P.Devi et al, /J. Pharm. Sci. & Res. Vol.2(2), 2010, 155-163

ISSN:0975-1459

Journal of Pharmaceutical Sciences and Research

www.jpsr.pharmainfo.in

Study of antioxdant, antiinflammatory and woundhealing activity of extracts of *Litsea glutinosa*

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Abstract:

Present work was undergone to investigate antioxidant and anti inflammatory and wound healing effects of Litsea glutinosa in rats. The aqueous extract of Litsea glutinosa (250 and 500 mg/kg body weight) was studied for ant inflammatory in animal models. The activity was studied in some acute models Viz carragenan , histamine and dextrin induced rats paw edema against indomethacin as standard, and it showed significant anti inflammatory activity in all the three models. The preliminary phytochemical analysis was carried out for different extracts. It was found that flavone glycosides, reducing sugars, aminoacids and tannins. The ethanolic extract was screened for wound healing activity by excision and incison wound model, in the form of an ointment having 2 concentrations(3 and 5 % w/w) of leaf extract in simple ointment base. Both the concentrations of the ethanolic extract showed significant response in both the wound types tested when compared with the control group. Nitrofurazone ointment (0.2%w/w) was used as an standard drug. Antioxidant activity was determined by two in vitro methods- DPPH and H_2O_2 radical scavenging . The extract having significant ($p<0.001$ and $p<0.0001$) antioxidant activity compared to control. BHT and Ascorbic acid were used as reference standard for antioxidant activity. On the basis of the results obtained suggest marked that extracts have significant Anti oxidant , Anti inflammatory and wound healing activity of Litsea glutinosa. The results supported the traditional use of this plant in some painful and inflammatory conditions.

Keywords: Litsea glutinosa, Lauraceae, Anti oxidant, Anti inflammatory, wound healing .

Introduction:

Litsea glutinosa (Lour.) C.B.Rob., Lauraceae is moderately-sized tree; bark thin, grey or pale brown; live bark 3 mm thick, pale brown, very slimy. Branchlets rather slender, stiff, minutely tomentellous towards apex, hairs very slender; terminal with dense layer of sub-appressed, glossy, long hairs. Leaves 7-15 x 3-7 cm, spirally arranged, very variable in size, usually oblong-oval or elliptic, shortly acuminate or obtuse, base acute, chartaceous to stiffly chartaceous, very densely, finely areolate-reticulate above, glabrous; midrib and slender lateral veins prominent, basal part of midrib often pilose, slightly impressed; paler beneath, minutely reticulate, very sparsely, minutely pilose, soon glabrous, midrib and lateral veins prominent, lateral veins slender, erect-patent, c. 8-12 pairs, secondary veins parallel, not horizontal. Petiole 1.5-3 cm long, slender, pilose. Flower umbels numerous, densely grey- tomentellous, 4-5 mm diameter; peduncles up to 5 mm long, slender, densely pilose on slender, short branches up to 14 mm long; perianth tube silky, funnel-shaped; tepals 0; stamens up to 20 with slender, very hairy filaments; glands on long stalks. Fruit 6 mm diameter, globose, purplish black, on flat, 4 mm diameter thin disc; fruiting pedicel slender"[1-5] . The seeds contain an aromatic oil which has been used to make candles and soap. Fibre: The roots yield fibres used in Thailand for rope manufacture and for paper pulp. Food: The fruits have a sweet creamy edible pulp. Fodder: The young leaves are eaten by livestock. Medicine: The pounded seeds are also applied medicinally against boils. The leaves and the mucilage in the gum from the bark have been used for poultices. The bark also acts as a demulcent and mild astringent in diarrhoea and dysentery. But adequate characterization of its anti-inflammatory and woundhealing activity has not been yet confirmed. The present study was undertaken for scientific evaluation of anti-inflammatory and wound healing activity in normal healthy rats. The extract was also studied for its acute toxicity effects and preliminary phyto chemical screening.

Materials and Methods:

Plant material

The entire herb of *Litsea glutinosa* has been collected in the month of December from Mangulam village near Madurai, and authenticated . A voucher specimen has been deposited in the herbarium of same department. The plant was shade dried and pulverized.

Extraction

The fresh plant material was washed under running tap water to remove adhered dirt, followed by rinsing with distilled water, shade dried and pulverized in a mechanical grinder to obtain coarse powder. The dried powdered plant material (500gms) was extracted successively with methanol by soxhlet for 72hours at a temperature not exceeding the boiling point of the solvent. Crude aqueous infusion prepared by adding boiling water to 500gms of powdered plant material in a 2500ml glass flask and incubated at room temperature for 2 hours on rotary shaker (200 rotation/minute) .The aqueous extract filtered using Whatman No 1 filter paper and concentration in vacuum at 40ºC using Rotary evaporator. The residue obtained were stored in freezer at -80ºC until use [6].

Preliminary phytochemical screening [7, 8]

Litsea glutinosa was studied for its preliminary phytochemical screening for the detection of various plant constituents.

Preparation of drug formulation

The drug formulations with different concentrations of the extract were prepared ,Viz 3% (w/w) ointment, where 3 gm of extract was incorporated in 100 g simple ointment base and 5% (w/w) ointment where 5g of extract was incorporated in 100 g of simple ointment base B.P.1993.[9]. Nitrofurazone (0.2% w/w) was used as standard drug for comparing the wound healing potential of the extract in different animal model.

Experimental Animals

Wistar rats (100-175 g) of either sex kept at the Laboratory Animal center and were used. The experimental protocol was initially approved from the Institutes animal ethics committee and then experimental studies were undergone according to their rules and regulations. The animals were housed under standard environmental conditions and standard pellet diet and water *ad libitum.*

Acute toxicity studies [10,11,12]

Animals were starved over night and divided into 5 groups (n=6) .They were fed orally with the leaf extracts of *Litsea glutinosa* in increasing does levels of upto 2000mg/Kg body weight.

Antioxidant Activity

Diphenylpicrylhydrazyl (DPPH) radical scavenging activity, Hydrogen peroxide (H_2O_2) radical scavenging activity was determined by following procedures [13-18].

DPPH radical scavenging activity: 0.1mm solution of DPPH in ethanol was prepared and 1 ml of this solution was added to 0.3 ml of extract solution in water at different concentrations (10-100µg/ml) .Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical and the standard

(BHT) was calculated using the following equation.

DPPH scavenged **(%)** = $\frac{A_{cont} - A_{test}}{A_{cont}}$ **x 100**

Where A_{cont} is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts.

H_2O_2 radical scavenging activity: A solution of H_2O_2 was prepared in phosphate buffer (PH 7.4). H_2O_2 concentration was determined spectroscopically measuring absorption with extinct coefficient for H_2O_2. Different concentrations of the extracts in distilled water were added to a H_2O_2 solution (0.6 ml, 40 mM). Absorbance of H_2O_2 at 230 nm was determined 10 min later against a blank solution containing the phosphate buffer without H_2O_2. Ascorbic acid was used as the standard. The % of H_2O_2 scavenging was calculated by the equation:

H_2O_2 scavenged **(%)** = $\frac{A_{cont} - A_{test}}{A_{cont}}$ **x 100**

Where A_{cont} is the absorbance of the control reaction and A_{test} is the absorbance in the presence of the sample of the extracts.

Anti Inflammatory Activity

Carrageenan-Induced Edema

Anti-inflammatory Activity of *Litsea glutinosa* was studied in adult albino rats of either sex weighing between 120-150gms by Carrageenan Induced hind paw oedema method [19]. Animal were divided into four groups containing 6 animals per each.

Group I (control) was given a 1% Sodium Carboxy methyl Cellulose solution. Group II of animals received 10mg/kg of Indomethacin which was considered as standard. Group III and Group IV were treated with 250 mg/kg and 500 mg/kg of methanol extract respectively dissolved in Sodium Carboxy methyl Cellulose solution. Doses were given orally with the help of an oral catheter. 0.1 ml of 1% solution of carragenan was administered to the rats into the plantar surface of the right hind limb to induce paw oedema. Paw volume was measured plethysmographically after 1h, 2h and 3h, 4h of carrageenan injection and paw swelling in groups of drug treated were compared with control. Percentage inhibition of oedema was calculated by using the following formula

$$\%\text{inhibition} = \frac{Vc - Vt}{Vc} \times 100$$

where

Vt- means increase in paw volume in rats treated with test compounds; **Vc**-means increase in paw volume in control group of rats.

Histamine –Induced Edema

For the study of Histamine – induced edema the animals were treated exactly the same method as in carrageenan induced method but instead of carrageenan ,here 0.1 ml of 1% w/w histamine in normal saline was used.

Dextran –Induced Edema [20]

For the study of Dextran – induced edema the animals were treated exactly the same method as in carrageenan induced method but instead of carrageenan , here 0.1 ml of 1% w/w dextran in normal saline was used.

Wound Healing Activity

The wound healing activity was investigated in ether anaesthetized rats in two different wound models (at 2 different concentrations 3 and 5%w/w).

Incision wound – The animals were divided in four groups of six animals each, the group I control (Simple ointment base B.P), the group II was reference standard and treated with 0.2% w/w Nitrofurazone ointment. The group III animals were treated with 3%w/w ethanolic extract ointment and the group IV animals were treated with 5% w/w ethanolic extract ointment of leaves. The animals were anaesthetized with anaesthetic ether. Paravertebral incision of 6cm long was made on either side of vertebral column of the rat. Care was taken to make the incision at least 1cm lateral to vertebral column. The

wounds were covered with interrupted sutures of 1 cm apart. The animals were caged separately according to groups. The tensile strength of the wound was measured on 10th day by tensitometer [21,22].

Excision wound – The animals were divided in four groups of six animals each, the group I control (Simple ointment base B.P), the group II was reference standard and treated with 0.2% w/w Nitrofurazone ointment. The group III animals were treated with 3%w/w ethanolic extract ointment and the group IV animals were treated with 5% w/w ethanolic extract ointment of leaves. A circular wound of about 2.5 cm diameter was made on depilated dorsal thoracic region of animals under light ether anaesthesia. The observation of percentage wound closure was made on 4th, 8th, 12th and 16th post woundings days. Number of days required for falling of the scar without any residual raw wound gave the period of epithelization [23,24]. The ointment of the leaf extract , reference standard and simple ointment (control) was applied to the wound twice daily, until recovery to the respective groups of animals.

Statistical analysis - Data are reported as the mean ± SD of three measurements. Statistical analysis was performed by students' t test [25].

Results:

Acute toxicity studies

The behaviour of the animal was carefully monitored for 1 day.

1.CNS activity : Tremors, Convulsions

2.CVS activity :Palpitation, increase in pulse rate.

3.Respiratory tract activity :Bronchial constriction, difficult in breathing

4.Reflex action :Loss of writhing reflex.

The number of deaths, if any were recorded after 24 and 72 h.

Preliminary phytochemical screening

Preliminary phytochemical screening of the plant extract showed the presence of flavanoids, tannins, aminoacids, glycosides and carbohydrates**.**

Antioxidant Activites

Antioxidant activity*:* The extract exhibited scavenging potential with IC_{50} value of 30.24µg/ml and 216.53µg/ml for DPPH and H_2O_2 radicals respectively. The value was found to be lesser than that of BHT (16.15µg/ml) and Ascorbic acid (123.84µg/ml),used as standards in respective assays (Table 1, 2).The extract showed dose dependant increase in reducing power that was comparable to standard BHT and Ascorbic acid.

Table 1 : DPPH radical scavenging activity

Drug Concentration	DPPH Scavenged %	
	BHT	*A. pungens*
10 µg/ml	26±0.21	18.4±0.17***
20 µg/ml	59.6±.67	49.8±0.68***
50 µg/ml	78.2±1.1	67.8±1.06***
100 µg/ml	93.08±1.54	85.46±1.54**
IC_{50}	16.15µg/ml	30.24µg/ml

Values are mean ± S.E., n = 6; data analysed by one way ANOVA and student's "t" test; **p< 0.001, *** p<0.0001 Vs control.

Table 2: H_2O_2 Scavenging activity

Drug Concentration	H_2O_2 Scavenged %	
	Ascorbic acid	*A.pungens*
50 µg/ml	29.17±0.45	13.14 ±0.29***
100 µg/ml	44.24±0.82	28.78±.65**
200 µg/ml	65.56±1.21	51.26±1.14***
300 µg/ml	77.72±1.54	60.21±1.28**
350 µg/ml	86.64±2.03	68.54±1.60***
IC_{50}	123.84µg/ml	216.53µg/ml

Values are mean ± S.E., n = 6; data analysed by one way ANOVA and student's "t" test; **p< 0.001, *** p<0.0001 Vs control.

Anti Inflammatory Activites

Carrageenan induced paw edema method

The aqeous leaf extract of *Litsea glutinosa* at the dose level of 250 and 500 mg/Kg body weight by oral route exhibited significant ($p<0.001$) anti-inflammatory activites against all the agents used and the inhibition of edema by 16.13% and 46.21% at 250mg/Kg and 500 mg/Kg body weight after 3h .(Table3)

Histamine induced paw edema method

The aqeous leaf extract of *Litsea glutinosa* at the dose level of 250 and 500 mg/Kg body weight by oral route exhibited significant ($p<0.001$) anti-inflammatory activites against all the agents used and the inhibition of edema by 22.21% and 35.08% at 250mg/Kg and 500 mg/Kg body weight after 3h . (Table 4)

Dextran induced paw edema method

The aqeous leaf extract of *Litsea glutinosa* at the dose level of 250 and 500 mg/Kg body weight by oral route exhibited significant ($p<0.001$) anti-inflammatory activites against all the agents used and the inhibition of edema by 15.36% and 43.14% at 250mg/Kg and 500 mg/Kg body weight after 3h . (Table 5).

There is a remarkable similarity of the dose dependent anti-inflammatory activity of the indomethacin with the extract.

Wound Healing Activites

The effect of ethanol extract on excision wound model, the wound healing contracting ability in different concentrations was significantly greater than that of control (simple ointment treated group). The 4% (w/w) extract ointment treated groups showed significant wound healing from the fourth day onwards , which was comparable to that of the standard drug, (Nitrosofurazone ointment treated group) of animals.The wound closure time was lesser as well as the percentage of wound contraction was much more with the 4% w/w extract ointment treated group (20 ± 1 days for 100% contraction which was almost similar to that of the Nitrofurazone treated group).The 2% (w/w) extract ointment treated group of animals showed significant wound contraction from the eigth day onwards and achieved 100% with the wound closure time of 22 ± 2 days.(Table 6)

The results of tensile strength wound model is shown in Table 7. The tensile strength of the 4% extract treated group were comparable to each other. The 2% extract treated group showed a lesser but significant increase in the tensile strength compared to control group. Thus the concentration of the extract as well as the standard drug showed a significant increase in tensile strength in the 10 days old wound.

The results of the present study revealed that both concentrations (2 and 4% w/w) of ethanol extract of *Litsea glutinosa* have significant wound healing activity in both incision as well as excision wound models.

Discussion:

Oxygen is vital for aerobic life. But the cellular biochemistry of dioxygen is Janius faced that comprise of both bright and dark side. The bright side includes numerous enzymes catalysed reactions of dioxygen that occur in respiration and normal metabolism while the dark side encompasses deletorius reactions of reactive species derived from dioxygen that inflict damages to the cellular components. Reactive oxygen species are ubiquitous and occur naturally in all aerobic organisms , arising from both endogenous and exogenous sources [26]. They are normally produce as a by product of cellular metabolism. They are capable of damaging biomolecules, provoking immune response, activating oncogenes and enchancing aging process [27]. Reactive oxygen species metabolites can be generated by the stepwise reduction of oxygen leads to the production of series of oxidant molecules such as superoxide (O_2) and other reactive nitrogen species like nitric oxides

Table 3: Antiinflammatory activity of methanol extract of *Litsea glutinosa* by Carrageenan induced paw oedema method

Group	Drug	Dose in mg/kg	No.of animals	Average weight in gms	%Inhibition			
					1 hour	2 hours	3 hours	4 hours
I	Solvent control	0.3 ml	6	146	0.24± 0.001	0.43 ± 0.001	0.50 ± 0.002	0.34 ± 0.001
II	Positive control	10 mg/kg	6	206	0.13 ± 0.001	0.27 ± 0.001	0.28 ± 0.002	0.15 ± 0.001
III	Aqueous extract	250 mg/kg	6	143	0.17 ± 0.002 28.06%	0.34 ± 0.002 16.37%	0.43± 0.002 13.83%	0.27 ± 0.001 16.13%
IV	Aqueous extract	500 mg/kg	6	215	0.16± 0.001 37.23%	0.27 ± 0.001 32.61%	0.31 ± 0.001 38.73%	0.19± 0.001 46.21%

Values are mean ± SEM; n =6; P < 0.001 significant against control

Table 4: Antiinflammatory activity of methanol extract of *Litsea glutinosa* by Histamine induced paw oedema method

Group	Drug	Dose in mg/kg	No.of animals	Average weight in gms	%Inhibition			
					1 hour	2 hours	3 hours	4 hours
I	Solvent control	0.3 ml	6	146	0.24± 0.001	0.43 ± 0.001	0.50 ± 0.002	0.34 ± 0.001
II	Positive control	10 mg/kg	6	206	0.13 ± 0.001	0.27 ± 0.001	0.28 ± 0.002	0.15 ± 0.001
III	Aqueous extract	250 mg/kg	6	143	0.17 ± 0.002 20.06%	0.34 ± 0.002 19.34%	0.43± 0.002 16.26%	0.27 ± 0.001 22.21%
IV	Aqueous extract	500 mg/kg	6	215	0.16± 0.001 43.34%	0.27 ± 0.001 33.36%	0.31 ± 0.001 28.74%	0.19± 0.001 35.08%

Values are mean ± SEM; n =6; P < 0.001 significant against control

Table 5: Antiinflammatory activity of methanol extract of *Litsea glutinosa* by Dextran induced paw oedema method.

Group	Drug	Dose in mg/kg	No.of animals	Average weight in gms	%Inhibition			
					1 hour	2 hours	3 hours	4 hours
I	Solvent control	0.3 ml	6	146	0.24± 0.001	0.43 ± 0.001	0.50 ± 0.002	0.34 ± 0.001
II	Positive control	10 mg/kg	6	206	0.13 ± 0.001	0.27 ± 0.001	0.28 ± 0.002	0.15 ± 0.001
III	Aqueous extract	250 mg/kg	6	143	0.17 ± 0.002 25.36%	0.34 ± 0.002 16.34%	0.43± 0.002 13.53%	0.27 ± 0.001 15.36%
IV	Aqueous extract	500 mg/kg	6	215	0.16± 0.001 34.02 %	0.27 ± 0.001 33.64%	0.31 ± 0.001 36.81%	0.19± 0.001 43.14%

Values are mean ± SEM; n =6; P < 0.001 significant against control

Table 6: Effect of ethanol extract ointment leaf extract of *Litsea glutinosa* on % wound closure of excision wounds

Treatment	**4th Day**	**8th Day**	**12th Day**	**16th Day**	**Period of epithelization in days**
Control (simple ointment base B.P)	14.82 ± 0.63	25.21 ± 1.01	46.12 ± 1.79	66.43 ± 2.36	26
Nitrofurazone 0.2% (Ref.Std.)	35.68 ± 0.12	73.08 ± 0.16	85.71 ± 0.43	95.31 ± 0.32	20
Ethanolic extract (4%)	33.30 ± 1.01	75.46 ± 1.34	82.12 ± 2.06	90.06 ± 2.07	20*
Ethanolic extract (2%)	21.06 ± 1.01	34.23 ± 1.73	59.30 ± 2.63	78.02 ± 2.23	22

Values are mean ± SEM; *P< 0.01 Vs control by Student's test

(NO [28]. Compounds with antioxidant activity categorized into three groups namely excellent, good and moderate. Excellent ones are those that perfectly quench excited state as well as ground state radicals. Good antioxidants strongly inhibit the peroxide formation but are less effective in quenching excited states. Moderate antioxidants fail to excel in both reactivates [29]. Antioxidants from plant products may fall under any of three catagories of antioxidants .The antioxidant activity may also depend on the type and polarity of the solvent that the plant is extracted. There are some synthetic antioxidants such as butylated hydroxy anisole and butylated hydroxy toluene. Reaction with the α,α di phenyl picryl hydrazyl (DPPH) will serve as

a method for the direct detection of radical scavenging as screening methods also reliable for bio assay guided Lactination. ROS contribute to a great variety of diseases. ROS including H_2O_2, superoxide radical anion, Nitric oxide, singlet oxygen react biological molecules leading to cell and tissue injury. In our work we have performed some popular methods that were easy to perform in our laboratory conditions. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component, but implies to the overall antioxidant capacity of the sample. H_2O_2 is highly important because of its ability to penetrate biological membranes. H_2O_2 is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Thus, removing of H_2O_2 is important for protection of food systems [30].

Table 7: Effect of ethanol extract ointments of fruit of *Litsea glutinosa* on tensile strength of incision wounds

Treatment	Tensile strength in g ± SEM
Control (simple ointment base B.P)	308 ± 3.2
Nitrofurazone 0.2% (Ref.Std.)	560 ± 1.5*
Ethanolic extract (4%)	532 ± 2.4*
Ethanolic extract (2%)	443 ± 4.1*

Values are mean ± SE; *P< 0.01 Vs control by Student's test

Three distinct phases are observed during inflammation which are the histamine and serotonin released in the first phase, Kinin and Prostoglandin are released in the second and third phases respectively [31]. The aqueous extract of Litsea glutinosa of 500 mg/kg body weight caused a significant inhibition only during the 3rd h (the phase of prostaglandin release), whereas the inhibiton it caused at other times was insignificant.

Wound healing is a fundamental response to tissue injury that results in restoration of tissue integrity. This is mainly achieved by the synthesis of the connective tissue matrix. Collagen is a major protein of the extracellular matrix and is the major component that ultimately contributes to wound strength. Tannins promote the wound healing through the several cellular mechanisms;chelating of the free radicals and reactive species of oxygen promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts [32,33].

Bark contains Laurotetanine, Actinodaphine, Boldine, Norboldine, Sebiferine and Litseferine. Leaves contain flavanoid naringenin, naringin, kaempferol-3 and 7-glucosides, quercitin and its 3-rhamnoside, pelargonidin-3 and 5 glucosides, cystine, glycine, L-alanine, and leucine. Seeds contain fatty acids and 20% tannins. The results of this study also revealed that tannins are one of the important phytoconsitutents responsible for wound healing mainly due to their astringent and antimicrobial property. Similar findings have been reported with the extracts of the plants containing tannins by earilier works [34].

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