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BIOACTIVE COMPOUND IDENTIFICATION, PHYTOCHEMICAL ESTIMATION, *IN-VITRO* ANTI-INFLAMMATORY AND ANTIOXIDANT ACTIVITY OF *PUPALIA LAPPACEA*

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ABSTRACT: *Pupalia lappacea* Juss belongs to the family Amaranthaceae useful in the bone fracture treatment, antimalarial, pyrexia, wounds, cough, toothache and boils. The present investigation was aimed to identify the phytocompounds by GC-MS, investigate phytochemically and to screen anti-inflammatory and antioxidant property by *in-vitro* methods. Bioactive compounds were identified using GC- MS; functional groups were identified using FT-IR, phytochemical estimation was done for the phenolics, flavonoids, tannins, and alkaloids, *in-vitro* anti-inflammatory activity by heat-induced hemolytic and albumin denaturation method, *in-vitro* antioxidant by DPPH, SOD, NO, PPO and CAT methods. GC-MS analysis of the methanolic extract confirmed the presence of bioactive principles like phenols, tetradecanoic acid, pentadecanoic acid, Eicosatetraenoic acid, Hexatriacontane, eicosapentaenoic acid, and chloroundecane. Methanolic extract of aerial parts of *Pupalia lappacea* was estimated for the presence of secondary metabolites and screened for *in-vitro* anti-inflammatory and antioxidant effects. Aerial parts were extracted and estimated for total phenolics 248.19 ± 4.84, flavonoids 123.47 ± 0.19, tannins 57.12 ± 2.45 and alkaloids 69.29 ± 1.84 quantitatively reaction equivalent per gram of the extract. The FT-IR report showed the presence of functional groups like amines, ester, alkyl and other carbonyl groups. Significant RBC membrane stabilisation effect in heat-induced haemolysis method and egg albumin protein inhibition was studied for *in-vitro* anti-inflammatory action. The phytochemicals like alkaloids, phenolics, flavonoids and tannins present in the extract might be responsible for the desired anti-inflammatory effect of the methanolic extract, which was confirmed by the preliminary phytochemical tests and by estimation. Also, the extract showed anti-oxidant properties in DPPH, Superoxide dismutase and nitrogen oxide, polyphenol oxidase, and catalase methods compared with the standard ascorbic acid.

INTRODUCTION: Inflammation is a bodily response to injury, infection or destruction characterized by heat, pain, redness, swelling and disturbed physiological functions. It is a protective response to tissue injury caused by physical trauma, noxious chemical or microbial agents. It is the body response to inactivate or destroy the invading organisms to remove the irritants and set the stage of tissue repair. It is released by the chemical mediators from injured tissues and migrating cells [1]. Natural sources like plants, herbs have been used as an alternative therapy for the ailment of various diseases since from ancient times.

Present years, a lot of research has been explored in medicinal plants in the treatment of various stress-related disorders caused by metabolism of oxygen leads to generate free radicals.

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Pupalialappacea belongs to the family Amaranthaceae is commonly known as Forest Burr or Creeping cock's comb. Itis an erect or straggling under shrub found in the edges of fields, fruit orchards, dry scrub forests and waste places of Kashmir to Kauman at an altitude of 300-1050 m and in all states of India [2].

The leaf paste of *Pupalialappacea* with edible oil (Sesamum or Carthamus) is an effective and inexpensive treatment of bone fracture for human beings as well as cattle. Stems used as a toothbrush, for treating toothache. A poultice of the fresh leaves is used in the treatment of boils, new and chronic wounds. A decoction of the black powder of the plant is drunk to cure piles and for enema, fever, and malaria [3].

MATERIALS AND METHODS:

Collection and Extraction: The aerial parts were purchased from Dr. Madhava Chetty, Sri Venkateswara University, Tirupathi and was air dried until free from moisture. Then they were subjected to size reduction to get coarse powder of desired particle size. The powdered drug was subjected to extraction with petroleum ether and methanol in a Soxhlet extractor, the temperature was maintained on an electric heating mantel with thermostat control.

The extracts were then concentrated to ¾ th of their original mass using rotary vapor apparatus. The concentrated extract was then transferred to a china dish and evaporated on a thermostat controlled water bath till it formed a thick paste. The thick mass was vacuum dried in a desiccator till it is free from moisture.

Phytochemical Test: Phytochemical tests on the extract and fractions were performed using standard procedures [4].

Estimation of Secondary Metabolites:

Determination of Total Phenolic contents: The amount of total phenolics in the extract was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard, and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentration of 0.1 and 1mg/ml of plant extract were also prepared in methanol and 0.5 ml of each sample were introduced into test tubes and mixed with 2.5 ml of a 10 fold dilute Folin- Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 30 min at room temperature before the absorbance was read at 760 nm spectrometrically.

All determinations were performed in triplicate. The Folin-Ciocalteu reagent being sensitive to reducing compounds including polyphenol is producing a blue color upon a reaction which is measured spectrophotometrically [5].

Determination of Total Flavonoids Content: The total flavonoids content of each plant extract was estimated by the method described by Zhishen *et al.* [6] Based on this method, each sample (1.0 ml) was mixed with 4 ml of distilled water and subsequently with 0.30 ml of a $NaNO_2$ solution (10%). After 5 min, 0.30 ml $AlCl_3$ solution (10%) was added followed by 2.0 ml of NaOH solution (1%) to the mixture. Immediately, the mixture was thoroughly mixed, and absorbance was then determined at 510 nm versus the blank. A standard curve of Rutin was prepared (0-12 mg/ml), and the results were expressed as Rutin equivalent (mg Rutin/gm dried extract).

Determination of Tannin Contents: Tannin content in each sample was determined using insoluble polyvinylpolypyrrolidone (PVPP), which binds tannins as described by Makkar *et al.* [7] Briefly, 1 ml of extract dissolved in methanol (1 mg/ml), in which the total phenolics were determined, was mixed with 100 mg PVPP, vortexed, kept for 15 min at 4 °C and then centrifuged for 10 min at 3,000 rpm. In the clear supernatant, the non-tannin phenolics were determined the same way as the total phenolics [10]. Tannin content was calculated as a difference between total and non-tannin phenolic content.

Determination of Alkaloids Content: 5 g of the sample was weighed into a 250 ml beaker, and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered, and the extract was concentrated on a water bath to one-quarter of the original volume.

Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was

complete. The whole solution was allowed to settle, and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed. Atropine Equivalent was used as a standard [8].

FT-IR Analysis: The FT-IR analysis of the plant extract in KBr pellets by using FT-IR spectroscopy Shimadzu, IR affinity 1, Japan) at a moderate scanning speed between 4000-400 cm^{-1}. The peak values (wave numbers) and the possibility of the functional group were shown.

GC-MS Analysis: GC-MS analysis of these extracts was performed using a Perkin-Elmer GC Clarus 500 system and Gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with an Elite-I, fused silica capillary column (30 mm × 0.25 mm 1D × 1µMdf, composed of 100% Dimethylpolysiloxane). For GC-MS detection, an electron ionization system with ionizing energy of 70eV was used.

Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min, and an injection volume of 2 µl was employed (split ratio of 10:1); Injector temperature 250 °C; Ion source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min.), with an increase of 10 °C/min, to 200 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5seconds and fragments from 45 to 450 Da.

Total GC running time was 36 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas; software adapted to handle mass spectra, and chromatograms was a Turbomass [9].

Interpretation on mass spectrum GC-MS was conducted using the database of national Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight, and structure of the components of the test materials were ascertained. The results were shown in **Table 2.**

***In-vitro* Pharmacological Screening heat Induced Haemolytic Method:**

Preparation of Red Blood Cells (RBC's Suspension): Fresh whole human blood 10 ml was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline.

The reaction mixture 2 ml consisted of 1ml of the test sample solution and 1ml of 10% RBC's suspension, instead of test sample the only saline was added to the control test tube. Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in water bath at 56 °C for 30 min [10].

At the end of the incubation, the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min, and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percentage membrane stabilization activity was calculated using the formula:

$$\text{Percentage of inhibition} = 100 \times \{Vt - Vc - 1\}$$

Where, Vt = Optical density of test, Vc = Optical density of control

Inhibition of Albumin Denaturation: The reaction mixture (5 ml) consisted of 0.2 ml egg albumin from fresh hen's egg, 2.8 ml of phosphate buffered saline (PBS, pH 6.4) and 2 ml of varying concentrations of extract so that so that final concentrations become 50, 100, 200, 400 and 800 µgm/ml. A similar volume of double distilled water served as control. Then the mixtures were incubated at 37 ± 2°C in a BOD incubator for 15 mins and then heated at 70 °C for 5 min. After cooling the absorbance was measured at 660 nm (SHIMADZU, UV 1800) by using the vehicle as blank.

Diclofenac sodium at the final concentration of 50, 100, 200, 400 and 800 µgm/ml was used as a reference as reference drug and treated similarly for absorbance [11]. The percentage inhibition of protein

denaturation was calculated by using the following formula:

$$\text{Percentage inhibition} = 100 \times [Vt - Vc/1]$$

Where, Vt = absorbance of the test sample, Vc = absorbance of control

Anti-oxidant Screening:

DPPH Radical Scavenging Assay: The free radical scavenging activity was measured *in-vitro* by DPPH assay. About 0.3mM solution of DPPH in methanol was prepared, and 1ml of this solution was added to 1 ml of both the extract at different concentrations (100, 200, 300, 400 and 500 µg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min, and the absorbance was measured at 517 nm using a spectrophotometer. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation.

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/ A_O] \times 100$$

Whereas, A_0 is the absorbance of the control reaction mixture and A_1 is the absorbance of the plant extract or standard at different concentrations [12].

Super Oxide Dismutase: The assay of superoxide dismutase was done according to the method of Das. In this method, 1.4 ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04 ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100 µl of the sample extract and incubated at 30 ºC for 5 min. 80 µl of 50 µM riboflavin was added, and the tubes were exposed for 10 min to 200 W-Philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (a mixture of an equal volume of 1% sulphanilamide in 5% phosphoric acid) was added, and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions [13].

Nitric Oxide Radical Scavenging Activity: 3 ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (250- 1250 µg/ml). After the incubation time, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride in 2 % H_3PO_4) was added. The absorbance of the chromophore formed was measured at 546 nm. Percentage of radical scavenging activity was calculated [14].

$$\text{\% NO radical scavenging activity} = (\text{Control OD-Sample OD} / \text{Control OD}) \times 100$$

Polyphenol Oxidase Activity: Assay of Polyphenol oxidase activity was carried out according to the procedure of Sadasivam and Manickam [15]. To 2.0 ml of plant extract and 3.0 ml of distilled water added and mixed. 1.0 ml of catechol solution (0.4 mg/ml) added to the above solution, and the reactants were quickly mixed. The enzyme activity was measured as the change in absorbance/min at 490 nm.

Catalase Activity: Catalase activity was assayed by the method of Sinha [16]. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for the different period.

The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 min, and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

Statistical Analysis: Data analyzed using One Way Analysis of Variance (ANOVA, SPSS Version 16) and expressed as mean ± SEM and comparisons were done using Tukey's test as post-hoc. Difference between means was regarded as significant at $P<0.01$.

RESULTS: The percentage yield of the methanolic extract was 20% w/v of methanol. The phytochemical evaluation showed the presence of alkaloids, terpenoids, flavonoids, carbohydrates, tannins, phenolic compounds, saponins, and phytosterols.

Phytochemical estimation showed the presence of total phenolics 248.19 ± 4.84 mg of tannic acid equivalent per gram of the extract, flavonoids 123.47/0.19 mg of rutin equivalent per gram of the extract, tannin 57.12 ± 2.45 mg of gallic acid equivalent per gram of the extract and alkaloids 69.29 ± 1.84 mg atropine sulphate equivalent per gm of the extract. The results were shown in **Table 1**. Also, The FT-IR showed the presence of functional groups like amines, ester, alkyl and other carbonyl groups. The results were shown in **Table 2.** GC-MS result was shown in **Table 3.**

TABLE 1: ESTIMATION OF SECONDARY METABOLITES

Total Phenolics mg Tannic acid/g extract	Flavonoids mg Rutin/g extract	Tannins mg of Gallic acid/gm of extract	Alkaloids mg Atropine RE/g extract
248.19 ± 4.84	123.47 ± 0.19	57.12 ± 2.45	69.29 ± 1.84

Values are means of three independent analysis of the extract ± standard deviation (n=3)

TABLE 2: FT-IR ANALYSIS OF THE METHANOLIC EXTRACT *PUPALIA LAPPACEA*

S. no.	Peak value nm^{-1}	Functional group
1	3404.87	Amine group or amide
2	2969	Alkyl C-H stretch
3	1753	Ester C=O Stretch
4	1571	Amines bending
5	1480	Amines bending
6	1226	Amines stretching
7	1262	Amines stretching
8	1119	Amines stretching
9	829	ortho substitution
10	764	ortho substitution

TABLE 3: COMPONENTS DETECTED IN AERIAL PARTS OF *PUPALIA LAPPACEA*

S. no.	R.T	Compound name	Formula	Peak area %	M.W
1	6.908	Phenol	C_6H_6O	4.69	94
2	8.964	1-Chloroundecane	$C_{11}H_{23}Cl$	0.09	190
3	10.440	Napthalene	C10H8	0.02	128
4	12.601	p-tert-Butyl cyclohexyl-acetamide	$C_{12}H_{22}O_2$	0.05	198
5	13.101	4-tert-butylcyclohexyl acetamide	$C_{12}H_{22}O_2$	0.11	227
6	14.757	1-Dodecanamine, N,Nidimethyl	$C_{15}H_{33}N$	1.26	-
7	14.909	Phenol, 2,4-BIS(1,1Dimethane, N,N-Dimethyl-1-Pentadecane	$C_{14}H_{22}O$	1.57	206
8	17.128	Undecanal,2-methyl	$C_{17}H_{37}N$	0.78	255
9	17.208	Ethanol,2-(dodecycloxy)-	$C_{12}H_{24}O$	0.93	184
10	17.294	Tetradecanoic acid	$C_{14}H_{30}O_2$	0.57	230
11	17.446	Hexadecanoic acid, methyl ester	$C_9H_9NO_3SSi$	0.58	249
12	17.742	Cis-10-Heptadecinoic acid	$C_{14}H_{28}O_2$	0.72	228
13	19.469	Ascorbic acid	$C_{16}H_{30}O_2$	1.09	254
14	20.145	Hexadecanoic acid	$C_{18}H_{36}O_2$	1.66	284
15	20.200	Diethylene glycol monodocecyl ether	$C_{16}H_{34}O_3$	0.68	274
16	21.399	Octadecanoic acid	$C_{19}H_{38}O_2$	0.86	298
17	21.597	Octadec-9-enoic acid	$C^{18}H_{34}O$	8.21	282
18	21.633	14-Petadecenoic acid	$C_{15}H_{28}O_2$	5.04	240
19	21.805	Stearic acid, Octadenoic acid	$C_{18}H_{36}O_2$	9.96	284
20	22.011	Methyl 17-methyl octadecanoate	$C_{20}H_{40}O_2$	2.35	312
21	22.054	Hexatriacontane	$C_{36}H_{74}$	0.58	506
22	22.781	5,8,11,14-Eicosatetraenoic acid	$C_{21}H_{34}O_2$	1.01	318
23	23.307	11,14,17-Eicosapentanoic acid	$C_{20}H_{30}O_2$	8.62	
24	23.450	Propyl 5,8,11,14,17 eicosapentaenoate	$C_{23}H_{36}O_2$	1.11	344
25	24.161	Dotriacontane	$C_{32}H_{66}$	2.20	450
26	24.999	Methyl 4,7,10,13,16,19 docosahexaenoate	$C_{23}H_{34}O_2$	2.51	342
27	25.538	Hexatriacontane	$C_{36}H_{74}$	3.61	506
28	25.846	5,8,11,14,17-Eicosapentanoic acid	$C_{21}H_{32}O_2$	4.61	316
29	25.930	Butyl 4,7,10,13,16,19-docosahexaenoate	$C_{26}H_{40}O_2$	2.15	384
30	27.253	Dotriacontane	$C_{32}H_{66}$	2.89	450

31	31.667	Phenol	C_6H_6O	1.11	450
32	32.729	1-chloroundecane	$C_{11}H_{23}Cl$	0.08	190
33	34.080	Napthalene	$C_{10}H_8$	0.24	128
34	34.886	Tritiobutylcyclohexyl acetate	$C_{12}H_{22}O_2$	0.08	198

The extract was tested for its anti-inflammatory action in *in-vitro* by a heat-induced hemolytic method in RBC and protein inhibition by egg albumin denaturation method. The methanolic extract inhibited membrane stabilization effect by inhibiting hypotonic induced lysis of erythrocyte membrane. Stabilization of lysosomal membrane was important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil such as bacterial enzyme and proteases which cause further tissue inflammatory and damage [17].

It showed the maximum inhibitory of 68.02 at 800 µg/ml. Diclofenac sodium showed the maximum inhibitory of 79.93 at 100 µg/ml. Proteinases have been implicated in arthritis and inflammatory reactions. Neutrophils are known to be a rich source of proteinases. The results were shown in **Table 3**.

Also, in the egg albumin inhibitory method the extract produced dose-dependent inhibitory of protein denaturation. Denaturation of tissue protein was one of the well-documented causes of the inflammatory and arthritic diseases. The extract produced 110.00 of a maximum of protein denaturation at 800 µg/ml compared with the diclofenac sodium 190.00 at 800 µg/ml of concentration. The results were shown in **Table 4**. All the enzymes studied shown significant antioxidant activity *in-vitro* method as they were compared with the standard drug ascorbic acid. The results were shown in **Table 5-9**.

TABLE 4: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY IN HEAT INDUCED HAEMOLYTIC METHOD OF METHANOLIC EXTRACT OF *PUPALIA LAPPACEA*

S. no.	Extract	Concentration (µg/ml)	Absorbance	% Inhibition of denaturation
1	Control	--	0.080 ± 0.00	--
2	*Pupalia lappacea*	100	0.123 ± 0.00	26.38 ± 0.00
3	*Pupalia lappacea*	200	0.136 ± 0.00	38.67 ± 0.01
4	*Pupalia lappacea*	500	0.156 ± 0.00	66.63 ± 0.11
5	Diclofenac sodium	20	0.183 ± 0.00	89.49 ± 0.12

TABLE 5: *IN-VITRO* ANTI-INFLAMMATORY ACTIVITY BY INHIBITION OF ALBUMIN DENATURATION OF METHANOLIC EXTRACT OF *PUPALIA LAPPACEA*

S. no.	Extract concentration (µg/ml)	Absorbance (nm)	% Inhibition of denaturation
1	*Pupalia lappacea* 50	0.113 ± 0.003	47.7 ± 0.003
2	*Pupalia lappacea*100	0.203 ± 0.003	52.7 ± 0.333
3	*Pupalia lappacea* 200	0.467 ± 0.003	75.7 ± 0.333
4	*Pupalia lappacea* 400	0.373 ± 0.086	85.3 ± 0.333
5	*Pupalia lappacea* 800	0.597 ± 0.117	110 ± 0.500
6	Diclofenac sodium 50	0.183 ± 0.003	105 ± 0.333
7	Diclofenac sodium 100	0.313 ± 0.003	110 ± 0.333
8	Diclofenac sodium 200	0.533 ± 0.066	142 ± 0.333
9	Diclofenac sodium 400	0.587 ± 0.074	119 ± 0.333
10	Diclofenac sodium 800	0.620 ± 0.030	190 ± 40.2

TABLE 6: NITRIC OXIDE RADICAL SCAVENGING ASSAY

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Pupalia lappacea extract	100	13.12 ± 0.14	391.5 ± 0.04
	200	25.22 ± 0.55	
	300	39.22 ± 0.52	
	400	42.21 ± 1.22	
	500	63.85 ± 1.20	
Ascorbic acid	5	22.72 ± 1.01	11.42 ± 3.17
	10	43.78 ± 1.06	
	15	36.67 ± 0.52	

Note: TRV – Total reaction volume

TABLE 7: DPPH RADICAL SCAVENGING ACTIVITY OF *PUPALIA LAPPACEA* EXTRACT

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Pupalia lappacea extract	100	17.28 ± 1.23	416.01 ± 0.11
	200	26.55 ± 1.05	
	300	37.85 ± 0.52	
	400	44.65 ± 1.01	
	500	60.08 ± 0.89	
Ascorbic acid	5	22.72 ± 1.01	11.42 ± 3.17
	10	43.78 ± 1.06	
	15	36.67 ± 0.52	

Note: TRV – Total reaction volume

TABLE 8: SUPEROXIDE OXIDE RADICAL SCAVENGING ACTIVITY OF *PUPALIA LAPPACEA* EXTRACT

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Pupalia lappacea extract	100	18.03 ± 1.42	401.6 ± 0.16
	200	32.90 ± 1.53	
	300	41.23 ± 0.52	
	400	53.13 ± 1.25	
	500	62.25 ± 1.52	
Ascorbic acid	5	22.72 ± 1.01	11.42 ± 3.17
	10	43.78 ± 1.06	
	15	36.67 ± 0.52	

Note: TRV – Total reaction volume

TABLE 9: POLYPHENOL OXIDASE ACTIVITY OF *PUPALIA LAPPACEA* EXTRACT

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Pupalia lappacea extract	100	16.87 ± 1.04	363.2 ± 0.23
	200	27.67 ± 1.53	
	300	38.93 ± 1.52	
	400	54.18 ± 1.51	
	500	68.85 ± 1.64	
Ascorbic acid	5	22.72 ± 1.01	11.42 ± 3.17
	10	43.78 ± 1.06	
	15	36.67 ± 0.52	

Note: TRV – Total reaction volume

TABLE 10: CATALASE ACTIVITY OF *PUPALIA LAPPACEA* EXTRA

Sample	Concentration (µg)	Percentage activity (%)	IC_{50} (µg/TRV)
Pupalia lappacea extract	100	15.43 ± 1.34	398 ± 0.40
	200	26.70 ± 0.93	
	300	37.35 ± 1.42	
	400	50.33 ± 1.11	
	500	62.75 ± 1.84	
Ascorbic acid	5	22.72 ± 1.01	11.42 ± 3.17
	10	43.78 ± 1.06	
	15	36.67 ± 0.52	

Note: TRV – Total reaction volume

DISCUSSIONS: The enzyme phospholipase A_2 Is known to be responsible for the formation of mediators of inflammatory such as prostaglandins and leukotrienes which by attracting polymorphonuclear leucocytes to the site of inflammation would lead to tissue damage probably by the release of free radicals.

Phospholipase A_2 comments phospholipids in the cell membrane into arachidonic acid, which is highly reactive and is rapidly metabolized by cyclooxygenase to prostaglandins, which are major components that induce pain inflammation. Erythrocytes have been used as a model system by some scientists to investigate the interaction of drugs in the membranes.

The erythrocyte membrane resembles lysosomal membrane and as seen the erythrocyte could be extrapolated to the stabilization of lysosomal membrane [18]. The vitality of cells depends on the integrity of their membrane, exposure of RBC'S to

a major substance such as hypotonic medium results in lysis of its membrane accompanied by hemolysis and oxidation of hemoglobin. An injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation [19]. It was therefore that phytochemicals present in the extract stabilize the RBC membrane should offer significant protection of the cell membrane against injurious substances. Compounds with the stabilizing properties as well known for their ability to interfere with the release of phospholipases that trigger the formation of inflammatory mediators.

Moreover, it has also been shown that the deformability and cell volume of RBC's are closely related to the intercellular content of the calcium stabilization of lysosomal membrane is important in limiting the inflammation response by preventing the release of lysosomal constituents of activated neutrophil such as a bactericidal enzymes and kills the proteases, which cause further tissue inflammation and damage upon extracellular release [20]. The membrane stabilizing the activity of extract may be due to the presence of flavonoids, alkaloids, tannins and or saponins present in the extract. The extract prevented protein denaturation effect on egg albumin solution. Denaturation of proteins is a well-documented cause of inflammation in conditions like rheumatoid arthritis.

Thus, protection against protein denaturation which was the main mechanism of action NSAID's. Production of auto-antigen in certain rheumatic diseases may be due to *in-vitro* denaturation of proteins. Mechanism of denaturation probably involves an alteration in electrostatic, hydrogen, hydrophobic and disulfide bonding. Neutrophils contain many neutral serine proteinases in their lysosomal granules. Leukocytes proteinases play an important role in the development of tissue damage during inflammatory reactions, and a significant level of protection was provided by proteinases inhibitors [21].

The components present in the methanol extract of aerial parts of *Pupalia lappacea* were identified GC-MS analysis. The active principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (%) in the methanol extract of leaves of *Pupalia lappacea* were presented in Table. Thirty-eight compounds were identified in the methanol extract of *Pupalia lappacea* aerial parts. Plants are a natural source of biologically active compounds known as phytoconstituents [22, 23, 24]. Preliminary analysis of *Pupalia lappacea* for chemical constituents showed the presence of alkaloids, amino acids, glycosides, flavonoids, glycosides, saponins, tannins, starch, steroids, terpenoids, and coumarins. The prevailing compounds were ascorbic acid (23.36%), phenol (4.69%), 10-heptadecenoic acid (3.74%), octadec-9-enoic acid (8.21%) stearic acid (9.96), 11,14,17-Eicosa pentanoic acid (8.62) and 14-pentadecanoic acid respectively.

In the present study, the methanolic extract was allowed to run in the GC – MS for 34 minutes, nearly 38 compounds were detected in the extract within the specified time limit. Among the identified phytochemicals ascorbic acid [25], Hexadecanoic acid [26], phenol [27], 1-Chloroundecane, Hexatriacontane, and Stearic acid, octadecanoic acid compounds have antioxidant activity. Thus, this type of GC-MS analysis is the first step towards understanding the nature of active principles in this medicinal plant, and this type of study will be helpful for further detailed study.

CONCLUSION: Hence, the methanolic extract of *Pupalia lappacea* possesses membrane stabilization properties, limiting protein denaturation process and white blood cell anti-migration properties. Therefore, the extract leads to effective RBC membrane stabilization and protein inhibition, denaturation both contributing to invitro anti-inflammatory activity. Further, definitive studies were necessary to ascertain the mechanism and constituents behind its anti-inflammatory actions.

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