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"Evaluation of Anti-inflammatory activity of *poly*herbal extract of *Ficus microcarpa and Adina cordifolia* by Human red blood cell membrane stabilization method"

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Abstract

Polyherbal extracts of Ficus microcarpa and Adina cordifolia were tested for their anti-inflammatory effects in this investigation. The in-vitro anti-inflammatory effect of a polyherbal ethyl acetate extract was studied using a technique that stabilises the membranes of human red blood cells. In a study using four different concentrations of extract, researchers found that HRBC membrane stabilisation methods yielded varying degrees of protection: 30% at 1 mg/ml, 42.8% at 2 mg/ml, 54.0% at 4 mg/ml, and 67.6% at 6 mg/ml in a hypotonic solution. Comparing the results of the conventional medication Indomethacin, which protected HRBC in hypotonic solution by 69.6%, to those of the polyherbal ethyl acetate extract reveals a substantial membrane stabilising effect on HRBC. Polyherbal extract may include one or more phytochemical components that are responsible for the action.

INTRODUCTION

The complicated process of inflammation includes events including increased vascular permeability, increased protein denaturation, and membrane change; it is often accompanied by discomfort. Cells in the body experience stress when they are injured by pathogens, physical agents, or chemical substances. The body's reaction to stress causes tissue inflammation. The damaged region will become red, painful, hot, swollen, and functionally impaired as a protective reaction. A tissue's reaction to an unintentional cut is comparable to that of other types of tissue injury, such as burns produced by heat, radiation, germs, or viruses, since inflammation is one of the body's nonspecific internal defence mechanisms.[1] Any potentially dangerous substances that enter the body are either neutralised or destroyed by inflammation. It initiates a cascade of

biological processes that repair the harm. A wide variety of immunological responses, infections, burns, and trauma are the leading causes of inflammation.

Enzyme activation, mediator release, cell migration, fluid extravasations, tissue breakdown, and healing are all components of the inflammatory response, which is often initiated in response to illness (Vane et al., 1995). There is a lot of interest right now in finding medicinal plants that have anti-inflammatory properties; this could lead to the development of a new therapeutic agent that can treat a wide range of diseases where inflammation plays a role in exacerbating symptoms.

Painful and unpleasant inflammations are common. However, rather of relying on chemical medications to alleviate inflammation, it is recommended to use natural therapies. This is due to the fact that, similar to fever, they really indicate that the body is attempting to recover from an issue, like an infection. Inflammation occurs when the immune system despatches extra "combat forces" to a region in an effort to "fight the enemies," if you will. When we try to reduce inflammation using synthetic and chemical methods, we are really hindering the body's natural healing processes. This is a concern with chemical medications in general and with anti-inflammatory pharmaceuticals in particular; the goal should not be to cure symptoms but rather to address the underlying causes and bring about complete and lasting recovery. This research set out to determine, using the HRBC assay, how effective a polyherbal extract of *Ficus microcarpa and Adina cordifolia* was in reducing inflammation in vitro.

Collection of plant material

Both the plants were collected from the near around Lucknow city and authenticated by Dr. Lal Bahadur, Deputy Director, CSIR-National Botanical Research Institute, University in Lucknow, Uttar Pradesh; voucher specimen was deposited in our department.

Preparation of plant powder

After being air-dried in the shade, the plants were mechanically crushed to a coarse consistency. After thoroughly mixing, the powder was filtered through a No. 40 sieve and stored in an airtight container.

Physico-Chemical Analysis

The powdered plant material was subjected to standard procedure for the determination of various physicochemical parameters.

Determination of ash values

The goal of measuring ash levels is to identify low-quality goods, exhausted pharmaceuticals, and earthy or sandy materials. Chemical components may potentially be identified by using water-soluble ash and acid-insoluble ash.

Total ash value

After being air-dried, about 3 grammes of the powder were placed in a tared silica crucible. They were then burned at a temperature of no more than 450 degrees Celsius until the mixture was carbon-free. After cooling, the weight was measured.

After weighing the air-dried powdered medicine, the percentage of total ash was measured. It was calculated what percentage of total ash was attributable to the air-dried drug.

Acid insoluble ash

The ash produced in the preceding procedure was heated with 25ml of diluted HCl for 5 minutes. On ash-free filter paper, the residue was collected, cleaned with hot water, ignited, and weighed. The proportion of acid-insoluble ash was estimated using the air-dried medication as a reference.

Water soluble ash

Whole ash was heated for five minutes with 25 millilitres of water. The insoluble debris was collected on ash-free filter paper, rinsed with hot water, and burned at a low temperature to maintain a consistent weight. Insoluble materials was eliminated from the ash's weight. The weight difference shows the water-soluble ash. The proportion of water-soluble ash in the air-dried medication was determined.

Determination of moisture content (Loss on drying)

After correctly weighing about 10 g of medication (without preparatory drying), it was put in a tared evaporating dish and heated at 1050 degrees Celsius for five hours before being weighed. The drying loss percentage relative to the air-dried medication was determined.

Determination of foreign organic matter

A thin layer of 100 g of accurately-weighed drug sample was spread out. The foreign object was found via a naked-eye examination with the use of a lens (6X). Foreign matter was separated and weighed, and the proportion present was computed.

Determination of swelling index

The presence of mucilage is evaluated to calculate the swelling index. In a 150 ml measuring cylinder, 1 g of the powdered plant portion was accurately weighed. This was then mixed with 50 ml of distilled water and set aside for 24 hours with periodic shaking. The volume of the seeds after 24 hours of soaking was determined.

Preparation of extracts:

Soxhlation was applied to about 250 grammes of polyherbal powder. After a Soxhlet defatting step using petroleum ether, it underwent a solvent extraction that lasted for 36 hours. Mild temperatures (40–50 degrees Celsius) were maintained. A rotary flash evaporator was used to vacuum-dry the semisolid mass that was obtained after solvent removal by distillation at reduced pressure in order to get the extract.

Phytochemical Screening of polyherbal extracts:

The various polyherbal extract obtained after extraction were subjected for phytochemical screening to determine the presence of following various phytochemical present in the extracts.

***In-vitro* Anti-inflammatory activity of different Polyherbal Extracts:**

Using the method for stabilising human red blood cell membranes, the in-vitro anti-inflammatory effectiveness of polyherbal ethyl acetate extracts was evaluated. Extracts were used at doses of 1 mg/ml, 2 mg/ml, 4 mg/ml, and 6 mg/ml for the anti-inflammatory experiment.

Preparation of drug

Standard medication (Indomethacin, 2.5 mg/ml) and extracts (1.0 -6.0 mg/ml) were produced to final concentration in isosaline (0.85% NaCl).

Preparation of Suspension (10% v/v) of Human Red Blood cell

A healthy human volunteer who had abstained from nonsteroidal anti-inflammatory drugs (NSAIDs) for at least two weeks before to the experiment had their blood extracted and then placed in a centrifuge tube containing heparin. At room temperature, blood samples were centrifuged at 3000 rpm for 15 minutes. Before washing the packed red blood cells with 0.85% w/w normal saline, the supernatant (plasma and leucocytes) was carefully removed. It took five rounds of washing and centrifugation to get the clear supernatant. Oyedapo et al. (2004) detailed the subsequent steps for creating a 10% v/v suspension of human erythrocytes.

Assay of Membrane stabilizing activity

Using a 10% (v/v) Human erythrocyte solution and Indomethacin as the reference medicine, the HRBC membrane stabilising activity test was carried out according to the methods given by Sadique et al. (1989) and Oyedapo et al. (2004). The test mixtures were prepared using the following ingredients: 2 millilitres of 0.25% (w/v) sodium chloride hyposaline, 1 millilitre of 0.15 M sodium phosphate buffer with a pH of 7.4, half a millilitre of a 10% (v/v) human erythrocyte suspension, 1 millilitre of medication (standard and extracts), and 4.5 millilitres of isosaline.

The HRBC membrane stabilisation method was used to determine the anti-inflammatory activity of the following solutions: .

Test solution (4.5ml) Contains 2ml of hypotonic saline (0.25%w/v), 1ml of phosphate buffer (pH7.4), 1ml of polyherbal ethyl acetate extract (1mg/ml - 6 mg/ml) in normal saline, and 0.5ml of 10% w/v human red blood cells in isotonic saline.

Test control (4.5ml) The solution contains 2ml of hypotonic saline (0.25%w/v), 1ml of phosphate buffer (7.4pH), 1ml of isotonic saline, and 0.5ml of human red blood cells in isotonic saline at a concentration of 10%w/v.

Standard solution (4.5ml) consists of 2ml of hypotonic saline (0.25%w/v) 1ml of phosphate buffer (7.4pH) and 1ml of Indomethacin (2.5mg/ml) and 0.5ml 10%w/v human red blood cells in isotonic saline.

There was no medicine in the blood control, and there was no erythrocyte suspension in the drug control. After 30 minutes of incubation at 37°C, the reaction mixtures were spun at 3000 rpm for 20 minutes. Spectrophotometry was used to ascertain the supernatant solution's absorbance at 560 nm. We performed each experiment three times and averaged the results. An equation was used to calculate the percentage of inhibition of hemolysis or membrane stabilisation.

$$\% \text{ Inhibition of haemolysis} = 100 \times (A_1 - A_2/A_1)$$

Where:

A_1 = Absorption of hypotonic buffered saline solution alone

A_2 = Absorption of test sample in hypotonic solution

RESULTS AND DISCUSSION

Physico-Chemical Analysis

A number of physicochemical parameters, including swelling index, moisture content (M.C.), foreign organic matter (F.O.M.), and ash values (total ash, acid insoluble ash, and water soluble ash), were assessed in a conventional manner using the dried polyherbal powder sections. (Table.1)

Table 1: Physico-chemical analysis of polyherbal powder of *Ficus microcarpa* and *Adina cordifolia*

Parameters (% w/w)	Results					
	R1	R2	R3	X	X±SD	X±SEM
Total ash (TA)	7.0532	7.1958	7.0972	7.1154	7.11±0.05	7.11±0.03
Water soluble ash (WSA)	4.9674	4.8260	4.9062	4.8998	4.89±0.05	4.89±0.03
Acid insoluble ash (AIA)	1.8856	1.8074	1.7988	1.8306	1.83±0.00	1.83±0.00

Moisture content (MC)	6.0	5.79	5.90	5.89666	5.896±0.07	5.89±0.04
Swelling index (SI)	0.21	0.17	0.23	0.203333	0.20±0.02	0.20±0.01
Foreign organic matters (FOM)	0.90	0.87	089	0.88666	0.88±0.10	0.88±0.06

Abbr.: R1=Reading 1, R2=Reading 2, R3=Reading 3, **X**=Mean, SD=Standard Deviation,SEM=Standard Mean Error

Phytochemical Screening

A preliminary phytochemical analysis was conducted on the powder extracts of the polyherbal plants that were produced. Several medicinally active components were identified in the extract after its extraction using water, ethanol, chloroform, ethyl acetate, and petroleum ether. Protein, amino acids, steroids, tannins, carbs, glycosides, and alkaloids are all present in the ethylacetate extract.

Table 2: Preliminary phytochemical screening of different extract of *Ficus microcarpa and Adina cordifolia*

Constituents	**polyherbal** *Ethyl Acetate Extract*
Carbohydrate	**- ve**
Glycosides	**- ve**
Fixed oil and fats	**+ ve**
Proteins and amino acids	**+ ve**
Saponins	**- ve**
Phenolic comp. and tannins	**+ ve**
Phytosterols	**+ ve**
Alkaloids	**+ ve**
Gums and mucilage	**+ ve**
Flavonoids	**+ ve**

(+ Present, - Absent)

***In-vitro* Anti-inflammatory activity of Extracts of polyherbal powder**

Several illnesses are associated with inflammation, which causes the production of lysosomal hydrolytic enzymes at sites where they harm neighbouring organelles and tissues. Several methods were used to test and study herbal mixtures, chemicals, and medicinal drugs that show anti-inflammatory properties or potential. Some examples of these methods include stabilised erythrocyte and lysosomal membranes, fibrinolytic testing, platelet aggregation, and uncoupling of oxidative phosphorylation (respiration-linked ATP production). The present experiment used the simple and repeatable method of stabilising erythrocyte membranes that had been exposed to heat and hypotonic-induced lyses. The efficiency of the polyherbal ethyl acetate extracts in reducing inflammation was tested in vitro using the HRBC membrane stabilisation method. One, two, four, and six milligrammes per millilitre of extract were used. In a hypotonic solution, PHEAE at a dosage of 6 mg/ml protected HRBC by 67.6%. Traditional indomethacin showed 69.6 percent protection at 2.5 mg/ml (Table3), and all results were compared to this. One or more of the phytochemical components in the extract could be responsible for the effect. Photomicrographs of the HRBC have confirmed the obtained findings (fig2-4). By preventing the lysis of erythrocyte membranes caused by hypotonicity, the polyherbal extract demonstrated a membrane stabilising action. Since the erythrocyte membrane is structurally similar to the lysosomal membrane (Chou, 1997), the stabilisation of the former suggests that the latter may also be stabilised by the extract. In order to reduce the inflammatory response, it is crucial to stabilise the lysosomal membrane. This is because active neutrophils release lysosomal components, including proteases and enzymes that kill bacteria, which may cause further tissue damage.

Figure 1: *In-vitro* Anti-inflammatory activity of poly herbal ethyl acetate Extracts of *Ficus microcarpa and Adina cordifolia*

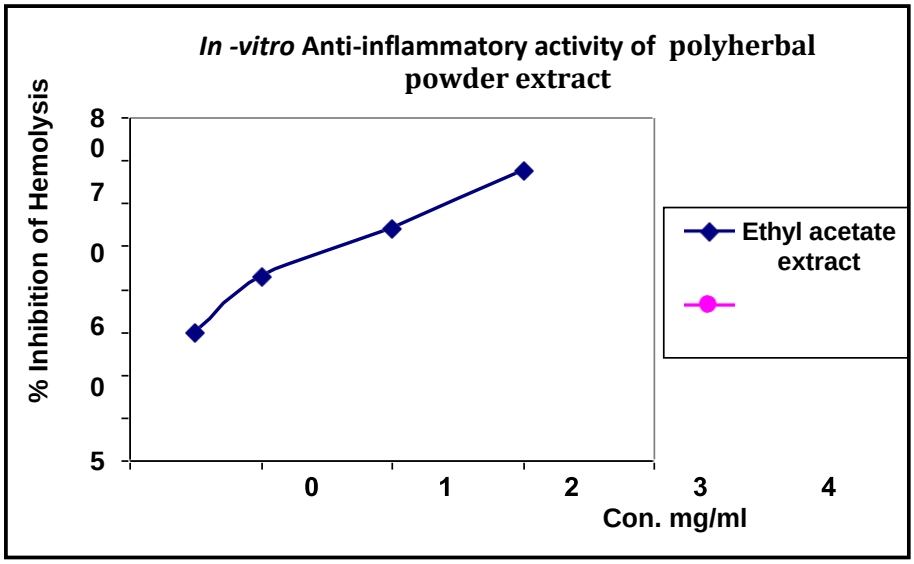


Table 3: *In-vitro* anti-inflammatory activity of Ethyl acetate extract of polyherbal powder by membrane stabilization method

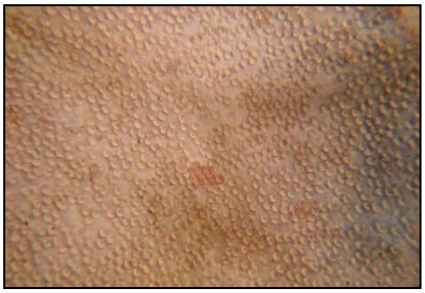
Treatment	Con(mg/ml)	Absorbance(560nm)	% of Inhibition
Control	-	0.250±0.29	-
Polyherbal Ethyl acetate extract	1.00	0.175±0.12[a]	30.0
	2.00	0.143±0.23[a]	42.8
	4.00	0.115±0.44[c]	54.0
	6.00	0.081±0.39[b]	67.6
Indomethacin (Standard drug)	2.50	0.070±0.18[b]	69.6

Values are expressed as X (Mean) ±SEM, n=3. (One way ANOVA followed by Student t-test). Statistically significance of [a]P < 0.05, [b]P<0.01, [c]P<0.001 and [d]NS in comparison to respective control.

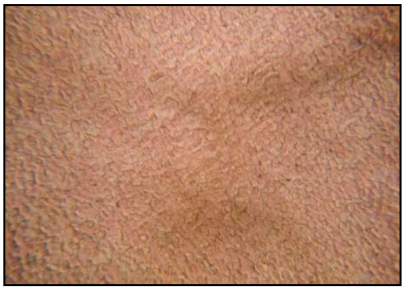
It is believed that the anti-inflammatory effects of several NSAIDs are enhanced by their ability to stabilise cell membranes. Cell shrinkage caused by osmotic loss of internal electrolyte and fluid components may lead to

hypotonicity-induced hemolysis, however the specific method by which the extract stabilises the membrane is yet unknown. The outflow of these intracellular components may be stimulated or enhanced by the activities that the extract inhibits (Iwueke, 2006).

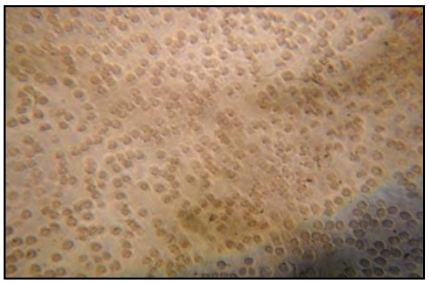
Figure 2-4 HRBC Membrane in isotonic and hypertonic solution



HRBC in Isotonic Solution Control



HRBC in Hypertonic Solution



RBC in Hypertonic solution with polyherbal extract (6mg/ml)

(Protection of Hypertonic induced HRBC membrane lysis)

CONCLUSIONS

An effort was undertaken to assess the anti-inflammatory system's topical therapeutic efficacy of polyherbal powder. The anti-inflammatory efficacy of the ethyl acetate polyherbal extract was investigated in vitro using the HRBC membrane stabilisation technique. The greatest anti-inflammatory activity and 68% protection of HRBC in hypotonic solution were shown by ethyl acetate polyherbal extract at a concentration of 6 mg/ml. When compared to normal indomethacin, the findings demonstrated a protection level of 69.6%. The extract may include one or more phytochemical components that are responsible for the action.

Traditional medicine's use of polyherbal powder to treat inflammation has also been well-supported by the study. Therefore, the plant might be seen as a natural source of membrane stabilisers and a potential alternative treatment for illnesses and conditions associated to inflammation.

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