection | Mean ± SD |
|-----------|---------------------|-------------|-----------|
| *Pinus brutia* extract  | 4 | 12.81 ± 1.51* | |
|                | 10 | 24.29 ± 0.00* | |
|                | 20 | 35.33 ± 2.28* | |
|                | 40 | 49.76 ± 3.01* | |
|                | 80 | 67.61 ± 0.49* | |
| Sodium Diclofenac | 40 | - | |
|                | 120 | 11.02 ± 2.70 | |
|                | 160 | 22.80 ± 1.30 | |

**p < 0.01 as compared to positive control (120 μg/ml), *p < 0.01 as compared to positive control (160 μg/ml).

The average licking time in Cedrus group was 76.6 ± 21.1 s with inhibitory effect of 66.2% (p < 0.01). However, In diclofenac group the licking time was 142.5 ± 20.7 with an inhibition rate of 30.49%.

In the chronic pain phase, the duration of paw licking in the control group was 619 ± 21.1 s with inhibition rate of 21.1 s with an inhibition rate of 21.1 s with inhibition rate of 2.73%

| Table 7. Effect of *Cedrus libani* extract on Albumin denaturation. |
|-----------------------------------------------|
| Treatment | Concentration μg/ml | %Protection | Mean ± SD |
|-----------|---------------------|-------------|-----------|
| *Cedrus libani* extract | 10 | 5.23 ± 2.05* | |
|                | 20 | 10.81 ± 3.07 | |
|                | 40 | 40.03 ± 1.29* | |
|                | 80 | 62.54 ± 6.13* | |
|                | 120 | 72.55 ± 4.94* | |
|                | 160 | 79.25 ± 0.59* | |
| Sodium Diclofenac | 40 | - | |
|                | 120 | 11.02 ± 2.70 | |
|                | 160 | 22.80 ± 1.29 | |

**p < 0.01 as compared to positive control (120 μg/ml), *p < 0.01 and #p < 0.001 as compared to positive control (160 μg/ml).
reduced licking time to 243 ± 17 s with inhibition rate of 57.9%, while Cedrus extract inhibited the licking response to 46.3% with licking time of 334.6 ± 35.5 s (p < 0.01). In diclofenac group the licking time was 345.7 ± 21.8 s with an inhibition rate of 43.9%.

3.5.2. Tail flick test

3.5.2.1. Acute skin irritation test. None of irritation signs, such as redness or edema, was shown on any rats after applying P. brutia or C. libani extract gels.

3.5.2.2. In comparison with lidocaine. As shown in Figure 2, %MPE of C. libani was higher than %MPE of Lidocaine at all time periods measurements except at minute 30. %MPE of C. libani reached the highest value at min. 20, while %MPE of P. brutia reached the highest value at min. 10, then %MPE of P. brutia decreased to become less than %MPE of lidocaine and C. libani extract at 20 and 30 min, after that it became more than Lidocaine %MPE at min. 40. At min 50, %MPE of Pinus brutia become more than %MPE of lidocaine and C. libani extract, then it decreased but still higher than %MPE of Lidocaine.

3.5.2.3. In comparison with diclofenac. As shown in Figure 3, %MPE of both extracts were higher than diclofenac. %MPE of P. brutia reached the highest value at min 10, then decreased gradually until minute 50 where it increased slightly, then continued to decrease over time. Whereas %MPE of C. libani reached the highest value at minute 20 then decreased gradually until minute 80 where it increased slightly, then it continued to decrease over the time.

Comparison between %MPE of P. brutia and %MPE of diclofenac showed that there is a statistically significant difference at min 10. Whereas there is a statistically significant difference at min 20, 60 and 70 when comparing %MPE of Cedrus libani with %MPE of diclofenac.

4. Discussion

4.1. Yield of plant extracts and qualitative analysis of phytochemicals of studied plants

Ethanolic extract yield of P. brutia leaves was higher than that of C. libani and this indicated that P. brutia leaves might be richer in active compounds. On the other hand the yield of C. libani leaves in this study was higher than the leaves yield of the same plant in Lebanon, where it was only 5% [43]. However, there was no reference study about the yield of P. brutia leaves ethanolic extracts, but the yield of other plant parts was determined instead, for instance, yields of p. brutia bark methanolic and aqueous extracts were 13.38% and 27%, respectively [44, 45].

These variations in yields in studies could be explained by several factors such as plant preparation process before extraction (dimension of
plant powder, using dried or fresh plants) [46, 47, 48], extraction method, temperature used during extraction and time of extraction as well as solvent: drug ratio [49, 50].

Results of phytochemical screening in the current study harmonized with reference studies, for example the bark of *P. brutia* grown in Turkey contains phenols and catechin tannins [51, 52]. In addition, flavonoids which may have a major role in the anti-inflammatory effects exist in several parts of *p. brutia* grown in Turkey such as: cones and bark [51, 53]. Regarding *C. libani*, Resveratrol and several flavonoids such as Rutin have been identified in the plant grown in Turkey [54]. As mentioned in another research on *C. libani* grown in Taurus Mountains, a peak of catechins was observed by FTIR scan and the proanthocyanidin content was determined in the same study and reached 41.77 mg/g [55].

4.2. Assay of erythrocyte membrane stabilization by heat-induced hemolysis

Erythrocyte membrane is similar to the lysosome membrane. During the inflammatory processes, lysosomes may have an important role in the tissue damage by oxidizing cell membrane lipids. A study in 1979 showed that lysosomes can inhibit the receptor Hsp 90 (asteroid anti-inflammatory receptor) by changing it to a smaller form, preventing it from binding steroidal anti-inflammatory drugs, thus increases the inflammatory state [56]. Therefore, the stabilization of the lysosome membrane can play an important role in preventing the release of proteases and reducing the inflammatory reactions.

The effectiveness of the extracts in stabilizing erythrocyte membrane indicates that they are effective in stabilizing lysosome membrane. Thus, the anti-hemolytic effect of plant extracts can be taken as evidence of the extract's anti-inflammatory effectiveness [35].

Several studies had proven that the presence of flavonoids in plants is responsible for their anti-inflammatory activity [57], and phytochemical screening in this study showed that *P. brutia* and *C. libani* extracts contained flavonoids.

Studies have shown that flavonoids possess anti-inflammatory effect by inhibiting prostaglandin synthesis such as Hesperidin and Diosmin [58], whereas, several biflavonoids such as ginkgetin inhibit phospholipase A2 enzyme [59]. Moreover, some flavonoids such as Quercetin, inhibit COX-2 and 5-LOX enzymes [60]. On the other hand, some flavonoids such as (Fisetin, Kaempferol, Myricetin, Quercetin, Rutin) inhibit histamine release from mast cells [61]. It is well known that during the inflammatory process, many kinase enzymes (like: tyrosine kinase and phosphatidylinositol kinase) are activated and several studies have demonstrated that flavonoids can inhibit protein kinase C enzymes competitively [62]. Other Studies showed that fisetin and luteolin could inhibit protein kinase C [63]. Moreover, the anti-inflammatory effect of some tannin derivatives such as epigallocatechin gallate EGCG can be explained by their antioxidant properties which reduces the production of free radicals [64].

In comparison with similar studies on plants belonging to pinaceae family, namely, *Pinus roxburghii*, *P. wellichiana* and *P. gerardiana*, it was found that the percentage of protection was the best for their bark ethanolic extracts with values of 89.92%, 81.24%, 85.23%, respectively, at the concentration of 2500 μg/mL. However, the protection of leaves extract in this study was better than bark extract and this could be due to the presence of more active compounds in the leaves [65].

4.3. Evaluation the extracts effects on albumin denaturation by heat

Researchers found that rheumatoid arthritis may be a result of protein denaturation and in specific arthritic illnesses protein denaturation may produce auto-antigen [66].

Polyphenols are one of the natural products which are known to have many biological properties and phytochemical screening confirmed that both plants contained tannins and flavonoids that have anti-inflammatory effect [57]. In addition, one study also demonstrated the efficacy of tannins isolated from the bark of *Myricaria bracteata*, such as tamarixinin A, in (croton oil-induced ear edema). The effectiveness of the previous compound in inhibiting edema formation reached 69.8% at a concentration of 200 mg/kg due to its antioxidant properties and its ability to capture free radicals [67, 68].

4.4. Evaluation of analgesic effect

In this study the analgesic potential of leaves hydroethanolic extracts of *P. brutia* and *C. libani* was evaluated using formalin test (chemical-induced pain stimuli) and tail-flick test (heat-induced pain stimuli). Formalin test is used as a model to assess the analgesic potential of substances [69]. In this test exist two phases of pain [70]. The first phase is the non-inflammatory or neurogenic pain and is due to direct pain receptors activation by formalin and the second phase is the inflammatory pain. In this phase many hyperalgesic and inflammatory mediators like prostaglandins, serotonin and histamine are released [71]. In this study both extracts exerted significant analgesic effects in both phases. Therefore, the analgesic effect of the extracts involved both neurogenic and inflammatory mechanisms.

Tail-Flick Test is a nociceptive essay based on the measurement of the latency of the avoidance response to thermal stimulus in rodents. Basically, a thermal stimulus is applied on the tail, when the animal feels discomfort, it reacts by a sudden tail movement. The tail flick reaction time is then measured and used as an index of animal pain sensitivity [72]. In this test both extracts showed also analgesic effect and could increase pain tolerance in rats.
The analgesic effect of both extracts could be due to their contents of various phytochemicals, such as flavonoids, tannins, and phenolic compounds. Flavonoids and tannins have demonstrated anti-inflammatory and analgesic activity in several studies [73, 74]. Flavonoids have been shown to reduce the production of arachidonic acid, prostaglandins, and leukotrienes, and to reduce the high levels of intracellular Ca²⁺. They may interact with 5-HT2A and 5-HT3 receptors which might be involved in the mechanism of analgesic activity [75, 76].

Whereas tannins inhibit cyclooxygenase [77], on the other hand, phenolic compounds have demonstrated anti-inflammatory activity in vivo and in vitro studies by controlling the levels of different inflammatory markers such as COX-2 [78]. Moreover, flavonoids and tannins also have antioxidant activity by removing free radicals that may be involved in stimulating pain [76].

The current study results were better than studies conducted on other plants families, such as Artemisia absinthium which gave a significant effect at concentrations 4% and 6%, where MPE% reached: 45%, 48%, respectively at minute 120 [79].

5. Conclusions

Both plants showed anti-inflammatory effect in the in vitro experiments and analgesic effect in was formalin and tail flick experiments that were conducted. Their effects were better than the positive control. Qualitative phytochemical analysis showed that extracts contained flavonoids and tannins which might be responsible for these effects. Results of this study were promising, therefore, further studies are now conducting to fractionate the extract, to evaluate their analgesic and anti-inflammatory activities using other tests and to define the active compounds.

Declarations

Author contribution statement

Lina Kharrat: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Mohammad Yaser Abajy; Ream Nayal: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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