In vitro anti-inflammatory activities of *Loranthus micranthus* (Linn.) parasitic on *Azadirachta indica*

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Abstract - The present work was aimed to evaluate the *in vitro* anti-inflammatory activity of crude extract of *Loranthus micranthus* and their phytochemical analysis. We have used four different solvents for extraction; the methanol extract was yielded all the potent phytochemicals at high percentage except saponins and athraquinones. The methanol extract possesses enough potential to reduce inflammation by *in vitro* and directs the importance of further research and development of novel anti-inflammatory activity.

Index Terms- *Loranthus micranthus*, anti-inflammatory, membrane stabilization, proteinase inhibitory, albumin denaturation

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1. Introduction

Inflammation is a recurring state of cells and tissues as a self defence mechanism in response to external stimuli like pathogens, heat, injury, chemicals, and toxic substances or due to improper metabolic activities. Inflammatory response and damage upon extra cellular release is initiated by the release of lysosomal constituents, activated neutrophil, bactericidal enzymes and proteases (Murugasan, 1981). Even inflammation is self defence mechanism; it is necessory to control to prevent further cellular damages because of free radicals, denatured proteins and DNA, pus cells and other toxins released during inflammation. Many herbals were recognized as important sources of medicinal copmounds from past centuries in treating inflammation. Despite of evolution in synthetic drugs, there has been more prominance was given to herbal medicines due to their fewer side effects and multiple therapeutic activities.

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Loranthus micranthus Linn (Loranthaceae) is a hemiparasitic plant growing on a variety of host trees and shrubs (Ali *et al.*, 2005). It depends on its respective host for water and mineral nutrition, even though it produces its own carbohydrates through photosynthesis. It is used in folkloric medicine in the treatment of epilepsy, hypertension, headache, infertility, cancer and rheumatism (Griggs, 1991), and has been reported to have antidiabetic, antimicrobial (Osadebe and Ukwueze, 2004), immunomodulatory and antimotility activities (Osadebe and Uzochukwu, 2006) and antimotility assay (Osadebe *et al.*, 2012).

The present work was aimed to carry out anti-inflammatory activities of *Loranthus micranthus* in four different solvent extracts *viz.*, hexane, ethyl acetate, mathanol and distilled water by hypotonicity induced Human Red Blood Cell (HRBC) membrane stabilization, heat induced HRBC membrane stabilization, proteinase inhibitory activity and albumin denaturation methods.

2. MATERIALS AND METHODS

2.1.Collection and processing of plant

On the basis of present literature review and slight experimental modifications, the fresh leaves of *Loranthus micranthus* (L.f) Ettingsh (Loranthaceae) growing on the host plant *Azadirachta indica* were collected in the month of April, 2009 during the flowering period at DC Bunglow, Tumkur, Karnataka, India and identified using authenticated herbarium from the Department of Studies in Botany, University of Mysore, Mysore and Government Ayurvedic College, Mysore. The plant material was washed with distilled water, shade-air dried (26 ± 2^0C) and pulverized to a coarse powder in a mechanical grinder, passed through a 40 mesh sieve and stored in air tight container for further work.

2.2. Preparation of crude extracts

25 g/100ml of powdered leaf of *Loranthus micranthus* was kept for solvent extraction in rotary shaker at 37^0C, 72 rpm for 48 h. The solvents hexane, ethyl acetate, methanol and distilled water were used with increasing order of their polarity. The solvent extract was cetrifuged at 6000 rpm for 10 min and then filtered with Whatman No. 1 filter paper and evaporated at a constant temperature of 62^0C in hot air oven until a very concentrated extract was obtained.

2.3. Phytochemical analysis

Phytochemical screening was performed according to

the standard procedures and detected different phytoconstituents like carbohydrates, alkaloids, glycosides, saponins, phytosterols tannins, flavonoids, resins and proteins present in the plant extracts **(**Sofowora, 1993; Trease and Evans, 1989; Siddiqui and Ali, 1997).

2.4. Membrane stabilization test

2.4.1. Preparation of Red Blood cells (RBCs) suspension

Fresh whole human blood (5 mL) was collected and transferred to the centrifuged tubes containing Heparin or EDTA or Sodium citrate to prevent clotting. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline (Sadique *et al.*, 1989).

2.4.2. Hypotonicity induced human red blood cell (HRBC) membrane stabilization method (Gandhisan *et al*., 1991)

The reaction mixture consists of 1.0 mL of test sample of different concentrations (40µg – 200 µg) in 1 ml of 0.2 M phosphate buffer and 0.5 mL of 10% HRBC suspension, 0.5 ml of 0.25 % hyposaline were incubated at 37^0C for 30 min and centrifuged at 3,000 rpm for 20 min. and the hemoglobin content of the supernatant solution was estimated spectrophotometrically at 560 nm. Diclofenac was used as standard and a control was prepared by distilled water instead of hypo saline to produce 100 % hemolysis without plant extracts. The percentage of HRBC hemolysis and membrane stabilization or protection was calculated by using the following Formula:

% of Hemolysis = (Optical density of test sample / Optical density of control) X 100
% Protection = 100 – [(Optical density of test sample / Optical density of control) X 100]

2.4.3. Heat induced human red blood cell (HRBC) membrane stabilization method (Shinde *et al*., 1999)

The reaction mixture in heat induced hemolysis consists of 1.0 mL of test sample of different concentrations (40µg – 200 µg) in normal saline and 1.0 mL of 10% RBC suspension. Diclofenac sodium was taken as a standard drug. Control was prepared by distilled water instead of normal saline to produce 100 % hemolysis without plant extracts. All the tubes containing reaction mixture were incubated in a water bath at 56^0 C for 30 min. After 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. The percentage of HRBC hemolysis and membrane stabilization or protection was calculated by using the following Formula:

% of Hemolysis = (Optical density of test sample / Optical density of control) X 100
% Protection = 100 – [(Optical density of test sample / Optical density of control) X 100]

2.5. Proteinase inhibitory activity

Anti-inflammatory activity was performed according to the modified method of Oyedepo *et al.* (1995). The reaction mixture contains 1.0 mL of test sample of different concentrations (40µg – 200 µg), 0.5 mL of 20 mM Tris HCl buffer (pH 7.4) containing 0.06 mg trypsin, and the mixture was incubated at 37^{o}C for 5 min and then 0.5 mL of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. 1.0 mL of 70% perchloric acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 280 nm against buffer as blank. Diclofenac sodium was used as standard drug. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was determined.

Percentage inhibition = 100 - [(optical density of sample) / (optical density of control) × 100]

2.6. Albumin denaturation method (Dey *et al.,* 2011; Chandra *et al.,* 2012)

Antiinflammatory activity involves inhibition of albumin denaturation. The reaction mixture consists of 1.0 mL of distilled water containing varied concentrations of plant extracts or standard (80µg – 400 µg), 0.2 mL of .05 % BSA and 1.8 mL of 0.2 M phosphate buffered saline (pH 6.4). The mixtures were incubated at 37ºC for 15 minutes and then heated at 70ºC for 5 minutes. After cooling, the absorbance was measured spectrophotometrically at 660 nm against a blank. Diclofenac sodium was used as standard drug and the percentage inhibition of protein denaturation was calculated by using the following formula:

Relative % of inhibitory activity = (A- A_{min}) / A_{max} - A_{min}) x 100

Where,
A – Abs of Sample
A_{min} – Abs of control
A_{max} – Highest Abs of Standard
The experiment was performed in triplicate

3. Results

3.1.Phytochemical analysis

Phytochemical analysis of different solvents yielded

different phytochemicls (Table. 1). Compared to other solvents, the methanol extracts yielded well distinguishable phytochemical compounds and revealed the presence of carbohydrates, cardiac glycosides, proteins, amino acids, phytosterols, alkaloids, phenols, tannins and reducing sugars at higher rate. Whereas, the hexane and ethyl acetate extracts yielded less quantity of phytochemicals. More phenolic substances observed in methanol extract followed by aqueous extract.

Table 1: Phytochemical screening of different extracts from *L. micranthus* leaves

Phytochemicals	Extracts			
	Hexane	Ethyl Acetate	Methanol	Distilled water
Carbohydrate	+	+++	+++	+
Cardiac Glycosides	+	++	+++	+
Fixed oils and Fats	--	-	-	-
Proteins and Amino acids	-	-	++	++
Saponins	-	-	-	++
Phytosterol	+	++	+++	+
Alkaloids	-	++	+++	++
Flavonoids	-	+	+++	+++
Phenol	+	+	++	++
Tannins	+	+	++	++
Gums and mucilages	-	-	+	++
Anthraquinones	++	-	-	-
Reducing sugars	-	++	+	+

Note: (-): not detectable. (+): Low quantities. (++): average quantities. (+++): high quantities. Repeated the each experiment thrice

3.2.Hypotonicity induced HRBC membrane stabilization method

In hyposaline condition, red blood cells undergo exosmosis leads to either cell shrinkage or membrane rupture causing haemolysis. The extent of haemolysis indicates the state of memrane stabilization. As the decrease in haemolysis, percentage of memrane stabilization increases and the absornance which can be read at 560 nm.

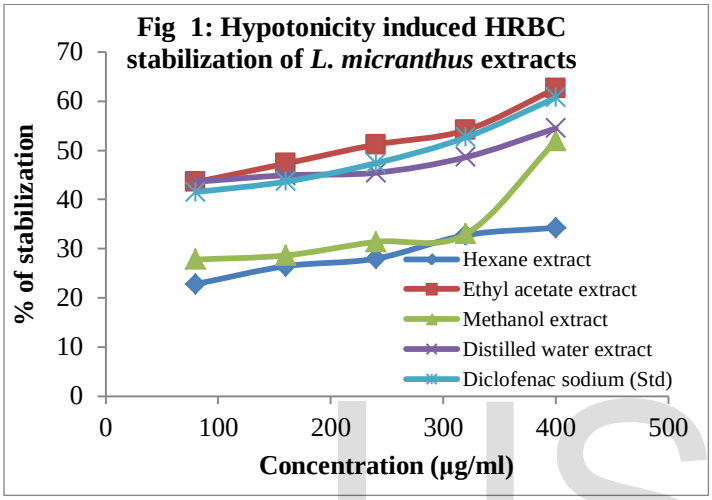
The percentage of hemolysis was found more in hexane extract (65.74 %) and it is further increased as concentration decreases. But ethyl acetate extract (37.44 %) and distilled water extract (45.52 %) were shown significant inhibition of hemolysis which in turn expressess the active membrane stabilization when compared to Diclofenac Sodium (39.28 %), the reference standard (Table. 2).

Table 2: Hypotonicity induced HRBC membrane stabilization method (% of hemolysis)

Concentration of Test / Std (µg)	% of hemolysis				
	Hexane extract	Ethyl acetate extract	Methanol extract	Distilled water extract	Diclofenac Sodium (Std)
80	77.202	56.461	72.209	56.387	58.480
160	73.568	52.679	71.365	55.066	56.350
240	72.026	48.825	68.612	54.552	52.606
320	67.254	45.888	67.033	51.395	47.356
400	65.748	37.444	48.091	45.521	39.280

*Repeated the experiment thrice

At final concentrations the percentage of membrane stabilization was found more in ethyl acetate extract (62.55 %) even efficient than the reference standard Diclofenac Sodium (60.71%). Hexane extract (34.25 %) was shown least stabilization activity (Fig. 1).



3.3.Heat induced HRBC membrane stabilization method

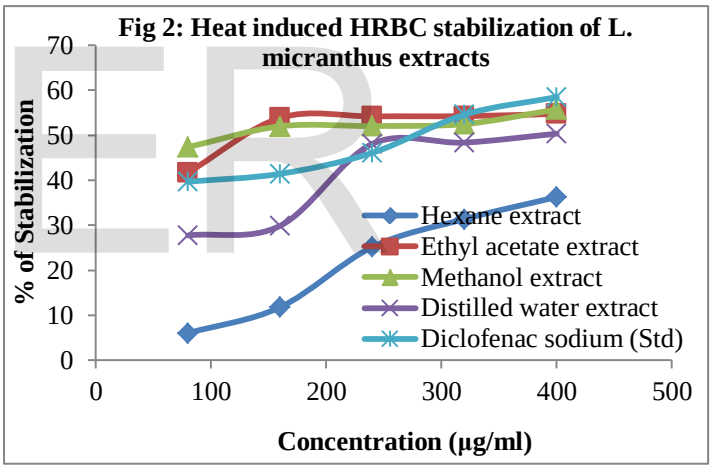
The percentage of hemolysis was found more in hexane extract (63.73 %) and it is further increased as concentration decreases. But methanol extract (44.28 %) and ethyl acetate extract (45.27 %) were shown significant inhibition of hemolysis when compared to Diclofenac sodium (41.53 %), the reference standard (Table. 3).

At final concentrations the percentage of membrane stabilization was found more in methanol extract (55.71 %) and ethyl acetate extract (54.72 %) when compared to reference standard Diclofenac sodium (58.46 %). hexane extract (36.26 %) was shown least stabilization activity (Fig. 2).

Table 3: Heat induced HRBC membrane stabilization method (% of hemolysis)

Concentration of Test / Std (µg)	% of hemolysis				
	Hexane extract	Ethyl acetate extract	Methanol extract	Distilled water extract	Diclofenac Sodium (Std)
80	93.956	58.296	52.637	72.252	60.329
160	88.186	46.153	48.021	70.109	58.571
240	74.835	45.824	47.967	51.923	53.901
320	68.626	45.769	47.527	51.648	45.439
400	63.736	45.274	44.285	49.670	41.538

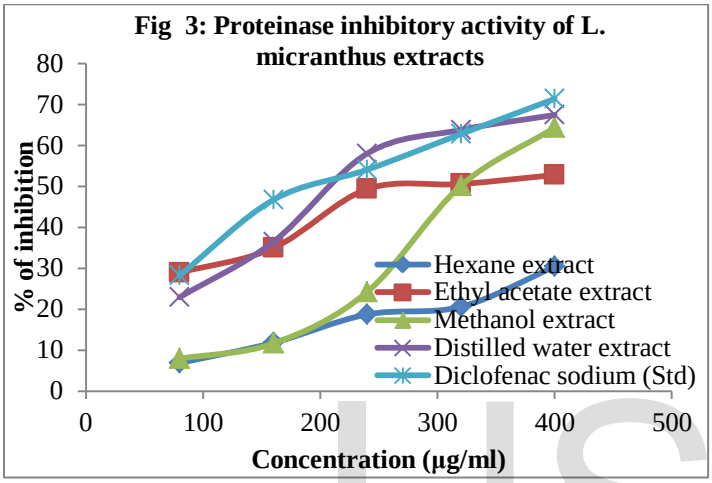
*Repeated the experiment thrice



3.4.Proteinase inhibitory activity

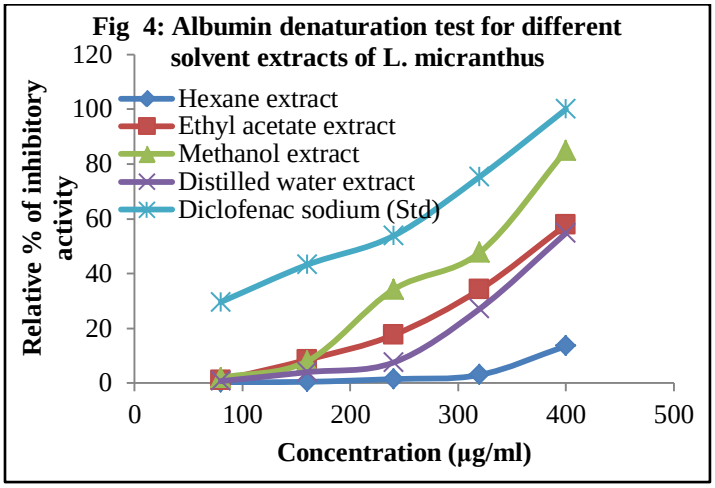
Trypsin is an endoprotease enzyme found in the pancreatic juice which catalyzes the hydrolysis of peptide bonds formed from the carboxyl groups of basic amino acids. The inhibition of trypsin activity was found to be dose dependent and shown more inhibitory activity in distilled water extract (67.43 %) and methanol extract (64.31 %). The inhibition shown

by reference standard Diclofenac sodium (71.37 %) was just higher than that of plant extracts. At final concentrations hexane extract (30.38 %) was shown least proteinase inhibitory activity compared to all extracts (Fig. 3).

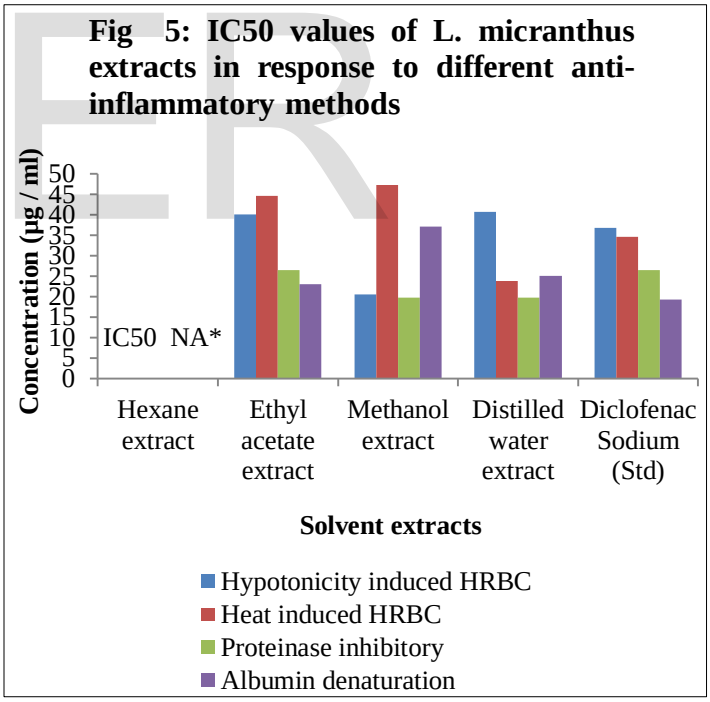


3.5.Albumin denaturation test

Anti-inflammatory activity involves inhibition of albumin denaturation by noncovalent change in the structure and loss of its solubility. The increased percentage of inhibition of albumin denaturation expressess the anti-inflammatory efficacy of plant extracts. Here the percentage of inhibition was carried out in realation with reference standard Diclofenac sodium. The realtive percentage of inhibition was significantly more in methanol extract (84.92 %) comapared to all other extracts. But hexane extract (13.56 %) was shown very least denaturation inhibitory activity (and Fig. 4).



3.6.IC_{50} values of *L. micranthus* extracts in response to different anti-inflammatory methods



*NA – 50 % inhibition not attained, repeated the experiment thrice

The half maximal (50 %) inhibitory concenatration has got more significance which shows the potency of drugs and helps in drug formulation and dose fixation.

The IC_{50} values of *L. micranthus* extracts in response to different anti-inflammatory methods were shown comparatively near values with few exptions except hexane extract. Hexane extract was failed to exhibit 50 % of effectiveness in all assays (and Fig. 5).

4. Discussion

Concentrating on the aims of preseent studies; the human red blood cell membrane is analogous to the lysosomal membrane (Chou, 1997) and its stabilization indicates the stabilization of lysosomal membranes. Stabilization of the membranes of these cells inhibits lysis and subsequent release of the cytoplasmic contents which in turn limits the tissue damage and exacerbation of the inflammatory response (Charles *et al.*, 2008). Exposure of red blood cells to hypotonic medium, heat, injurious substances such as methyl salicylate or phenylhydrazine results in the lysis of membranes accompanied by haemolysis and oxidation of haemoglobin (Feirrali *et al.*, 1992). At final concentrations the percentage of membrane stabilization by hypotonicity induced was found more in ethyl acetate extract (62.55 %) even efficient than the reference standard Diclofenac Sodium (60.71%). In heat induced, methanol extract (55.71 %) was shown significant effect when compared to reference standard Diclofenac sodium (58.46 %).

Trypsin is well recognized as an activator of Proteinase-Activated Receptor-2 (PAR2). Studies have demonstrated that trypsin and PAR-2 induces inflammatory responses through p65-NF-*k*B pathway in many cell types **(**Liang *et al.*, 2011) and via the production of cytokines, such as IL-6, IL-8, and prostaglandin (Asokananthan *et al.*, 2002; Johansson *et al.*, 2005). PAR-2 activation results in proinflammatory effects including vasodilatation, edema, reflux esophagitis, leukocyte-endothelial interactions and damage the lining of gastrointestinal tract (Busso *et al.*, 2007; Saifeddine., 1996; Georgie *et al.*, 2007). Even trypsin plays an important benificial role in gastro intetstinal tract system, it is necessory to control the trypsin activity in an unconditional events. In our studies trypsin inhibitory activity was dose dependent and shown comparable effect in distilled water extract (67.43 %) and methanol extract (64.31 %) when compared to reference standard Diclofenac sodium (71.37 %).

Maintenance of structural heirachy of proteains by cell system is necessory for proper functioning of metobolic activities. In humans, albumin is the most

abundant plasma protein, accounting for 55–60% of the measured serum protein (Gosling, 1995). Modification of the amino, carboxyl or disulfide groups of the albumin may results in its deanturation (Norman *et al.,* 1971). Denaturation of protein constituents and intercellular fluid is a well referable cause of inflammation in rheumatoid arthritis (Mizushima *et al.,* 1964). Extracellular proteases from plasma transudate infiltrating leukocytes, chondrocytes and synovial cells may degrade albumin (Barnnart *et al.,* 1968) and it may be possible in conditions like tissue injury, redox reactions due to free radicles, activation of trypsin induced proteolysis, heat, chemicals, antigens and by metabolic impairments. Chronic inflammation leads to cardiovascular disease, cancer, diabetes, degenerative joint diseases and neurodegenerative diseases (Lucas *et al.,* 2011). Relatively methanol extract (84.92 %) has inhibited albumin denaturation and can be choosen for further optimal studies by purification.

IC_{50} values of *L. micranthus* extracts were very less and few crude extracts were shown even lesser IC_{50} values than the reference standard towords some assays. The effective crude extracts can be concetrated more on *in vivo* anti-inflammatory studies with purification.

5. Conclusion

Phytochemical and *in vitro* anti-inflammatory studies of *Loranthus micranthus* were shown good results. Among hexane, ethyl acetate, methanol and distilled water, methanol was the good choice as a solvent in extraction of well distinguishable phytochemicals. Methanol extract has shown comparatively significant anti-inflammatory effects for all anti-inflammatory tests. It can be considered in future for optimal studies and also for isolation and identification of the exact anti-inflammatory phytochemical in *Loranthus micranthus.*

6. Acknowledgement

7. References

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