Research Article

Formulation and evaluation of Anti-inflammatory activity of Lemon grass oil Liniments on wistar rats

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Abstract

Objective: The main objective of the present research is to formulate lemon grass oil liniment and evaluate its anti-inflammatory activity on Wistar rats. **Materials and methods:** Lemon grass oil liniment and turpentine oil liniments were formulated using tween 80 as surfactant in different ratios. After evaluation of various physical parameters like globule size, pH, viscosity, the optimized formulations namely LL5 and TL5 were selected for pharmacological evaluation. These formulations were subjected for evaluation of anti-inflammatory effect using carrageenan induced paw edema method. **Results:** The edema in paw was measured using plythesmometer. LL5 reduced 63.15% of oedema which was induced by carrageenan on sub-plantar administration when compared with untreated control groups, whereas TL5 reduced 56.84% of oedema. A significant increase in the percentage inhibition of heat induced haemolysis and protein denaturation were observed with LL5 when compared to that of the standard group which clearly indicates the anti-inflammatory activity of lemon grass oil. **Conclusion:** Thus from the present study, it was concluded that the lemon grass oil liniment made with 10% of tween 80 shows significant anti-inflammatory activity.

***Keywords*:** Lemon grass oil, Turpentine oil, liniment, anti-inflammatory.

Introduction

Medicinal plants are believed to be therapeutically important as they are enriched with various phytochemical constituents, which treats many diseases (Farnsworth, 1989). Several traditional folklore studies revealed the therapeutic importance of plants (Brekhmann and Dardimov, 1969). Inflammation is a general protective mechanism in which the body reacts to any injury, infection or irritation which can be identified by some key features like redness, warmth and swelling at the site. It is considered to have different mechanisms for each type of pathogen (Abul and Andrew, 2009). As the traditional NSAIDS have some adverse effects like formation of ulcers, plant derived products are gaining importance (Mahesh et al., 2009). Topically applied plant preparations in the form of paste or liniment are made available which are useful in treating several ailments like inflammation (Lee, 2012). Liniment is a medicated topical preparation meant exclusively for application to skin. They are less viscous than lotions and are rubbed to create friction while applying (James and Adams, 2012). These are typically formulated from acetone, alcohol or suitable solvents and contain counter-irritant aromatic chemical compounds. Stability is the major hurdle, which is to be specifically considered while formulating a liniment.

Cymbopogon citratus belonging to the family Poaceae, is a shrub distributed throughout India. It is commonly called as Lemon grass which is widely used as a medicinal herb in India. Lemon grass oil is used as a pesticide, mosquito repellent and preservative (Warrier et al., 2006). It is supposed to help in relieving cough and nasal congestion. It has anthelmintic, anti-fungal, anti-inflammatory, anti-convulsant and analgesic properties (Vaibhav et al., 2013). Due to its extensive medicinal properties, it is extensively used in Ayurvedic medicine. The present study was aimed to formulate and evaluate the anti-inflammatory effect of Lemon grass oil liniment.

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Materials and methods

Indomethacin was a gift sample from M/s. Life Line Formulations, Vijayawada. Turpentine oil was procured from Loba Chemic Pvt. Ltd., Mumbai. Carrageenan was procured from Ozone International, Mumbai. Tween 80 was procured from Merck Specialities Pvt. Ltd., Mumbai. All other substances used were of analytical grade and commercially procured.

Experimental animals

Adult healthy male albino rats (*Wistar strain*) weighing 250–300g, housed in proper cages, maintained under standardized condition i.e., 12:12 hour light/dark cycle at 25 ± 2^0C at the animal house, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Guntur, India were procured for the study. The animals were provided with standard diet and had free access to purified drinking water. The guidelines were followed according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India and prior permission was soughted from Institutional Animal Ethics Committee (IAEC) with an approval number of 1529/PO/Re/11/CPCSEA./CHIPS/IAEC4/PRO-01/2015-16 for conducting the study.

Plant materials

The leaves of *Cymbopogon citratus* belonging to family *Poaceae* were collected from the medicinal plant garden of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur and was identified and authenticated by Dr. P. Ammani, Acharya Nagarjuna University, Guntur and a voucher specimen is preserved in the Department of Pharmacology, Chebrolu Hanumaiah Institute of Pharmaceutical Sciences, Chowdavaram, Guntur, India.

Extraction of plant materials

Freshly cut lemon grass was made into pieces and placed in a round bottomed flask along with water and fixed to Clevenger apparatus. It was refluxed for 4 hours and the oil isolated was collected from the fluorentine and stored in a well closed container

Preparation of Turpentine Oil Liniment

Turpentine oil liniment was prepared by measuring the calculated amount of turpentine oil and then mixing it with tween-80 using bottle shaking method. Six different formulations were made by keeping the amount of oil constant and increasing the surfactant concentration. Then the solution was made up to required quantity by adding distilled water with proper shaking.

Preparation of Lemon grass Oil Liniment

Lemon grass oil liniment was prepared by measuring the calculated amount of lemon grass oil and then mixing it with tween-80. Six different formulations were made by keeping the amount of oil constant and increasing the surfactant concentration. Then the solution was made up to required quantity by adding distilled water with proper shaking.

Evaluation of physical parameters for Liniments

Determination of average globule size of Liniments

After proper calibration of eye-piece micrometer with stage micrometer, 1ml of emulsion is diluted with 10 ml of distilled water. A small amount of diluted turpentine oil liniment was placed on a glass slide and focused under microscope by placing over the stage micrometer in 10X lens. By using the magnification lens of 45X, the size of 100 globules were measured using eye piece. Similarly, the globule size was measured for lemon grass oil liniment (Subramanyam, 2002). The average diameter of the globules was calculated using mean and standard deviation. Similar procedure was adopted for lemon grass oil liniment formulations. Out of them, the formulation which shows less Standard deviation was considered as an optimised formulation and further physical parameters and stability studies were performed.

Dilution test

This test depends upon the fact that when a dispersion medium is added to a liniment, a phase separation is possible or not. This test was performed by mixing 1 ml of liniment with 1ml of water. Then the miscibility of liniment with water is observed. This was done for both turpentine oil liniment and lemon grass oil liniment which gives the type of liniment.

Estimation of pH

The 50 ml of liniments were taken in a beaker. The electrode was placed in suitable buffers for calibration. After calibrating the instrument, the electrode was placed in liniment and the pH reading was noted down.

Determination of viscosity by Brookfield Viscometer

The 100 ml of homogeneous liniment was prepared at room temperature and kept aside for 24 hours for homogenization. Spindle 61 was selected on the basis of viscosity. The instrument was cleaned and the spindle was attached to Brookfield viscometer. The beaker was adjusted such that the spindle is just at the surface of liniment. Required speed was selected and the spindle was rotated for at least 20 minutes in the liniment till get to find constant dial reading. To determine viscosity of a sample, constant electric supply as well as constant temperature was maintained. Care must be taken while fixing or removing the spindle.

Determination of stability

50 ml of liniment was taken in a measuring cylinder and kept aside for 24 hours. After 24 hours, the volume of external phase which was separated was noted. The phase volume ratio was calculated from which the stability was measured. Phase volume ratio was calculated by the following formula;

$$\text{Phase Volume Ratio} = \frac{\text{Volume of internal phase}}{\text{Volume of liniment}}$$

Anti-Inflammatory Activity

***In-vivo* Anti-Inflammatory activity by Carrageenan induced Paw Oedema method**

The liniments were tested for anti-inflammatory activity by carrageenan (inflammagen) induced paw oedema method in rats (Kulkarni, 2002). The animals were grouped into four with five animals in each group. The control group was treated with normal saline and the standard group was treated with Indomethacin at a dose of 10 mg/kg orally. Whereas the test groups were treated with turpentine oil liniment (TL5) and lemon grass oil liniment (LL5) separately to paw. The animals were pre-treated with vehicle/Indomethacin 30 minutes before the injection of 0.1 ml of 1% carrageenan into the s.p (sub-plantar) region in right hind paw to the rats. Paw volumes were measured by the dislocation of water column in a plythesmometer at 1, 2, 3 and 4 hours after the administration of test materials. The liniments were rubbed to rat paw. Reduction in the paw volume compared to the control animals was considered as the anti-inflammatory response. Finally, the anti-inflammatory activity was calculated by using the percentage inhibition of oedema which can be given by the formula:

$$\%\ \text{Inhibition of oedema} = \frac{\text{Paw oedema in control group} - \text{Paw oedema in test group}}{\text{Paw oedema in control group}} \text{X } 100$$

***Ex-vivo* Anti-inflammatory activity**

Heat induced haemolysis

The 20µL of uncoagulated fresh rat blood was added to vials containing 1 mL of 0.1 M PBS (Phosphate Buffered Saline, pH 7.4). TL5, LL5 and standard drug (Indomethacin) were added to the vials (in triplicate), so as to achieve the final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5ml in 1ml of each sample. PBS (1ml) and rat blood was added to control vials. Then the solutions were subjected to centrifugation at 3000 rpm in centrifuge for 10 minutes to make sure that all the materials had dissolved completely. After centrifugation, no residues were observed which indicates complete solubility of the drug preparations. After mixing, the contents in vials and pre-incubating at 37^{0}C for 15 minutes. Then the mixtures were heated for 25 minutes at 54^{0}C. After spinning down the precipitate, the absorbance of the supernatant was measured at 540 nm in a spectrophotometer. The percentage inhibition of haemolysis of test was compared to that of the control (Shinde et al., 1999).

$$\%\text{Inhibition of haemolysis} = \frac{\text{Absorbance in control group} - \text{Absorbance in test group}}{\text{Absorbance in control group}} \text{X } 100$$

Protein denaturation

The 0.2 mL of egg albumin was added to vials containing 2.5 mL of 0.1 M PBS (Phosphate Buffered Saline, pH 6.4). 2 ml of TL5, LL5 and standard drug (Indomethacin) were added to the vials (in triplicate), so as to achieve the final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5ml in 1ml of each sample. PBS (2.5 ml) and egg albumin was added to control vials. Then the mixtures were incubated at 37 ± 2^{0}C in a BOD incubator for 15 minutes and then heated at 70^{0}C for 5 minutes. After cooling, their absorbance was measured at 660 nm using vehicle as blank. The percentage inhibition of protein denaturation of test should be compared with respect to the control (Elias and Rao, 1988).

$$\%\text{Inhibition of denaturation} = \frac{\text{Absorbance in control group} - \text{Absorbance in test group}}{\text{Absorbance in control group}} \text{X } 100$$

Statistical analysis

In determination of average globule size of liniments, the mean and standard deviations were measured. In *in-vivo* pharmacological evaluation, the averages of animals were calculated in individual groups to get the mean data along with standard deviation. Similar statistical parameters were calculated for *ex-vivo* pharmacological screening. All the statistics were done using basic Microsoft excel 2010 software.

Results

The present study was intended to prepare a stable lemon grass oil liniment using the best polymer concentration (tween 80) and to evaluate the anti-inflammatory activity of such formulation.

Preparation of Liniments

Liniments were prepared using suitable oil and polymer in different ratios. The polymer used in this study is tween 80. Various formulations were prepared by increasing the polymer quantity and by keeping the oil concentration same in every formulation. All the formulations were prepared under identical conditions to minimize the processing variables. Further these were subjected to various physical stability studies.

Evaluation of physical stability parameters

Determination of Globule Size

The globule sizes were estimated for all the formulations using eye piece micrometer and stage micrometer. Of all the formulations, TL5 of turpentine oil liniment and LL5 of lemon grass oil liniment with oil to surfactant ratio of 1:0.5 were found to have optimised globule size with less standard deviation. The LL5 has less Standard deviation (1.182) when compared to that of TL5 (1.486). This indicates that the lemon grass oil liniment and turpentine oil liniment prepared using 10% of tween 80 as surfactant showed best stability.

Determination of emulsion type

Using dilution method, the emulsion type was determined for the liniments. Both the liniments showed complete miscibility when mixed with water without any phase separation. This clearly indicates that the liniments TL5 and LL5 are Oil in Water type of emulsion

Estimation of pH

The pH of liniments was estimated using pH meter. The formulation TL5 showed a pH of 6.14 which is slightly acidic, whereas LL5 showed a pH of 7.2 which is slightly alkaline in nature.

Determination of viscosity

The viscosity of liniments was measured using Brookfield Viscometer. TL5 showed a viscosity of 2.15 Centi-poise is less than LL5, which showed a viscosity of 5.37. This clearly indicates that LL5 is highly stable than that of TL5, as viscosity increases, the stability of liniment also increases.

Determination of stability

The stability of liniments was determined by Phase volume ratio measurement. After 24 hours, the phase volume ratio was calculated. LL5 showed more phase volume ratio which shows higher stability when compared to that of TL5. All the results of physical stability parameters were indicated in table 1.

Pharmacological evaluation

***In-vivo* Anti-inflammatory activity using Carrageenan induced paw oedema**

Carrageenan-induced inflammation is most commonly equipped experimental model for evaluation of anti-inflammatory property of compounds because it produces reproducible results (Winter et al., 1962). Development of oedema in the paw of the rats is due to the release of histamine, serotonin and prostaglandin like substances (Umesh et al., 2011). The effect of TL5 and LL5 on carrageenan-induced paw oedema in albino rats is shown in the table 2 and figure 1. The results obtained indicate that both of the liniments had significant anti-inflammatory activity in albino rats when compared with that of the control groups. The potency was found to be inversely proportional to the time taken for reduction in paw volume. The inhibition obtained with LL5 was 63.15%, whereas TL5 showed 56.84% inhibition in oedema. This clearly

Table 1. Parameters for TL5 and LL5 Liniment Formulations

Parameter	Formulations											
	TL1	TL2	TL3	TL4	TL5	TL6	LL1	LL2	LL3	LL4	LL5	LL6
Average Globule Size (µm)	14.45	14.50	10.40	9.37	8.90	13.25	12.50	7.90	9.20	8.60	7.80	11.35
Emulsion Type	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w	o/w
pH	5.82	5.91	5.98	6.02	6.14	6.12	6.20	6.54	6.87	6.95	7.20	7.04
Viscosity (cps)	9.34	7.01	5.64	5.12	2.18	1.06	10.44	9.82	7.31	6.49	5.37	4.11
Stability	Unstable	Unstable	Unstable	Unstable	Stable	Unstable	Unstable	Unstable	Unstable	Unstable	Stable	Unstable

Table 2. Effect of TL5 and LL5 on Carrageenan Induced Paw Oedema in rats

Treatment Groups	Dose	Paw Oedema (ml) Mean ± S.E.M (% Inhibition)			
		1st Hour	2nd Hour	3rd Hour	4th Hour
Control	Saline	0.6 ± 0.422	0.95 ± 0.214	1.02 ± 0.785	0.9 ± 0.104
Standard (Indomethacin)	10 mg/Kg	0.25 ± 0.332 (58.33)	0.31 ± 0.012 (67.36)	0.37 ± 0.003 (63.72)	0.30 ± 0.106 (58.88)
TL5	1ml	0.34 ± 0.005 (43.33)	0.41 ± 0.184 (56.84)	0.53 ± 0.042 (48.03)	0.39 ± 0.722 (41.11)
LL5	1ml	0.29 ± 0.042 (51.66)	0.35 ± 0.842 (63.15)	0.42 ± 0.071 (58.82)	0.37 ± 0.006 (53.33)

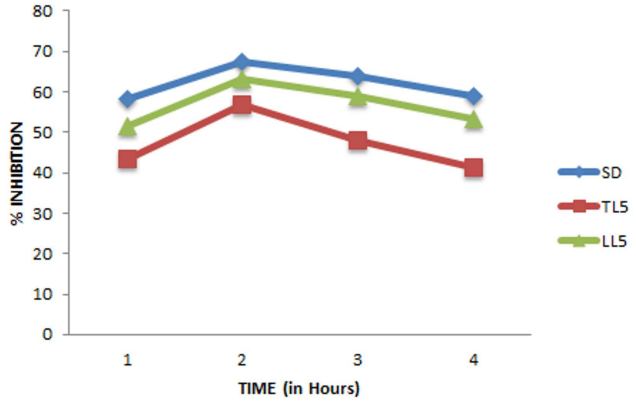

Figure 1. Effect of TL5 and LL5 on Carrageenan Induced Paw Oedema in rats

indicates that LL5 has more anti-inflammatory activity when compared to that of traditional turpentine oil liniment. Based on the reports, it can be inferred that the inhibition effect of Liniments on carrageenan induced inflammation on rats may be due to inhibition of mediators that are responsible for inflammation.

Ex-vivo Anti-inflammatory activity

Heat induced haemolysis

In this, lysosomes play a major role in the inflammatory reaction (Gokani et al., 2011). The vitality of cells depends upon the integrity of their membrane, exposure of RBCs to injurious substances such as hypotonic medium results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin (Augusto et al., 1982). The liniments, TL5 and LL5 were subjected to heat induced haemolysis test in various concentrations using fresh rat blood. LL5 showed 89.24% inhibition of haemolysis on erythrocyte membrane at a maximum dose of 500µl which is higher than that of TL5 which showed 84.32% inhibition. LL5 is more effective when compared to that of the control and as the dose increases, there is an increase in the % inhibition. Compounds with membrane stabilizing properties are well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators. These results were indicated in table 3 and figure 2.

Table 3. Effect of TL5 and LL5 on Heat Induced Haemolysis of Erythrocyte Membrane

Concentration (µl)	% Inhibition of Heat Induced Haemolysis (Mean ± SEM)		
	SD (Indomethacin)	TL5	LL5
100	42.26 ± 1.0022	56.43 ± 1.0174	60.81 ± 1.0015
200	58.74 ± 0.8845	68.75 ± 1.2356	72.45 ± 1.5224
300	67.62 ± 1.5841	72.79 ± 1.2150	77.44 ± 1.2443
400	75.98 ± 1.6427	79.73 ± 1.0714	81.32 ± 0.8462
500	82.22 ± 0.7425	84.32 ± 1.2124	89.24 ± 0.9511

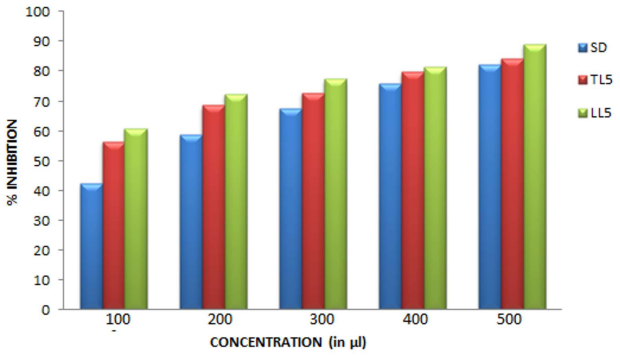

Figure 2. Effect of TL5 and LL5 on Heat Induced Haemolysis of Erythrocyte Membrane

Protein denaturation

Denaturation of proteins is a well-documented cause of inflammation and rheumatoid arthritis (Grant et al., 1970). TL5 and LL5 formulations were subjected to protein denaturation in various concentrations using fresh egg albumin. LL5 showed 94.28% inhibition of protein denaturation at a maximum dose of 500 µl which is higher than that of TL5 which showed 85.90% inhibition. It is more effective when compared to that of the control and as the dose increases, there is an increase in percentage inhibition. Several anti-inflammatory agents had already shown dose dependent action to reduce protein denaturation which induced thermally. Ability of LL5 to bring down thermal denaturation of protein is possibly contributing factor for its anti-inflammatory activity. These results were indicated in table 4 and figure 3.

Table 4. Effect of TL5 and LL5 on Protein Denaturation

Concentration (µl)	% Inhibition of Protein Denaturation		
	SD (Indomethacin)	TL5	LL5
100	46.58 ± 1.4222	55.63 ± 0.9088	69.27 ± 0.6876
200	60.44 ± 0.4055	69.09 ± 0.9333	77.08 ± 1.2890
300	71.98 ± 1.5371	77.23 ± 0.9295	80.21 ± 0.6037
400	77.55 ± 1.0142	82.64 ± 0.9388	88.45 ± 0.8536
500	81.04 ± 1.8534	85.90 ± 0.8249	94.28 ± 0.4309

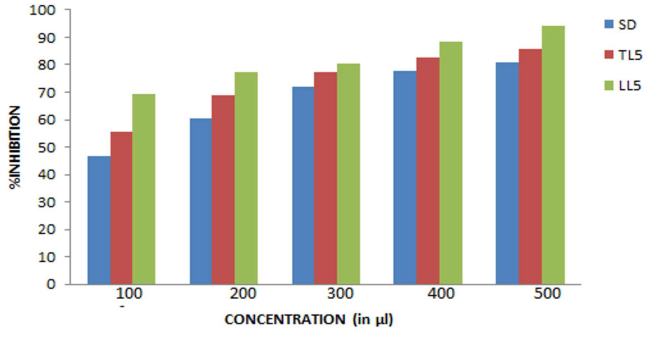

Figure 3. Effect of TL5 and LL5 on Protein Denaturation

Conclusion

Lemon grass oil liniment prepared using tween 80 as surfactant in the ratio of 1:0.5 was found to be a stable formulation after performing suitable physical stability studies. This formulation LL5 along with stable turpentine oil liniment TL5, were further subjected to pharmacological evaluation. From the obtained results, it was concluded that LL5 reduced oedema significantly. There is also a significant inhibition of heat induced haemolysis of and protein denaturation which clearly indicates the anti-inflammatory effect. Thus, from the current work, it was concluded that the lemon grass oil liniment showed significant anti-inflammatory action.

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Conflict of Interest

The authors declare no conflicts of interest.

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