

COMPARATIVE PHYTOCHEMICAL INVESTIGATION AND EVALUATION OF *IN VITRO* ANTI-INFLAMMATORY ACTIVITY OF DIFFERENT HERBAL EXTRACTS

*Guttikonda Udaya, ¹Vemula Yamini, ²Sk. Moheeth and ³Mallam Salma

*Assistant Professor, Dept. of Pharmacognosy, Narayana Pharmacy College, Nellore, Andhra Pradesh-524003.

^{1,2,3}Student, Final B. Pharm, Narayana Pharmacy College, Nellore, Andhra Pradesh-524003.

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***Corresponding Author: Guttikonda Udaya**

Assistant Professor, Dept. of Pharmacognosy, Narayana Pharmacy College, Nellore, Andhra Pradesh-524003.

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ABSTRACT

In Ayurveda, many natural plant compounds are used to inhibit inflammatory pathways for centuries with fewer side effects. Inflammation is a defense mechanism that enables the body to protect itself against infection, burn, toxic chemical allergens or any other harmful stimuli. Denaturation of protein causes production of auto-antigens in conditions such as rheumatoid arthritis, cancer and diabetes which are conditions of inflammation. Hence, by inhibition of protein denaturation, inflammatory activity can also be inhibited. Egg albumin method provides a cheap alternative method of testing the anti-inflammatory activity of herbal medicine. Hence in the present study different aqueous extracts, from plants- *Lantana camara* (leaf), *Tagetes erecta* (flower), *Azardirachta indica* (leaf) and *Muntingia calabura* (leaf) and Hydroalcoholic extract from *Calotropis procera* (leaf) were prepared and evaluated for presence of various phytochemicals and in vitro anti-inflammatory activity. Aqueous extract of *Lantana camara* showed the presence of carbohydrates, proteins, Tannins, phenols, flavonoids and glycosides. Aqueous extract of *Azardirachta indica* exhibited the presence of carbohydrates, proteins, alkaloids, Tannins, phenols and glycosides whereas *Muntingia calabura*, *Tagetes erecta* and *Calotropis procera* were reported to contain all these phytoconstituents. In the present investigation, two types of drugs-NSAID-Ibuprofen and steroid-Prednisolone were used as reference. Concluding the results, among all the extracts tested for activity the highest % inhibition rates were observed for Neem extracts followed by Calotropis and Marigold at a concentration of 1000µg/ml. Extracts exhibited % inhibition in a dose dependent manner. The incorporation of these three extracts in the polyherbal formulation could be a novel treatment strategy for various inflammatory ailments.

KEYWORDS: Denaturation, in vitro, Anti-inflammatory, Egg Albumin, Phytochemicals.

I. INTRODUCTION

Ancient texts form the highly esteemed source of traditional medicine and are an asset to the mankind. Traditional medicines are a divine and versatile gift from nature to the human to counteract and alleviate various ailments leading to the resurgence of interest of the investigators in the drug discovery and development of Natural products.

In Ayurveda, many natural plant compounds are used to inhibit inflammatory pathways for centuries with fewer side effects. Inflammation is a defense mechanism that enables the body to protect itself against infection, burn, toxic chemical allergens, or any other harmful stimuli. Inflammation is a substantial reaction to damage, disease or destruction portrayed by heat, redness, pain, swelling and disturbed physiological functions.^[1,2,3]

Phytochemicals from different secondary metabolites category viz. flavonoids, terpenoids, poly phenols, saponins, tannins, etc. have been reported from different medicinal plants to possess Anti – inflammatory activity Preliminary Phytochemical studies indicate the phytoconstituents present in the extracts. Phytochemical methods mainly involve Extraction, Isolation and Purification of the active constituents from the extracts.

Most common techniques employed for the extraction includes Maceration, Decoction, Infusion both cold and hot and Soxhlet /hot continuous percolation.

Various solvents would be employed to extract the different phytoconstituents, depending on the polarity and solubility of the phytoconstituent the appropriate solvent would be selected for the extraction process. Some phytoconstituents may or maynot exhibit the pharmacological activity when tested as an isolated compound when compared to the mixture of compounds in the plant extract owing to the synergistic nature of the certain phytoconstituents.^[4]

Inflammation is a defense mechanism that enables the body to protect itself against infection, burn, toxic chemical allergens or any other harmful stimuli. Inflammation is a substantial reaction to damage, disease or destruction portrayed by heat, redness, pain, swelling and disturbed physiological functions. When an invader (like a virus) tries to enter your body, or you get injured, your immune system sends out its first responders. These are inflammatory cells and cytokines (substances that stimulate more inflammatory cells). These cells begin an inflammatory response to trap germs or toxins and start healing injured tissue. Inflammation is a normal and important process that allows your body to heal. There are two main types of inflammation: acute and chronic.^[5,6]

Denaturation of protein has an unpredictable mechanism which includes modification in electrostatic hydrogen, hydrophobic and disulfide bonding.^[13] Protein denaturation is defined as a process wherein due to external factors such as heat, strong acid or strong base; an organic solvent or a concentrated inorganic salt causes the protein to denature that means the protein's tertiary structure and secondary structure is disoriented.^[7,8] Enzymes lose their activity since the substrates are able to no longer attach to the active site.^[4] Denaturation of protein causes production of auto-antigens in conditions such as rheumatic arthritis, cancer and diabetes which are conditions of inflammation as mentioned above. Hence, by inhibition of protein denaturation, inflammatory activity can also be inhibited. Egg albumin method provides a cheap alternative method of testing the anti-inflammatory activity of herbal medicine using denaturation technique.^[9,10]

NSAIDs has accounted for prevention of the protein denaturation, which acts as antigens and prompts autoimmune diseases.^[11] Conventional treatment for the inflammation includes NSAIDs, and opioids. NSAIDs act by the reduction of pain & inflammation by the inhibition of the metabolism of Arachidonic acid by COX (cyclo oxygenase enzyme

COX-1 and COX-2), hence decreasing the prostaglandin production but by the usage of medicinal plants possessing the anti-inflammatory activity these side effects can be very minimal or completely absent.

Most of the drugs from these classes produce side effects like kidney damage, gastrointestinal disturbances, and respiratory depression. These drugs contain several adverse effects, particularly gastric irritation prompting the development of gastric ulcers.^[12,13]

Ibuprofen is a nonselective NSAID which is a derivative of propionic acid and it is prescribed as an analgesic, anti-inflammatory and antipyretic agent. Prednisolone is considered as oral steroid utilized as an alternative because of its mitigating impact, especially in asthma and allergic condition and infections.^[10]

Some of the in vitro anti-inflammatory activity methods include Egg Albumin protein denaturation, Anti-proteinase and Anti-lipoxygenase activities, Human red blood cell (HRBC) membrane stabilization method.^[14,15] Among these methods Egg albumin protein denaturation method is one of the easily affordable and accessible method and hence used in the present study.

Flavonoids play a significant role in the inhibition of inflammation that occurs by inhibition of both capillary permeability and Arachidonic acid metabolism so that the production of prostaglandins is reduced.

Flavonoids are known to reduce the secretion of lysosomal enzymes which are mediators of inflammation and inhibit phosphodiesterase, aldol reductase, monoamine oxidase, protein kinase, DNA polymerase and lipoxygenase.

Tannins are known for their anti-inflammatory, astringent, anti diarrheal, antiseptic and diuretic properties. . Tannins are known for their anti-oxidant activity which in turn act as anti-inflammatory agents in many ways viz. by inhibition of production of oxidants by neutrophils, monocytes, macrophages which in turn reduces the formation of hydrogen peroxide which results in the production of hypochlorous acid and hydroxyl group being inhibited.

Saponins act as cleansers and antiseptics that helps to kill or prevent the growth of micro-organism and also helps in the formation of collagen, a structural protein having a profound role in the wound healing process. Anti-inflammatory action of saponins is to inhibit the increase in the vascular permeability.

Inhibition of these inflammatory mediators decrease the proliferation of the inflammatory process. They inhibit directly reactive oxidants like hydroxyl radicals and hypochlorous acid.^[1]

Hence in the present study different aqueous extracts from plants- *Lantana camara* (leaf), *Calendula officinalis* (flower), *Azadirachta indica* (leaf) and *Muntingia calabura* (leaf) and Hydroalcoholic extract from *Calotropis procera* (leaf) were prepared and evaluated for presence of various phytochemicals and *in vitro* anti-inflammatory activity.

II. MATERIALS AND METHODS

2. 1 Collection and Authentication of Plant Material

The leaves of *Lantana camara*, *Azadirachta indica*, *Muntingia calabura*, *Calotropis procera* and flowers of *Tagetes erecta* were collected from local surrounding areas and authenticated by Mr. G. Prabhakar, Botanist, TNC Govt. Junior College, Kovur, Nellore district.

2. 2 Chemicals used

Ethanol, H₂SO₄, HCl, lead acetate, sodium chloride, sodium mono phosphate, sodium ortho phosphate dibasic and other chemicals for preliminary phytochemical screening etc.

2.3 Preparation of Extracts by different extraction techniques

2.3.1 *Lantana camara* by decoction

50g of crude powder was boiled in 500ml of distilled water and then filtered, the filtrate was then concentrated to get the crude extract.

2.3.2 *Azadirachta indica* by hot infusion

50 g of crude powder was soaked in 500ml hot water 3-4 hr and then filtered, the filtrate was then concentrated to get the crude extract.

2. 3. 3 *Muntingia calabura* by maceration

50g of crude powder was soaked in 500ml of cold water and put aside for 24hr and then filtered, the filtrate was then concentrated to get the crude extract.

2. 3. 4 *Tagetes erecta* by maceration

50 g of crude powder was macerated in 500ml of water and put aside for 24hr and then filter.

2.3.5 *Calotropis Procera* by Soxhletion

50g of crude powder was taken for soxhletion by using 70% ethanol to get hydro-alcoholic extract



Fig. 1: Photo gallery of Extracts prepared by different Extraction techniques.



Calotropis Hydroalcoholic Ext.by Soxhlation

2.4 Qualitative Phytochemical Investigation

The obtained extracts were subjected to phytochemical tests to detect the presence of various phytoconstituents viz. Carbohydrates, Proteins, Alkaloids, Tannins and Phenols, Flavonoids and Glycosides.^[16]

2.5 *In vitro* anti-inflammatory activity by egg albumin protein denaturation assay

- Incubate the reaction mixture for 30 min at $37 \pm 2^{\circ}\text{C}$ and then heated on water bath for 15 min at $70 \pm 2^{\circ}\text{C}$.
- Then allow it cool, after cooling measure the absorbance at 280 nm by using suitable uv visible spectrophotometry,
- Here we using distilled water as blank.^[2]
- And then measure the absorbance of the each denaturated concentrations (1000 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$) each one test is repeated three time and then noted the absorbance.
- The percentage of protein denaturation inhibition was analysed by using percentage basis with respect to control by using following formula.^[3]

2.5.1 Formula

$$\text{Percentage inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test} \times 100}{\text{Absorbance of control}}$$

2.5.2 Preparation of Reference drug

NSAID (Ibuprofen) and one steroid (Prednisolone) were used as standard drugs for this study. Here Prednisolone was blended into a fine powder. Weighed accurately 0.2 g of Prednisolone by using a digital balance and dissolved in 20ml of distilled water, respectively. The solution was dissolved perfectly by using vortex and same procedure was used for preparation of Ibuprofen standard solution.^[1]

2.5.3 Preparation of Phosphate Buffer

Take 800ml of distilled water dissolve 20.21g of sodium phosphate dibasic and then add 3.3 g of sodium phosphate monobasic and make up to 1000ml with distilled water.

2.5.4 Preparation of Egg Albumin solution

The egg albumin solution was prepared by using egg albumin powder or hen's egg, the egg albumin powder is readily available in stores. Take 2g of egg albumin powder in 100ml of w/v distilled water in a 250 ml beaker and boil on water bath and the kept under the sonicator until a clear solution appears.^[3]

2.5.5 Preparation of Blank

The distilled water was used as blank for this experiment.^[2]

III. RESULTS AND DISCUSSION

The percentage yield of the drug accounts for the quantity of different phytoconstituents present in the extracts. The study of the nature of the extract gives an approximate idea of the various phytoconstituents that may be present in the extract.

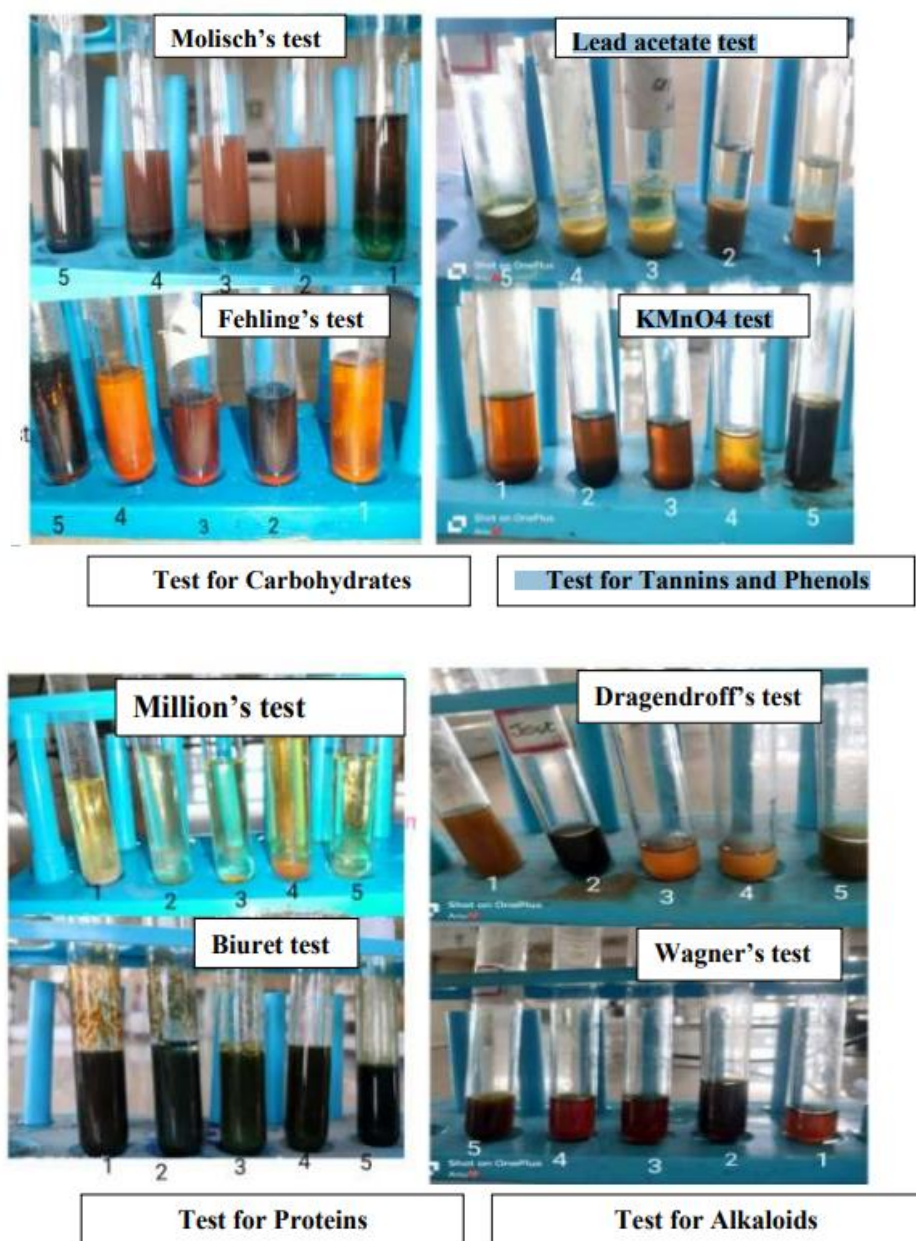
The Percentage yield of different herbal extracts along with the nature and colour of the extract is summarised in the table below.

Table 1: Percentage yield of different extracts and its nature.

Name of the Plant	Part of the Plant	Solvent	% Yield (%w/w)	Colour and nature of extract
<i>Lantana camara</i>	Leaf	Water	11.26%	Black and sticky substance
<i>Azadirachta indica</i>	Leaf	Water	23.16%	Dark brown and sticky
<i>Muntinga calabura</i>	Leaf	Water	16.58%	Dark green and sticky
<i>Tagetes erecta</i>	Flower	Water	23.00%	Dark brown and viscous
<i>Calotropis procera</i>	Leaf	70% Ethanol	11.48%	Dark green and viscous

Table 2: Qualitative Phytochemical screening of different Herbal extracts.

S. No.	Tests	Different Herbal extracts				
		<i>Lantana camara</i>	<i>Azadirachta indica</i>	<i>Muntinga calabura</i>	<i>Tagetes erecta</i>	<i>Calotropis procera</i>
1.	Carbohydrates					
	Molisch's	+	+	+	+	+
	Fehling's	+	+	+	+	+
2.	Proteins					
	Million's	+	+	+	+	+
	Biuret test	+	+	+	+	+
3.	Alkaloids					
	Dragendroff's	-	+	+	+	+
	Wagner's	-	+	+	+	+
4.	Tannins and Phenols					
	Lead acetate	+	+	+	+	+
	KMnO ₄ test	+	+	+	+	+
5.	Flavanoids					
	Sulphuric acid	+	-	+	+	+
	KMnO ₄ test	+	-	+	+	+
6.	Glycosides					
	Foam test	+	+	+	+	+
	Bromine water	+	+	+	+	+



1-Marigold extract, 2-Neem extract, 3- Mutingia extract, 4-Lantana extract, 5- Calotropis extract.

Fig. 2: Photogallery of Phytochemical analysis of different Herbal extracts.

Aqueous extract of *Lantana camara* showed the presence of carbohydrates, proteins, Tannins, phenols, flavonoids and glycosides and absence of alkaloids. Aqueous extract of *Azardirachta indica* exhibited the presence of carbohydrates, proteins, alkaloids, Tannins, phenols and glycosides and absence of flavonoids. Whereas *Mutingia calabura*, *Tagetes erecta* and *Calotropis procera* were reported to contain all these phytoconstituents. These results co-relate with the previously reported phytoconstituents.^[1,2,17,18, 19, 20]

In vitro anti-inflammatory activity by egg albumin protein denaturation assay: The *in vitro* anti-inflammatory effects of different herbal extracts was investigated against the egg albumin protein denaturation. Denaturation of protein leads to the generation of auto-antigens in ailments like Rheumatoid arthritis, Diabetes, Cancer which are some of the ailments associated with inflammation. Denaturation may lead to either modification in electrostatic hydrogen, hydrophobic and

disulfide bonding which is highly unpredictable hence by the inhibition of the denaturation of protein the inflammatory activity can be inhibited.^[22,23] In the present investigation, two types of drugs-NSAID-Ibuprofen and steroid Prednisolone are used as reference. NSAIDS are known to prevent inflammation by the inhibition of the COX enzyme activity. Prednisolone act as anti-inflammatory agent by suppressing the migration of the polymorphonuclear leucocytes and decreases the increased capillary permeability. It works by blocking the immune response to inflammation. These categories of drugs causes adverse effects of ulcer formation, perforation, haemorrhage and obstruction.^[24]

Table 3: % Inhibition of the Denaturation of protein for Reference Drugs.

Name of Reference drug	% inhibition at Concentration (µg/ml)				
	1000	100	10	1	0.1
Prednisolone	16.32%	12.68%	11.71%	11.09%	9.89%
Ibuprofen	14.86%	11.06%	10.12%	6.76%	5.43%

Table 4: % inhibition of the denaturation of protein for various herbal extracts.

Name of the Plant	Extract	Part of the Plant	% inhibition at Concentration (µg/ml)				
			1000	100	10	1	0.1
<i>Lantana camara</i>	Aqueous	Leaf	35.23%	29.67%	16.23%	12.31%	8.32%
<i>Azarditcha indica</i>	Aqueous	Leaf	45.43%	40.21%	29.81%	15.47%	9.38%
<i>Mutinga calabura</i>	Aqueous	Leaf	33.85%	25.21%	11.82%	8.25%	6.89%
<i>Tagetes erecta</i>	Aqueous	Flower	42.69%	58.9%	28.78%	14.78%	8.61%
<i>Calotropis procera</i>	Hydro-alcoholic	Leaf	43.62%	45.67%	30.62%	18.24%	9.12%

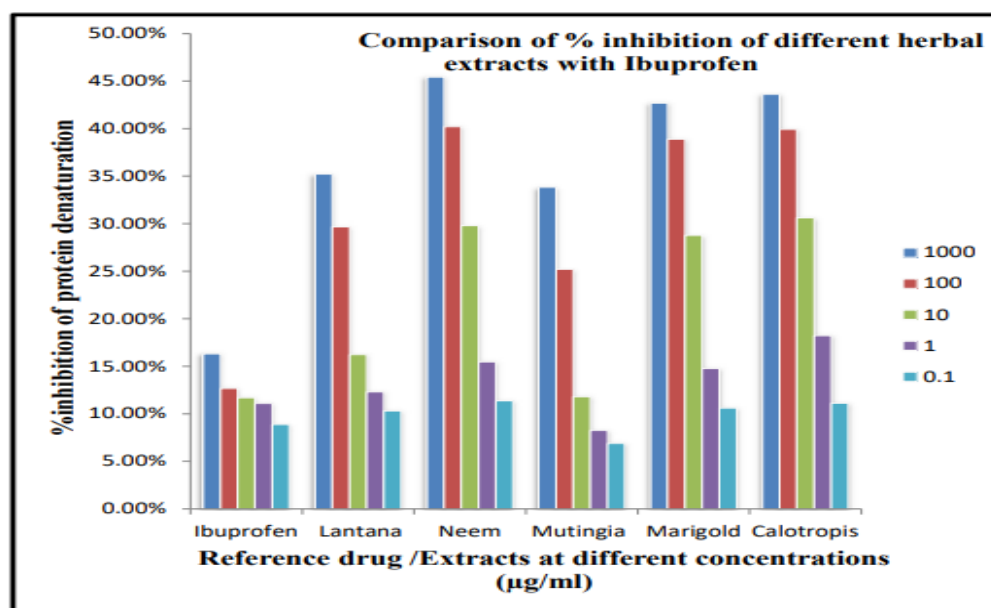


Figure 3: Comparison of % Inhibition of Different Herbal Extracts with Ibuprofen.

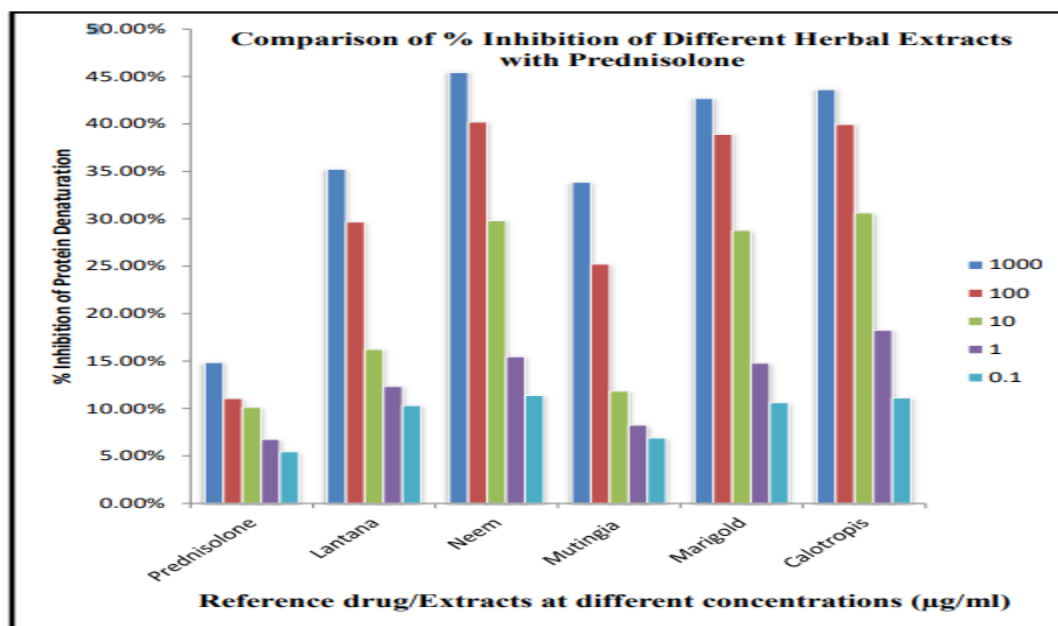


Figure 4: Comparison of % Inhibition of Different Herbal Extracts with Prednisolone.

in vitro anti-inflammatory activity of different herbal extracts was investigated by the egg albumin protein denaturation inhibition assay. The results infer a concentration dependent inhibition of egg albumin denaturation by different herbal extracts. The highest inhibition rate was observed for Neem extracts at the concentration of 1000 µg/ml. There was significantly higher inhibition of denaturation of protein in extracts of Calotropis and Marigold when compared to Muntingia and Lantana at 1000 µg/ml. When compared to the aqueous extracts and hydro-alcoholic extracts, the inhibition rates of the two reference drugs were found to be low and was found to be dependent on concentration. The enhanced absorbance in extracts and standard drug indicates the protein stabilizing activity that implies that denaturation is inhibited with the increasing concentration or dose of the extract. The Neem extracts exhibited the albumin denaturation in a dose dependent manner of 9.38% at lowest dose to 45.43% at highest dose. Similarly, Calotropis extract exhibited 9.12% at lowest dose to 43.62% at highest dose and Marigold exhibited 8.61% at lowest dose to 42.69% at highest dose. Concluding the results, among all the extracts tested for activity the highest inhibition rates were observed for Neem extracts followed by Calotropis and Marigold at a concentration of 1000µg/ml. These findings about the % inhibition of various herbal extracts in this study agrees with previous reports.^[1,17,19,20,24]

IV. CONCLUSION AND FUTURE PERSPECTIVE

Inflammation is known to be associated with certain processes viz. enhanced Protein denaturation, increase in the vascular permeability and rearrangement of the membranes which is the cause of the discomfort during inflammation. Therefore the present investigation has been focussed on the comparative exploration of the various phytoconstituents present in various herbal extracts along with the anti-inflammatory activity exhibited by the respective extracts when compared to the standard drugs.

The phytoconstituents mostly responsible for the anti-inflammatory activity include Phenols, Tannins, Flavonoids, etc., the extracts screened for the phytochemical analysis were found to contain them in Lantana, Neem, Muntingia, Marigold and Calotropis.

The herbal extracts of Neem leaf, Marigold flower and hydroalcoholic extract of Calotropis leaf exhibited higher anti-inflammatory activity in the egg albumin denaturation method and were found to be more potent than that of reference drugs. Egg albumin is one of the most affordable tests to check the anti-inflammatory activity, however this needs further validation by other methods. Other in vitro tests like membrane stabilization and in vivo studies using carrageenan-induced paw edema can be performed on various concentrations of these extracts to establish their effectiveness.

The findings of the study provides valuable insights about the therapeutic activity of these three extracts among the five tested for the activity and has an tremendous potential as the natural remedy for the ailments associated with the inflammation. The incorporation of these three extracts in the polyherbal formulation could be a novel treatment strategy with no or minimal adverse effects in contrast to those reported by their synthetic counterparts.

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