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A wide array on anti-inflammatory study in an ethanolic extract of *Pupalia lappaceae* juss. (amaranthaceae) by using wistar rats

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Abstract

Inflammation causes debilitating mandatory to the human physic, also the marketed allopathic drugs exhibits a side effects. Main background to minimize the side effects by treating ayurvedic drugs which are available in the naturals. The study was intended to evaluate the anti-inflammatory activity of ethanolic extract of leaves of *Pupalia lappaceae* Juss. (Amaranthaceae) (EEPL) in Wistar strain rats and the toxicity studies was intended in Swiss Albino mice. Phytochemical analysis was carried out by using standard methodology to analyze the major phytoconstituents. The anti-inflammatory activity was carried out in different methods such as carrageenan, cotton pellet, croton oil, histamine and serotonin induced oedema. Different doses (100 and 300 mg/kg/i.p.) of EEPL was injected to rats and the results compared with standard drug indomethacin (10 mg/kg). Paw volume was measured using digital plethysmometer. The plant has alkaloids, amino acids, glycosides, flavonoids, saponins, starch, steroids, tannins and terpenoids. The EEPL showed the maximum inhibitory activity at 300 mg/kg/i.p. in a dose dependent manner. These inhibitions were statistically significant ($p < 0.01-0.001$). The significant values are intended to investigate the chemicals present in the extract of EEPL. EEPL was analyzed its chemical compounds by GC-MS (Gas Chromatography and Mass Spectrum). The major chemical compounds were eluted as phytol, mesitylene and etc. These results indicated that EEPL is a bioactive agent and having significant results in anti-inflammatory action by inhibition of the exudation, and leukocytes recruitment into the inflamed tissues.

Keywords: Anti-inflammation, *Pupalia lappaceae*, GC-MS

Introduction

Herbal medicines are being used by about 80% of the world population mainly, in the developing countries for primary health care. Ancient literature also mentions herbal medicines for various diseases, for which no scientific proof is available. The plant is an erect or straggling under shrub found in the hedges of fields, Diffuse, hispid herbs, fruit orchards, and dry scrub forests and waste places from Kashmir to Kumaun, at altitudes of 300-1050 m., densely near white-tomentose or velvety, the leaves smaller and usually orbicular-without locality (Wight) [1]. They are widely distributed in Western Australia, Northern Territory, Alien to Australia, alien to Western Australia, Tropical Asia, Africa and India, Particularly in Tamil Nadu: All districts and commonly, in waste places, in fields [2]. The plant extract is given in the form of soup for cough and fever. Ashes after burning the plant is mixed with water and given for flatulence, also applied to leprosy sores and used as a rat poison [3]. Fruit is an ingredient in enema preparations; mixed with palm oil applied to boil. In Africa, forms an ingredient in enema preparation; mixed with palm oil, it is applied as a dressing for boils and also applied to leprosy sores after making them bleed. In Senegal, the plant is employed as bait to attract fish. Ayurvedic, Unani drug manufacturers are the major users of this drug [4].

Materials and Methods

Plant collection and authentication

The plant *P. lappaceae* Juss. was collected from Ariyamangalam and the surrounding area, Tiruchirappalli districts of Tamil Nadu, and authenticated by Botanical Survey of India, Coimbatore, India. Plant authentication was given by Botanical survey of India, Agriculture University, Coimbatore. Voucher no: BSI/ SC/5/23/06-07/Tech-1821.

Preparation of extract

500 g of shade dried coarsely powdered leaves of *P. lappaceae* Juss. was extracted exhaustively for 72 hours in a distillation apparatus with the double quantity of ethanol, which was previously distilled off before extraction. The excess ethanol from the crude extract was distilled off under reduced pressure and the concentrated crude extract was stored in a desiccator for further analysis was reported by Harborne [5], Kokate [6] and Wagner and Roth [7].

Experimental animals

The toxicity studies was performed in Swiss albino mice (25-30 g) and anti-inflammatory activity only in Wistar strain rats (180-200 g). The animals were purchased from Kings Institute, Guindy, Chennai. They were housed in large spacious polypropylene cages and supplied with pellet feed and water *ad libitum*. The animals were acclimatized for at least one week in lab condition before commencement of the experiment in standard laboratory conditions 12±1 h day and night rhythm, maintained at 25±2°C and 35-60 % humidity. The study was approved by the Institutional Animal Ethical Committee (IAEC) of Committee for the purpose of control and supervision of Experiment on Animals (CPCSEA).

Acute and subacute toxicity studies

The acute oral toxicity study was carried out in Swiss Albino mice as per OECD guidelines [8]. The LD₅₀ cut-off dose was found to be in EEPL 5000 mg/kg body weight. They did not show any sign of toxicity to animals.

Anti-inflammatory activity

The anti-inflammatory activity of EEPL was assessed by carrageenan, cotton pellet, croton oil, histamine and serotonin induced oedema models.

Carrageenan induced paw oedema

The anti-inflammatory activity was evaluated by the carrageenan induced paw oedema in rats [9,10]. Animals were anesthetized with sodium pentobarbital (40 mg/kg i.p.) and with 0.1 ml carrageenan in isotonic saline (3.0 mg/ml) injected subplantarily into the right hind paw. The animals were treated with EEPL in saline and administered intraperitoneally 1 hour prior to the subplantar injection of 0.1 ml carrageenan (10 mg/ml). Oedema measurements were made using a modified digital plethysmometer as reported by Winder et al., [11] before injecting carrageenan and 1, 2, 3, 4 and 24 hour after carrageenan injection. The results were expressed as percentage inhibition in relation to the control group.

Cotton pellet induced granuloma

Cotton pellets weighing about 10±1 mg were autoclaved up to 20 minutes. Cotton pellets were aseptically implanted in the interscapular distance under the skin on the previously shaved back of the rats which were anesthetized with 25 mg/kg sodium pentobarbital intraperitoneally [12]. Inflammation induced animals were treated with EEPL intraperitoneally groups up to 7 days. After 7 days the animals were sacrificed and the pellets together with the granuloma tissues were carefully removed, dried in an oven at 60°C. The pellets were weighed both moist and dry. Mean weight of the granuloma tissue was recorded. The weight of the pellets taken out from drug administered rats was compared with the weight of the pellets taken out from the control group and indomethacin administered rats was reported by earlier workers [13,14].

Croton oil-induced ear inflammation

Croton oil irritant solution prepared was applied (0.1 ml) to the inner surface of the right ear of rats. The rats were sacrificed after 4 h and 7 mm punches were made in the ear using cork borer. Each ear disc was weighed and compared with control. Extract or vehicle was administered intraperitoneally 30 minutes before croton oil application. Mean weight of the ear granuloma tissue was recorded. The weight of the ear granuloma taken out from drug administered rats was compared with the weight of the ear granuloma taken out from the control group and indomethacin i.e., standard drug was administered to rats reported by Brooks et al., [15].

Histamine serotonin induced oedema

Oedema was induced by subcutaneous injection of 0.05 ml 1% freshly prepared solutions of histamine, serotonin individually (20µg in 0.05 ml) into the hind paws of the rats after 30 min of intraperitoneal administration of EEPL. The volume of the injected and counter-lateral paws was measured 3 h after administration of the phlogistic agents and the paw volume was measured after one hour [16].

Statistical analysis

ANOVA followed the Student–Duncan-test was used to determine significant differences between groups and $P < 0.01$ and $P < 0.001$ was considered significant.

Results and Discussion

Table. 1. Anti-inflammatory activity of EEPL in Carrageenan induced oedema method paw volume (in ml) and percentage inhibition (in %)

Groups	Normal Paw Volume	0 th Hour	1 st Hour	2 nd Hour	3 rd Hour	4 th Hour	24 th Hour
Positive Control	0.54±0.04	1.43±0.04	1.53±0.06	1.66±0.05	1.77±0.03	1.82±0.02	1.93±0.03
Indomethacin (10 mg/kg)	0.49±0.01	1.12±0.11	1.06±0.11 ^a (30.944)	0.94±0.0 ^a (43.01)	0.87±0.04 ^a (50.66)	0.78±0.05 ^a (56.81)	0.49±0.01 ^a (74.19)
EEPL 100 mg/kg	0.55±0.03	1.24±0.04	1.20±0.04 ^b (21.56)	1.17±0.0 ^c (29.51)	1.14±0.05 ^c (35.59)	1.10±0.03 ^c (39.82)	1.07±0.04 ^c (44.55)
EEPL 300 mg/kg	0.55±0.04	1.23±0.06	1.11±0.03 ^a (27.45)	1.07±0.03 ^b (35.54)	1.01±0.03 ^a (42.93)	0.96±0.02 ^b (47.48)	0.94±0.02 ^a (51.036)

Values are means ± SD of the six numbers of animals. Rats were induced with carrageenan adjuvant 14 days prior to 100 & 300 mg/kg/i.p. of drug administration. ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$ vs. control group with one way analysis i.e., DMRT.

Carrageenan is an early exudative phase of inflammatory pathology was reported by Ozaki *et al.*, [17] that involved in the action of vasoactive amines, such as histamine, serotonin, and kinins on vascular permeability [18-20]. Subcutaneous injection of carrageenan into the rat paw produces plasma extravasations and inflammation characterized by increased tissue water and plasma protein exudation with neutrophils extravasations and metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase enzyme pathways [21]. It was observed by the increased paw volume under the plethysmometer in experimental rats of this study. Winter and Porter [9] suggested that the early hyperemia of carrageenan-induced oedema results from the release of histamine and serotonin.

Thus Carrageenan-induced paw oedema in rats appears to be a biphasic events and the early phase (2.5–3 h) of the inflammation is due to the release of vasoactive amines such as histamine and serotonin. In the later phase (4.5–6 h) is due to the activation of kinin-like substances such as prostaglandins, proteases and lysosome [23]. (Table 1) shows the anti-inflammatory effect of the EEPL on carrageenan induced oedema in rats. The extracts caused (44.55 and 51.03%) inhibition of contractions at doses of 100 and 300 mg/kg b. wt, i.p., respectively a maximum inhibitory effect at 24th hour. In the carrageenan induced oedema test (Table 1), inhibition occurred predominantly during the second phase of the response and thus, after i.p., administration, in which, EEPL caused a (51.03%) response inhibition of the second phase at dose level on 300 mg/kg b.wt/i.p., The second phase showed that the EEPL reduced the inflammation by control the proliferation of the histamine and serotonin and in second phase controlled the stimulation of kinin like substances.

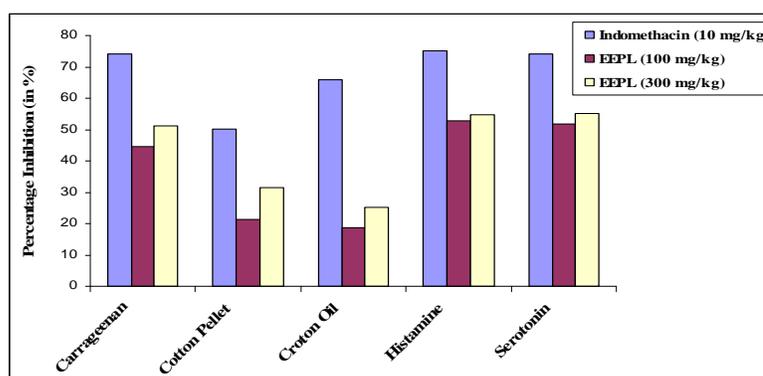
Table. 2. Anti-inflammatory activity of EEPL in Cotton pellet, Croton oil, Histamine, Serotonin induced oedema method. Percentage inhibition (in %)

Models	Cotton pellet induced model	Croton oil induced model	Histamine induced model	Serotonin induced model
Groups	Cotton pellet weight (in mg)	Ear Disc weight (in mg)	Paw volume (in ml)	Paw volume (in ml)
Positive Control	39.66±1.84	16.15±1.26	1.52±0.08	1.31±0.04
Indomethacin (10 mg/kg)	19.79±2.01 ^a (50.10)	5.53±0.89 ^a (65.75)	0.38±0.02 ^a (75.00)	0.34±0.02 ^a (74.04)
EEPL (100 mg/kg)	31.19±1.09 ^c (21.35)	13.12±0.22 ^c (18.76)	0.72±0.04 ^c (52.63)	0.63±0.02 ^b (51.90)
EEPL (300 mg/kg)	27.16±1.25 ^a (31.51)	12.05±0.46 ^b (25.38)	0.69±0.01 ^b (54.60)	0.59±0.02 ^a (54.96)

Values are means ± SD of the six numbers of animals. Rats were induced with adjuvants 14 days prior to 100 & 300 mg/kg/i.p. of drug administration. ^a*p* < 0.001, ^b*p* < 0.01, ^c*p* < 0.05 vs. control group with one way analysis i.e., DMRT.

In the second method known as the cotton pellet induced granuloma (Table 2), shows the percentage activity (21.35 and 31.51%) at doses of EEPL 100 and 300 mg/kg b.wt., i.p., this is exhibit negligible inhibitory effect when compared to other methods used. In this cotton pellet induced granuloma method the tissues were recovered and it was reproduced after seven days by the EEPL treatment. Wagner and Roth [24] reported that leukocyte adhesion represents one of the first steps in the inflammatory response initiation and it is essential for accumulation of active immune cells at sites of inflammation. It is observed that the cotton pellet granuloma tissues were reduced (from 31.19 to 27.16 mg). By which the EEPL extracts acting on the immune cells and controlled the release of immune cells to respond the vasoactive amines.

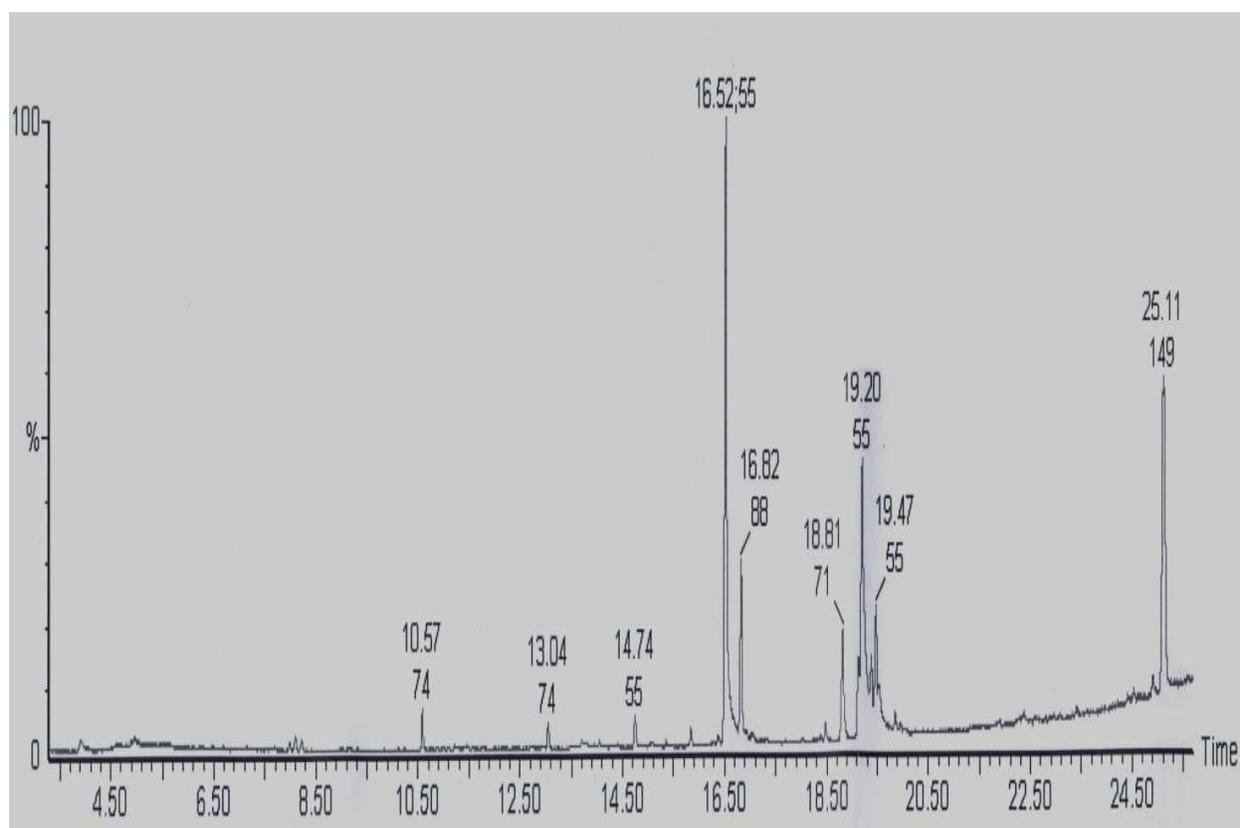
In third, croton oil induced method (Table 2), although a moderate significant effect was observed, the percentage inhibition (18.76 and 25.38%) in the latency to healing stimuli were observed at doses of 100 and 300 mg/kg b.wt., i.p., after 30 min of drug administration, respectively. EEPL 300 mg/kg b.wt, i.p., showed maximum inhibitory effect. Ear disc weight was reduced (from 13.12 to 12.05 mg) at the doses 100 to 300 mg/kg doses. Interestingly, in these cases, the effect was long lasting. The increase in stimuli could be observed 30 min after drug administration, at the doses of 100, 300 mg/kg respectively. The method has certain advantages for natural product testing [25]. So, the herbal treatment is very suitable to this ear oedema method.

Figure. 1. Comparative evaluation of the percentage inhibition

In the fourth and fifth methods (Table 2), i.e., histamine and serotonin those are the initial mediators for creating the inflammation in our body by extravasations and permeability. They induced the neutrophils and water on inflamed area, that's causing the elevated paw volume by plethysmometrically. Histamine showed the inhibition (52.63 and 54.63%) at doses of 100 and 300 mg/kg b/wt., i.p., respectively. Serotonin showed that the inhibition (51.90 and 54.96%) at doses of 100 and 300 mg/kg b/wt., i.p., respectively.

The above said five methods are compared with a technical graph sheet in excel software. They observed that the percentage inhibition decreased in the order serotonin, histamine, carrageenan, cotton pellet and croton oil induced method respectively.

Figure. 2. GC-MS analysis of EEPL



As expected, indomethacin was very efficient during both the first (50.66% inhibition) and second (74.19% inhibition) phases and its effect was totally responses in carrageenan method, (50.10% inhibition) in cotton pellet method, (65.75% inhibition) in croton oil method, (75% inhibition) in histamine induced method and (74.04% inhibition) in serotonin induced method. But peripherally acting drugs such as aspirin, indomethacin and dexamethasone only inhibit the later phase [26]. Although the direct evidence of mechanism of action of extract is not clear. Flavonoids exhibit anti-inflammatory activity was reported [27] it will be useful to elucidate the phytochemicals from EEPL in further analysis. Thus the oral and intraperitoneal administration of the drugs, this constitutes the activity of the herbal or standard marketed drugs, in a dose dependent manner.

Table 3. GC-MS Analysis on EEPL-Chemical Compounds Identification

No	RT	Name of the Compound	Molecular formula	Molecular weight	Peak area %	Structures
1.	3.91	Benzene, 1,3,5-trimethyl- (Synonyms: Mesitylene)	C ₉ H ₁₂	120	1.13	
2.	10.57	Dodecanoic acid, methyl ester	C ₁₃ H ₂₆ O ₂	214	1.50	
3.	13.04	Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	242	1.08	
4.	14.74	Oxirane, decyl-	C ₁₂ H ₂₄ O	184	1.58	
5.	15.84	Tridecanoic acid, methyl ester	C ₁₄ H ₂₈ O ₂	228	0.82	
6.	16.52	n-Hexa decanoic acid	C ₁₆ H ₃₂ O ₂	256	31.53	
7.	16.82	Hexa decanoic acid, ethyl ester	C ₁₈ H ₃₆ O ₂	284	7.58	
8.	18.81	Phytol	C ₂₀ H ₄₀ O	296	6.58	
9.	19.20	Oleic acid	C ₁₈ H ₃₄ O ₂	282	19.53	
10.	19.47	Ethyl oleate	C ₂₀ H ₃₈ O ₂	310	4.92	
11.	25.11	1,2-Benzenedi carboxylic acid, diisooctyl ester	C ₂₄ H ₃₈ O ₄	390	23.74	

The study was extended with GC-MS analysis and recruits the various chemical constituents. The plant has benzene, 1,3,5-trimethyl- (Synonyms: Mesitylene); dodecanoic acid, methyl ester; methyl tetradecanoate; oxirane, decyl-; tridecanoic acid, methyl ester; n-hexa decanoic acid; hexa decanoic acid, ethyl ester; Phytol; Oleic acid; ethyl oleate; 1,2-benzenedi carboxylic acid;

diisooctyl ester. One among the chemical constituents has been showed that the anti-inflammatory activity.

Conclusion

EEPL showed that the anti-inflammatory activity by inhibition of the exudation, and leukocytes recruitment. Statistical analysis showed that the oedema inhibitions of preparations containing extract is significantly different from the control group at all the concentrations tested and the activity is dose-dependent. From the above study we can be concluded that the ethanolic extracts of *Pupalia lappaceae* Juss. showed the anti-inflammatory activity and the inhibitory effect in a dose dependent manner.

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