

Anti-Inflammatory and Anti-Nociceptive Activity of Bioactive Fractions from Leaves of Citrus Sinensis

Ajay Kumar¹, Kislaya Mishra²

Department of Pharmacology, Hygia Institute of Pharmaceutical Education & Research, Lucknow, India

Abstract: *Citrus sinensis* (*C. sinensis*) has been traditionally used for the diagnosis of many diseases which includes- asthma, hypertension, microbial infections, diabetes, tuberculosis, etc. Pain and inflammation cause unnecessary discomfort, suffering and also lower productivity of the victims. Conventional drugs for these conditions are expensive, not easily available and have adverse side effects. Therefore it need to develop alternative therapeutic agents, such as medicinal plant derivatives, that are cheaper and have lesser side effects. *Citrus sinensis* used in traditional medicine to treat pain and inflammation but there is no scientific evidence to confirm these ethno-medicinal claims. The antinociceptive and anti-inflammatory activities of the extract were compared to those of Phenylbutazone. The phytochemical secondary metabolites tested for include alkaloids, cardiac glycosides, flavonoids, phenols, saponins, steroids and terpenoids. *Citrus sinensis* leaves extract demonstrated significant antinociceptive and anti-inflammatory effects in a dose-dependent manner. The extract at the dose level of 200mg/kg bw exhibited the highest antinociceptive and anti-inflammatory activities and its activities were comparable to those of the respective reference drugs. The BFLCS in doses of 50, 100 and 200 mg/kg caused a dose-dependent inhibition of swelling caused by carrageenin equivalent to 30.2–63.2% protection ($P < 0.05$ – $P < 0.001$) and in cotton pellet granuloma, 47.2–45.4% protection ($P < 0.01$ – $P < 0.001$) was observed from inflammation. There was a significant increase in analgesio meter force induced pain in rat equivalent to 98.1–146.5% protection ($P < 0.01$ – $P < 0.001$) and 7.19–37.8 % ($P < 0.05$ – $P < 0.001$) protection against Acetic acid induced writhing.

1. Introduction

Traditional System of Medicine

Medicinal plants and traditional knowledge Nature made human and bestowed countless favours. Ironically, sickness, diseases, complications, inconsistencies and ailments grew slowly. The creator has not made any disease without any cure for it and graced the earth with numerous plants, especially for healing. It has been the necessity of man, which made him trace out the cure from the nature itself. Due to the safe status of herbal medicine Medicinal plants constitute an effective source of traditional (Ayurvedic, Chinese, Homeopathic and Unani).

Pain: In medicine, pain is considered as highly subjective. A definition that is widely used in nursing was first given as early as 1968: "Pain is whatever the experiencing person says it is, existing whenever he says it does" (McCarffery, 1968). It is a major symptom in many medical conditions, significantly interfering with a person's quality of life and general functioning. Diagnosis is based on characterizing pain in various ways, according to duration, intensity, type (dull, burning, throbbing or stabbing), source, or location in body.

Nociceptive pain: Nociceptive pain is a transient pain in response to a noxious stimulus at nociceptors that are located in cutaneous tissue, bone, muscle, connective tissue, vessels, and viscera. Nociception may be thermal, chemical, or mechanical

Neuropathic pain: Neuropathic pain is defined as spontaneous pain and hypersensitivity to pain associated with damage to or pathologic changes in the peripheral nervous system as in painful diabetic peripheral neuropathy (DPN), acquired immunodeficiency syndrome (AIDS)

Inflammation: Inflammation is considered to be a morbid process affecting some part of the body, characterized by excessive heat, swelling, pain, and redness. It is a common factor in arthritic diseases or osteoarthritis (Andrejus, 1988) and a cardinal host defense response to injury, tissue ischaemia, autoimmune responses or infectious agents. Many diseases are now recognized to have an inflammatory component as part of the pathophysiology (e.g. rheumatoid arthritis). Inflammation often elicits a generalized sequence of events known as the acute phase response which is characterized by classical features of swelling, redness, heat and often pain. The essence of inflammation is to contain and eradicate local injury

2. Materials and Methods

2.1 Materials

Collection of the Plant

The Leaves of *Citrus sinensis* (willd.) Ding Hou (Family: Rutaceae) was collected from Central Institute of Medicinal and Aromatic Plant (CIMAP), Lucknow, India in month of Sep 2018. The plant materials were authenticated in Lucknow.

2.2 Method

Preparation of Hydroalcoholic extract of *Citrus sinensis*

The freshly collected plant parts of *Citrus sinensis* (Leaf, Peel, Stem and root) were washed with distilled water, air-dried at a temperature of $30 \pm 2^\circ\text{C}$ and dried in tray drier under the control conditions and powdered. The powdered plant materials was percolated with petroleum ether to remove fatty substances, the marc was further exhaustively extracted with of 50% ethanol for 3 days. Extractives were

concentrated below 50 °C and further drying was carried out under reduced pressure. The dried extractives were stored in desiccators for further evaluation

Phytochemical Screening

The following tests were carried out on the herbal extractives of various parts of plant to detect various phytoconstituents present in them. (Kokate *et al.*, 1997, Trease *et al.*, 1983).

Test for Alkaloids

Dragendroff's Test (Potassium bismuth iodide solution): Alkaloids give reddish brown precipitate with Dragendroff's reagent.

Mayer's Test (potassium mercuric iodide): Alkaloids give yellow color precipitate with Mayer's reagent.

Wagner's Test (Solution of iodine in potassium iodide): Alkaloids give reddish brown precipitate with Wagner's reagent

In-vitro antioxidant studies of different parts of *Citrus sinensis* Total Flavonoid Content (TFC).

Total flavonoid content of different parts of *Citrus sinensis* were measured by the colorimetric assay with some modification and expressed as mg of quercetin equivalents (QE)/g of extract (Oyaizu, 1986). The crude extracts of each plant (25 mg) were dissolved in 10 ml of 50% methanol at room temperature.

Animals and Drug Treatment

The adult Sprague Dawley albino rats of either sex weighing 140-180 g were fasted for 48h with free access to water and divided into six groups. They were placed in cages with grating floor to avoid coprophagy.

The BFLCS (suspended in 1% carboxy methyl cellulose) at the dose levels of 50, 100, 200 mg/Kg body wt., *p.o* was administered once daily for three consecutive days. Phenylbutazone (100 mg/Kg; *p.o*) was used as standard anti-inflammatory and anti-nociceptive activity. Control group of animals (n=6) received suspension of 1% CMC in distilled water (10 ml/Kg). Experiments were conducted on day 3, 60 minute for anti-inflammatory and 30 minute for anti-nociceptive activity (Amresh *et al.*, 2007).

Group I:	Normal Control
Group II:	Disease control
Group III:	BFLCS (50 mg/kg <i>b.w. p.o.</i>)
Group IV:	BFLCS (100 mg/kg <i>b.w. p.o.</i>)
Group V:	BFLCS (200 mg/kg <i>b.w. p.o.</i>)
Group VI:	Phenylbutazone (100 mg/Kg; <i>p.o.</i>)

Anti-Inflammatory Activity

Carrageenin-induced paw oedema

Rats were injected with 0.1 ml of 1% λ carrageenin into the sub-planter side of the left hind paw (Winter *et al.*, 1962). The paw was marked with ink at the level of lateral

malleolus and dipped in perspex cell up to this mark. The paw volume was measured immediately with an Ugo Basile Plethysmometer and 3 hrs after injecting the λ carrageenin suspension. The BFLCS extract and phenylbutazone was administered orally by gavage, 1 hr before the λ carrageenin injection. Significant reduction in the paw volume compared to vehicle treated control animals were considered as anti-inflammatory response. Percentage inhibition of oedema was calculated as follows:

$$\% \text{ Inhibition} = (1 - V_T / V_C) \times 100$$

V_T = Paw volume in drug treated rats.

V_C = Paw volume in control group of rats.

Cotton pellet induced granuloma formation

The rats were anesthetized with ether and incision was made on the lumbar region (Winter *et al.*, 1957). By a blunted forcep subcutaneous tunnel was formed and cotton (100 mg \pm 1 mg) was inserted in the groin area. Groups of 6 animals received either test drug (50, 100, 200 mg/Kg body wt., *p.o.*) or reference drug (100 mg/kg body wt.) for seven consecutive days from the day of cotton pellet insertion. The animals were sacrificed and the pellets were removed and dried until the weight remained constant on 8th day according to the procedure described and the net dry weight was calculated (Sheth *et al.*, 1972).

Antinociceptive activity

Analgesio- meter induced pain

The analgesic effect of BFLCS tested in rat of either sex, using an Ugo Basile Analgesy meter (Rodriguez *et al.*, 1990). This method involves the application of force to the paw of the rat using the analgesy-meter, which exert a force that increase at constant rate. The rat was gently placed between plinth and plunger. The instrument was switched on and a constant motor rate was used to drive the plunger on to the paw of the mice. When the mice struggle, the instrument was switched off and the force at which animal felt pain was read on a scale calibrated in grams x 10 by a pointer. The pre and the post treatment weight causing pain were determined for each mouse. The doses of test drug or reference drug were administered 60 minutes before testing.

Acetic acid induced writhing

Animals received BFLCS extract (50, 100, 200 mg/kg) and standard drug orally 30 minute before the injection of 0.6% acetic acid (10ml/kg, *i.p*) (Witkin *et al.*, 1961). The number of abdominal contractions (writhing) and stretching with a jerk of the hind limb were counted for 15 minutes after administering acetic acid and % inhibition was calculated.

3. Result

Extractive values of *Citrus sinensis*

The percentage yield of freshly collected plant parts of *Citrus sinensis* (Leaf, Peel, Stem and root) were shown in table 2.

Table 2: % Yield of different parts of *Citrus sinensis*

S. No.	Plant Parts	Hydroalcoholic extract	Nature	% yields
1	Leaf	Ethanol:water (1:1)	Greenish brown solid	18.32
2	Peel	Ethanol:water (1:1)	Greenish brown solid	10.20
3	Stem	Ethanol:water (1:1)	Brown semisolid	14.21
4	Root	Ethanol:water (1:1)	Brown semisolid	13.51

Phytochemical Screening

Qualitative phytochemical screening of *C. sinensis* showed the presence of carbohydrate, tannins, protein & amino acid, triterpenoids, glycoside, alkaloid and flavonoids (Table 3).

λ Carrageenin-induced paw oedema

The BFLCS at the dose level of 50, 100 and 200 mg/kg *b.w* produced a dose-dependent inhibition of swelling caused by the λ Carrageenin at 3 hrs equivalent to 30.2–63.2% ($P < 0.05$ – $P < 0.001$) protection (Table 10).

Cotton pellet induced granuloma formation

BFLCS at a dose level of 50, 100 and 200 mg/kg *b.w* significantly decreased the granuloma weight from 47.2–45.4% ($P < 0.01$ – $P < 0.001$) respectively compared to reference compound phenyl butazone 34.6% ($P < 0.001$) (Table 11).

Anti Nociceptive activity

Analgesio- meter induced pain

The BFLCS at a dose level of 50, 100 and 200 mg/kg *b.w* caused a significant increase in the analgesio-meter-induced force ($P < 0.01$ to $P < 0.001$) and exhibited resistance against pain after 30 min equivalent to 98.1–146.5% protection respectively (Table 12)

Total Phenolic content present in different parts of *C. sinensis*

S. No.	Sample	TFC (GA equivalent in mg/g*)
1.	Leaf	32.80 \pm 0.50
2.	Peel	13.20 \pm 0.80
3.	Stem	28.30 \pm 0.30
4.	Root	14.24 \pm 0.20

* All values are average of three determinations, mean \pm SEM

Table 5: Total Phenolic content present of different parts of *C. sinensis*

S.No.	Sample	Phenolic Content (Gallic acid mg /g*)
1	Leaf	4.3 \pm 0.16
2	Peel	1.3 \pm 0.22
3	Stem	3.3 \pm 0.50
4	Root	3.4 \pm 0.42

* All values are average of three determinations, mean \pm SEM

Effect of BFLCS on λ Carrageenin-induced pawoedema in rats

Treatment	Dose (mg/kg <i>b.w</i>)	Paw volume (ml) at 3 hrs	
		λ Carrageenin	% inhibition
Control	0.1 ml	1.85 \pm 0.11	-----
BFLCS	50	1.29 \pm 0.07*	30.2
BFLCS	100	0.96 \pm 0.10**	48.1
BFLCS	200	0.68 \pm 0.04***	63.2
Phenylbutazone	100	0.59 \pm 0.03***	68.1

*Values are mean \pm SEM for six rats.

* $P < 0.05$ compared to control group.

** $P < 0.01$ compared to control group.

*** $P < 0.001$ compared to control group.

Effect of BFLCS on cotton pellet-induced granuloma in rats

Treatment	Dose (mg/kg <i>b.w</i>)	Dry weight (mg)
Control	-----	49.5 \pm 2.6
BFLCS	50	47.2 \pm 0.60*
BFLCS	100	38.7 \pm 0.71**
BFLCS	200	35.4 \pm 0.41**
Phenylbutazone	100	34.6 \pm 0.24**

*Values are mean \pm SEM for six rats.

* $P < 0.01$ compared to control group.

** $P < 0.001$ compared to control group.

4. Discussion

As fresh plant materials (samples) were collected and foreign matter was discarded before the preparation of the sample hence it may be considered as no foreign organic matter was present in the samples. Moisture content is important parameter of physiochemical analysis. Low moisture content reduces errors in the estimation of the actual weight of drug material, reduces components hydrolysis by reducing the activities of hydrolytic enzymes which may destroy the active components, and also reduces the proliferation of microbial colonies and therefore minimize the chance of spoilage due to microbial attack (Shellard, 1958).

The present study establishes the anti-inflammatory and anti-nociceptive activity of bioactive fraction from leaves of *C. sinensis* (BFLCS). The BFLCS showed significant anti-inflammatory activity against λ carrageenin and cotton pellet induced granuloma in rats.

Carrageenan is a natural carbohydrate derived from a number of seaweeds of the class Rhodophyceae (Necas and Bartosikora, 2013). Sub plantar injection of carrageenan in the rat hind paw induces a biphasic edema; the early and late phases (Kapewangolo *et al.*, 2015; Nivsarkar *et al.*, 2009). The key inflammatory mediators detectable during the early phase (1 hour) of the carrageenan-induced edema include; serotonin, histamine and kinins (Chatterjee *et al.*, 2015). The late phase occurs after the first one hour of the carrageenan-induced edema and the key mediators detectable in this phase include prostaglandins and inducible cox-2 (Necas and Bartosikora, 2013).

Non-steroidal anti-inflammatory drugs such as indomethacin, aspirin and diclofenac are the conventional drugs used to manage inflammation (Mwangi *et al.*, 2015). The anti-inflammatory effect of NSAIDs is attributed to their inhibitory effect on the activity of COX-2 enzyme that converts arachidonic acid to the inflammatory mediator prostaglandins (Shukla and Mehta, 2015). Two types of COX enzymes exists; COX-1 enzyme that produces prostaglandins that are responsible for supporting platelets and protecting the stomach and COX-2 enzyme that produces prostaglandins that are responsible for inflammation (Mitchell *et al.*, 1993). Therefore, NSAIDs inhibit only the late phase of carrageenan-induced inflammation where prostaglandins and COX-2 enzymes

are the detectable mediators (Necas and Bartosikora, 2013). It can therefore be suggested that the BFLCS reduced the carrageenan-induced paw edema by inhibiting the activity of COX-2 enzyme.

In cotton pellet induced granuloma model of sub-acute inflammation, the BFLCS significantly reduced the weight of granulation tissue²³. This method shown that foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. This method has been useful for evaluation of steroidal and nonsteroidal anti-inflammatory drugs.

The phytochemical screening of the hydroalcoholic extract of leaves of *C. sinensis* revealed the presence of various phytochemicals in the extract some of which could have been responsible for its anti-inflammatory activity. Flavonoids inhibit the activity of the enzyme prostaglandin synthetase (Chatterjee *et al.*, 2015). Flavonoids have also been reported as potent anti-inflammatory agents in another study (Tapas *et al.*, 2008). Steroids reduce inflammation by inhibiting phospholipase A2 which is responsible for the hydrolyzation of arachidonic acid from the membrane phospholipids leading to the formation of prostanoids and leukotrienes (Mencarelli, 2009)

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6. Conclusion

The data obtained in this study revealed significant anti-inflammatory and antinociceptive properties of bioactive fraction from leaves of *C. sinensis* (BFLCS) which may be due to the presence of bioactive ingredients with a pharmacological potential. The extract demonstrated a dose dependent response to the carrageenan-induced inflammation, cotton pellet induced granuloma, analgesio-meter induced pain and acetic acid induced writhing. At the dose level of 200mg/kg *b.w*, the extract exhibited the highest anti-inflammatory and antinociceptive activities. The anti-inflammatory and antinociceptive activities of the extract at the dose level of 200mg/kg *b.w* was comparable to the anti-inflammatory and antinociceptive activities of the respective reference drugs.

Our result suggests that the administration of BFLCS showed inhibition of inflammation and pain in the experimental animals. Further studies are in progress to find out exact mechanism of action and responsible active constituents for anti-inflammatory and anti-nociceptive activity

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Author Profile

Ajay Kumar, Master Degree in Pharmacy from HIPERLucknow.