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Development and Evaluation of Topical Formulation with Chloroform Extract of *Pisonia grandis* leaves for Anti-inflammatory effect

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Abstract:
Objectives: The chloroform extract of *Pisonia grandis* leaves has been scientifically proved for its anti-inflammatory activity and the present study was conducted to develop a novel herbal formulation in the management of inflammation.
Methods: Topical formulations (cream) have been developed containing the chloroform extract of *Pisonia grandis* leaves (PG I to IV) at different concentrations 2%, 3%, 4% and 5% w/w. These topical formulations were tested for pH, homogeneity, appearance, spread ability, viscosity, after feel, type of smear, removal. All the formulations were evaluated for its acute skin irritancy activity and stability. The formulation was evaluated for anti-inflammatory activity using Carrageenan induced paw edema models in rats.
Results: The formulations showed good spreadability, no evidence of phase separation and good consistency during this study period. It was found that the viscosity of the formulation increases when decreasing the rate of shear so that the viscosity of the cream is inversely proportional to rate of shear (rpm). These formulations did not produce any skin irritation for about a week when applied over the skin. The creams were found to be stable during stability study according to ICH guidelines for 2 months. In anti-inflammatory activity, the PG I and II showed slight inhibition and PG III and IV have showed significant inhibition of carrageenan induced rat paw edema compared to control group in which only cream base was used.
Conclusion: The anti-inflammatory activity of PG IV was comparable with reference standard 1% hydrocortisone. Therefore the developed cream would possibly for an alternative treatment for inflammation.
Key words: *Pisonia grandis,* anti-inflammatory, herbal cream, chloroform extract.

Introduction

Inflammation is a pathophysiological reaction that occurs in tissues when they are exposed to injury and this reaction leads to accumulation of plasmatic fluid and blood cells in injured tissues. At the same time these processes help the body in the protection against infection, burns, toxic chemicals, allergens or other noxious stimuli.[1] Inflammation is triggered by the release of chemical mediators from the injured tissues and migrating cells. The specific chemical mediators vary with the type of inflammatory process and include amines such as histamine, serotonin, lipids such as prostaglandins and small peptides such as kinins.[2]

Conventional anti-inflammatory drugs such as steroidal and nonsteroidal anti-inflammatory drugs (NSAID) are used in the treatment of most of the acute and chronic pain and inflammatory disorders including rheumatoid arthritis. However, long-term use of these agents may produce serious adverse effects. Thus, it is

worth developing new plant-derived anti-inflammatory agents with fewer adverse effects. *Pisonia grandis* R.Br (Nyctaginaceae) commonly known as Leechai kottai keerai in Tamil is a wide spread evergreen tree distributed throughout India.[3] The folklore claims include analgesic, anti-inflammatory, diuretic, anti-rheumatic and hypoglycemic activity.[4] This plant is pharmacologically studied for its antifungal, antioxidant, antimicrobial, anti-inflammatory, antidiabetic, diuretic, analgesic and wound healing properties.[5-9] The plant contains octacosanol, β-sitosterol, α-spinaterol glucoside, dulcitol, quercitrin, allantoin, pinitol.[10-11] Earlier work on the plant reported that the chloroform extract of the plant showed significant anti-inflammatory activity when compared with alcoholic extract.[12] Hence the present study was aimed to formulate and evaluate topical cream containing chloroform extract of *Pisonia grandis* leaves for its anti-inflammatory activity on Carageenan induced rat paw edema model.

Materials and Methods

Plant material

The plant material proposed for the study, *Pisonia grandis* leaves were collected from SRM University campus, Potheri, and authentified by Prof. P. Jayaraman, Taxonomist, Plant Anatomy Research Center (PARC), Tambaram and a voucher specimen No. PARC/2012/1309 was kept in the department museum for future reference.

Extraction

The air dried, powdered leaves were extracted by maceration with chloroform for 72 hrs. Then the extract was vacuum dried using rotary vacuum dried evaporator to yield solid residue of chloroform extract. The percentage yield of the chloroform extract was found to be 7.5% w/w.

Preliminary phytochemical screening

The chloroform extract was subjected to preliminary phytochemical screening using standard procedure.[13]

High performance thin layer chromatography[14]

It is mandatory to investigate the quality of *Pisonia grandis* chloroform extract by densitometric analysis. In order to study the possible composition, TLC plate (4 x 8cm), precoated with silica gel 60 F254 (E.Merck) with aluminum sheet support were used. The spotting device was a CAMAG Linomat V Automatic Sample Applicator. The developing chamber was a CAMAG glass twin trough chamber (20 x 10cm) the densitometer consisted of a CAMAG TLC scanner 3 linked to WINCATS software for interpretation of data. Chromatogram was developed using Toluene: Ethyl acetate: Formic acid (5:4:1) as mobile phase. The developed chromatogram was inspected under UV light at 254 nm. The developed plate was then scanned to record the peak areas.

Development of formulation [15]

Four batches of cream PG I to IV were prepared containing varying concentration of chloroform extract (2%, 3%, 4% and 5% w/w respectively). The oily phase that consisted of stearic acid (15 g), Isopropyl myristate (3 g) and cetyl alcohol were mixed, blended and warmed to melt to 70˚C. The aqueous phase consisted of chloroform extract, glycerin (6g), water (74.7 g) containing methyl paraben (0.15g) as preservative and heated to 70˚C. Aqueous phase was added to oily phase and mixed with a blender for approximately 20 min. (300 rev/min). The mixture was neutralized using KOH (0.5g) and NaOH (0.15g).

Evaluation of Herbal Formulation [16, 17]

Physical evaluations

Preliminary evaluation of formulations was carried out as follows:-

pH

The pH of various formulations was determined by using Digital pH meter (Digital pHmeter 335, Systronics, Noroda, Ahmedabad). One gram of cream was dissolved in 100 ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate.

Viscosity

The measurement of viscosity of prepared creams was carried out with Brookfield Viscometer (model LV-DV-II, Helipath-spindle type S-96).

Spreadability

Spreadability denotes the extent of area to which the gel readily spreads on application to skin or the affected part. The bioavailability efficiency of a cream also depends on its spreading value. The spreadability was expressed in terms of time in seconds taken by two slides to slip off from the cream, placed in between the slides, under certain load. Lesser the time taken for separation of the two slides, better the spreadability. Two sets of glass slides of standard dimensions were taken. The cream formulation was placed over one of the slides. The other slide was placed on the top of the cream, such that the cream was sandwiched between the two slides in an area occupied by a distance of 6.0 cm along the slide. 100 gm weight was placed upon the upper slide so that the cream between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of cream adhering to the slides was scrapped off. The two slides in position were fixed to a stand without slightest disturbance and in such a way that only the upper slide to slip off freely by the force of weight tied to it. A 20 gm weight was tied to the upper slide carefully. The time taken for the upper slide to travel the distance of 6.0 cm and separated away from the lower slide under the influence of the weight was noted. The experiment was repeated by three times and the mean time taken for calculation. Spreadability was calculated by using the following formula:

$$S = \frac{m \times l}{t}$$

Where,

S - Spreadability
m - Weight tied to the upper slide (20gm)
l - Length of the glass (6 cm)
t - Time taken in seconds.

Appearance

The appearance of the cream was judged by its colour, pearlscence and roughness and graded.

After feel

Emolliency, slipperiness and amount of residue left after the application of fixed amount of cream was checked.

Type of smear

After application of cream, the type of film or smear formed on the skin were checked.

Removal

The ease of removal of the cream applied was examined by washing the applied part with tap water.

Acute skin irritation study[18]

The primary skin irritation test was performed on albino rats and weighing about 150-200 gm. The animals were maintained on standard animal feed and had free access to water *ad libitum*. The animals were kept under standard laboratory condition. The total mass was divided into four batches, each batch containing seven animals. Two batches of each were used for control and test. Dorsal hairs at the back of the rats were clipped off one day prior to the commencement of the study. Animals showing normal skin texture were housed individually in cages with copography meshes to avoid contact with the bedding. 50 mg of the each formulation of different concentrations were applied over one square centimeter area of intact and abraded skin to different animals. Aqueous solution of 0.8% formalin was applied as standard irritant. The animals were observed for seven days for any signs of oedema and erythema.

Stability studies [19]

The stability studies were carried out in all formulations at different temperature conditions (4˚, 25˚, and 37˚C) for 60 days. All the evaluation parameters i.e. pH, viscosity, spreadability, consistency, extrudability, appearance, after feel, type of smear, removal were studied at different time intervals viz., 15th, 30th and 60th days.

Pharmacological activity

In the present study, the topical preparation of chloroform extract of *Pisonia grandis* leaves was examined for anti-inflammatory activity by carrageenan induced rat paw edema model.

Carrageenan induced rat paw edema [20]

Animals

All experiments were carried out on Wistar rats 150-200 g, under a 12 h light/dark cycle in a room with controlled temperature (22±1°C). Rats were divided into six experimental groups, each comprising 6 animals. All experiments followed the guidelines on ethical standard for investigation of experimental pain in animals.

Method

Animals were allowed to free access to feed and water before experiment. Approxiamtely 50µl of a 1% suspension of carrageenan in saline was prepared 1h before each experiment and was injected into the plantar side of the right hind paw of rat. 0.3g of cream containing 2-5% of Herbal cream PG I-IV were applied to the plantar surface of the hind paw by gently rubbing 50 times with the index finger. Rats of the control groups received only the cream base and standard groups received 1% Hydrocortisone. Paw volume was measured immediately after carrageenan injection and at 1, 2, 3 and 4 h intervals after the administration of the noxious agent using a plethysmometer. The percentage inhibition in paw volume is calculated using the formula,

$$\text{Percentage inhibition} = \frac{\text{Control paw volume} - \text{Test paw volume}}{\text{Control paw volume}} \times 100$$

Statistical analysis

Level of significance of all the parameters was expressed as the arithmetic mean ± SEM and was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's "t" test. *P* value less than 0.05 ($P < 0.05$) was the critical criterion for statistical significance.

Results and discussion

The chloroform extract of *Pisonia grandis* leaves has been scientifically proved for its anti-inflammatory activity and the present study was undertaken to develop a novel herbal formulation in the management of inflammation. Preliminary phytochemical screening of chloroform extract showed presence of sterols, alkaloids and saponins. The choloroform extract was further subjected to HPTLC. Chromatography is essentially a group of techniques used for separation of the constituents of mixture by continuous distribution or adsorption of analyte between two phases. Among various chromatographic analytical techniques HPTLC has a firm place as a reliable method for analysing several samples of divergent nature and composition at the same time.[21] HPTLC analysis of the extract revealed the presence of 9 components in the chosen solvent system and corresponding ascending order of Rf value starts from 0.06-0.75 in which highest concentration of phytoconstituents was found to be 41.45 % and its corresponding Rf value was found to be 0.57 and was recorded in Table. 1. The corresponding HPTLC chromatogram was presented in Figure 1.

Table 1: HPTLC profile of the chloroform extract of *Pisonia grandis* leaves

Peak	Start position (Rf)	Start height (AU)	Max position (Rf)	Max height (AU)	Max %	End position (Rf)	End height (AU)	Area (AU)	Area %
1	0.01	14.4	0.01	105.1	20.99	0.06	1.6	2322.6	18.46
2	0.06	0.4	0.05	17.3	3.45	0.09	1.9	209.1	1.66
3	0.14	10.4	0.17	32.9	6.57	0.18	27.4	930.3	7.39
4	0.18	27.7	0.20	40.9	8.18	0.23	0.0	1360.0	10.81
5	0.23	0.7	0.26	19.1	3.81	0.27	18.2	423.9	3.37
6	0.30	24.1	0.30	30.4	6.07	0.34	16.0	1008.0	8.01
7	0.52	3.1	0.57	207.5	41.45	0.58	0.3	5064.2	40.24
8	0.65	13.7	0.67	25.0	5.00	0.68	22.5	754.6	6.00
9	0.71	20.7	0.71	22.4	4.47	0.75	6.1	512.2	4.07

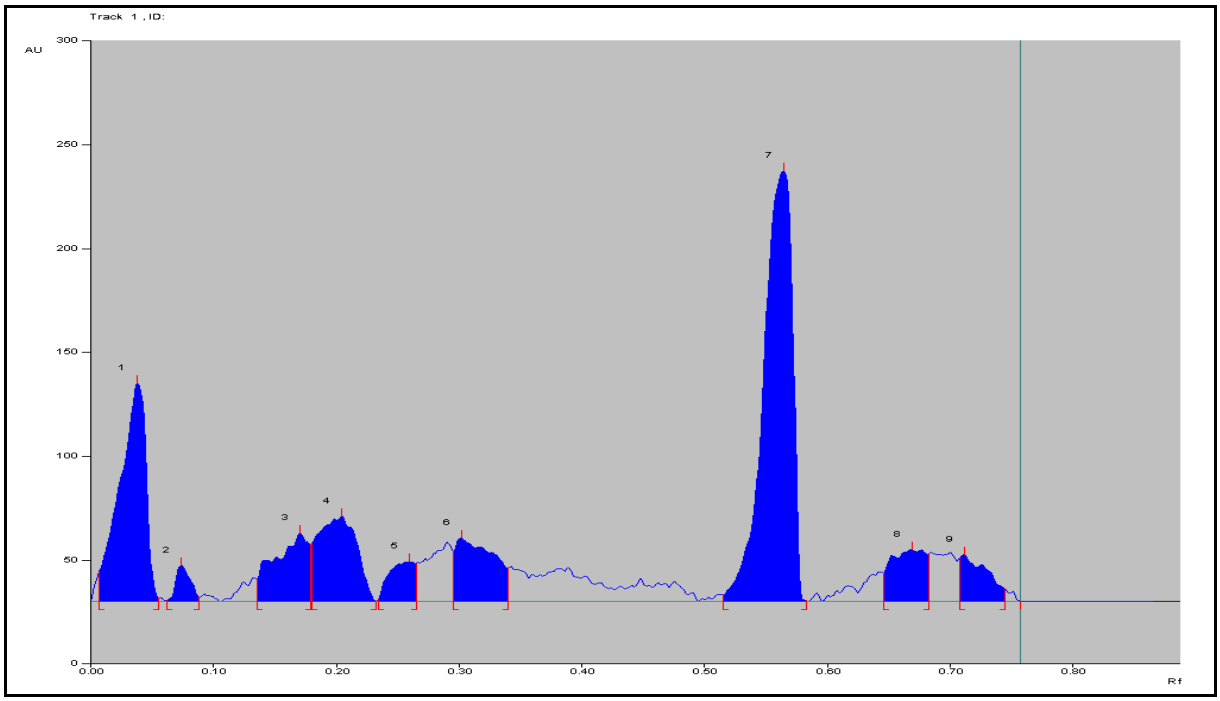

Figure 1: HPTLC Peak densitogram of the chloroform extract of *Pisonia grandis* leaves

Herbal creams have been developed containing chloroform extract of *Pisonia grandis* leaves (2%-5% w/w). The developed cream evaluated using various physicochemical parameters shown in Table. 2. The viscosity of the formulation ranges from 7000 to 7800 cps. The pH of the formulations ranges from 6.36 to 6.56, which lie in the normal pH range of the human skin. All the developed formulations showed excellent homogeneity and there were no lumps in the formulation. Spreadability values showed that formulations spread with an ease and the type of smear is non greasy. Skin irritation study revealed that all developed creams were safe for topical application and did not induce any skin reaction. The rheological behaviours of the different formulation of the creams were studied using Rotational Brookefield Viscometer. The results indicated that the torque and shear stress increases whereas viscosity decreases. From the stability studies, creams PG I-IV showed no changes in viscosity, pH, spreadability, extrudability, viscosity, consistency and phase separation after keeping at different temperatures for 60 days. The pH and viscosity of the formulation found to decrease at higher temperature. There was no rancid smell which showed the effectiveness of the antioxidants in formulation.

Table 2: Physiochemical evaluation of developed cream

Formulation	P^H	Viscocity (cps)	Homogeneity	Spreadability gm.cm/sec	Irritation	Appearance	After feel	Type of smear	Removal
PG-I (2%)	6.36	7000	***	10.58	No	Green	E	NG	ES
PG-II (3%)	6.39	7400	***	10.52	No	Green	E	NG	ES
PG-III (4%)	6.48	7600	***	9.52	No	Green	E	NG	ES
PG-IV (5%)	6.56	7800	***	9.69	No	Green	E	NG	ES

*****:** Excellent ,****:** Good, ***:** Satisfactory, **E:** Emollient, **NG:** Non greasy, **ES:** Easy

The results of anti-inflammatory activity revealed that the formulation PG I-IV containing chloroform extracts (2% - 5% w/w) exhibited significant inhibition of carrageenan induced rat paw edema when compared to control group and the activity is dose dependent. Among the developed cream, the cream PG III (**p<0.01) and PG IV (***p<0.001) have shown significant activity at 4^{th} hr after carageenan induction. The herbal cream PG IV (5%) showed maximum inhibition of 85.99% after 4 hours of treatment which is comparable to the standard drug hydrocortisone effect (90.84%) The results were shown in Table. 3. Carragennan induced paw edema method is a standard and most commonly used technique to screen the acute inflammatory activity.[22] The development of Carrageenan induced inflammation is a biphasic event. First phase occurs within an hour of injection of phlogistic agent and is mediated through release of histamine, serotonin and kinins while the second phase which can be measured around 3 to 4 hours is related to release of prostaglandins.[23]

Table 3: Effect of cream on carrageenan-induced paw edema in rats

Treatment	Paw volumes (ml) at time after carrageenan injection				
	Initial	1h	2h	3h	4h
Base	1.582±0.064	1.527±0.012	1.670±0.005	1.75±0.009	1.69±0.003
Standard	1.015±0.022	1.038±0.006***	1.107±0.014***	1.106±0.020***	1.118±0.022***
PG-I (2%)	1.012±0.009	1.308±0.011*	1.405±0.007*	1.423±0.018*	1.395±0.004*
PG-II (3%)	1.065±0.048	1.275±0.018*	1.343±0.009*	1.405±0.006*	1.368±0.016*
PG-III (4%)	1.267±0.088	1.215±0.017*	1.265±0.007**	1.322±0.006**	1.287±0.012**
PG-IV (5%)	1.062±0.009	1.05±0.003**	1.193±0.012***	1.217±0.012***	1.12±0.021***

Each value is represented in mean ± S.E.M, significant values are represented as *p<0.05,**p<0.01 & ***p<0.001 compared with control (ANOVA followed by Dunnets 't' test)

In the present study herbal cream showed slight inhibition of inflammation in first phase and maximum inhibition is observed in second phase, which is mainly due to release of prostaglandins. The possible anti-inflammatory effect may be due to inhibition of cyclooxygenase enzyme which catalyzes the biosynthesis of prostaglandins and thromboxane from arachidonic acid. The anti-inflammatory activity of plant sterols has been already established.[24-25] Alkaloids are known by their anti-cholinergic, anti-inflammatory and anti-histamine activities and inhibiting pro-inflammatory cytokine production and their receptors**.** In addition, alkaloids have been shown to affect many enzyme systems involved in allergic and inflammatory responses such as tyrosine and serine-threonine protein kinases, phospholipases A2, phospholipase C, and lipoxygenase.[26] The Phytochemical investigations revealed the presence of sterols and alkaloids in chloroform extract of *Pisonia grandis* leaves. The present activity may be due to presence of sterols and alkaloids.

Conclusion

From these overall results, we can conclude that the topical formulation containing 5% chloroform extract of *Pisonia grandis* possess significant anti-inflammatory activity, which can be used as an herbal remedy for the treatment of acute pain and local inflammation. The preliminary phytochemical investigation of chloroform extract showed the presence of alkaloids, saponin and phytosterols, which might be in part responsible for anti-inflammatory effect. The assessment of observed pharmacological effects with isolated

individual chemical constituent merit further investigation for better understanding of the molecular mechanisms underlying anti-inflammatory activity of the developed formulation.

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