PHARMACOLOGICAL EVALUATION OF *IN- VITRO* AND *IN-VIVO* ANTI-RHEUMATOID ARTHRITIS ACTIVITY OF POLYHERBAL EXTRACT OF SELECTED TRADITIONAL INDIAN PLANTS IN *WISTAR* RATS.

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Abstract

Arthritis is leading cause of disability in adults all over the world, limiting day-to-day activities for billion of people. WHO reports that 1% of the world population is affected by arthritis. The present study aims to evaluate the effectiveness of a polyherbal extract *Imperata cylindrica, Abroma augusta, Feronia limonia* and *Abelmoschus esculentus* for the treatment of rheumatoid arthritis by *in-vitro* and *in-vivo* methods. **Materials and Methods:** Poly herbal ethanolic extract of different fractions are used to investigate *in vitro* by human red blood cell (HRBC) membrane stabilization assay, inhibition of protein denaturation assay using egg albumin, and inhibition of protein denaturation assay using bovine serum. Furthermore, we evaluated i*n-vivo* anti-arthritic activity of the ethanolic extract of two different formulations containing the 300 mg/kg orally and 600 mg/kg body weight, it was evaluated using different methods such as complete Freund's adjuvant-induced arthritis, turpentine oil and formaldehyde induced arthritis.

Results: The result revealed that the formulations PHF3 and PHF4 possessed significant *in-vitro* anti-arthritic activity by protein denaturation inhibition and HRBC membrane stabilization comparing with the standard drug diclofenac sodium. In turpentine oil, formaldehyde, and complete Freund's adjuvant-induced arthritis models, the polyherbal extract formulations significantly ($P < 0.001$) reduced joint and paw swelling and markedly improved body weight, haematology profile and parameters in complete Freund's adjuvant model

Conclusion: It could be concluded that the above two polyherbal ethanolic extracts are an effective for anti-arthritic activity, supporting its traditional use in the treatment of rheumatoid arthritis.

Keywords: -Polyherbal, Imperata cylindrica, Abroma augusta, Feronia limonia and Abelmoschus esculentus, in-vitro and in-vivo methods.

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INTRODUCTION

Traditional awareness of plants is responsible for mainly of the medicine and food utilized in modern society. Herbs have been used during time as food and as a primary tool for maintaining physical condition and helping in the recovery of ailments. About 80% of the world population use plant based medicines and about one third of the world countries depend on herbal medicines. The *Ayurvedic* literature "*Sarangdhar Samhita"* highlighted the concept of polyherbal formulation to achieve greater therapeutic effectiveness. When multiple herbs are combined in a particular proportion, it will give better therapeutic effect and reduce toxicity. [1-2]

Rheumatoid arthritis (RA) is an autoimmune system disease characterized by pain, synovial membrane inflammation, peripheral joint inflammation, morning stiffness, destruction of articular tissues and restricted joint movement. RA is treated with anti- inflammatory and immune suppressive drugs, whose side effects are well known. [3-6]

It would therefore be highly desirable to find less toxic alternatives. Some medicinal botanicals may be possibilities for such substitutes. Hence, the present study was envisaged to evaluate the poly herbal fractions containing different proportion of ethanolic extract of *Imperata cylindrica, Abroma augusta, Feronia limonia, Abelmoschus esculentus* for its anti-arthritic activity.

Imperata cylindrica (Family: Poaceae (Gramineae) commonly recognized as dabh (darbh) cogon grass, kunai grass, blady grass, alang-alang, lalang grass, cotton wool grass, kura-kura that constitute vital ingredient in various Vedic sacrifices (Yagnas) and rituals. Cogon grass is a therapeutic plant native to southwestern Asia and the tropical and subtropical regions. Chemical constituents saponins, flavonoids, phenols, and glycosides have been identified from I. cylindrica. Various parts of *limonia acidissima* have been utilized include relieving fever, stopping vomiting, improving renal function, clearing heat and toxins, pulmonary heat and asthma increasing urination and promoting hemostasis.[7-8]

Abroma augusta (Family: Malvaceae) is commonly known as devil's cotton and in hindi called as ulatkambal. It has a long history of medicinal used in Homoeopathic System of Medicine. Ulatkambal is generally grown in Australia, South Africa, Eastern Africa, Asia and India. Phytochemicals of different parts of the plant showed the presence of various essential phytochemicals like alkaloids, abromin, abromasterol, sterol, taroxerylacetate, taraxeral and β-sitosterol. This plant has been claimed to possess major pharmacological activities such as anti-diabetic, thrombolytic, analgesic, hypolipidemic, anti-inflamatory, antioxidant etc. [9-10]

Feronia limonia (Family: Rutaceae) is commonly recognized as kaitha and kavit monkey fruit, curd fruit, wood apple, elephant apple, in India. It has planted in Pakistan, Bangladesh and Sri Lanka as well as India. The medicinal plants are main sources of different phytochemicals alkaloids, phenolics, saponins, steroid, flavinoids, glucoside, terpenoids, tannins, aliphatic alcohols, acids and esters, etc. Various parts of *limonia acidissima* have been utilized for more than thousands of years in traditional medicines. In traditional system it is used to cure dysentery, diarrhea, asthma, wounds, tumors, hepatitis and cardiac debility. [11-12]

Abelmoschus esculentus *L.* (Family: Malvaceae) is commonly known as lady's finger, bhindi, okra or gumbo. Bhindi is a multipurpose crop due to it's a variety of utilization of the fresh leaves, flowers, pods, buds, stems and seeds. Besides the nutritional benefit, the different parts of the plant are used extensively in traditional medicine around the world. Phytochemicals are tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and phenols are responsible for their bioactivities such as antimicrobial, antidiabetic, antipyretic, diuretic, antispasmodic and antioxidant. Lady's finger has therapeutic purposes when utilized as a plasma replacement or blood volume expander. [13-14]

MATERIALS AND METHODS

Plant Source and Authentication

Imperata cylindrica roots, *Abroma augusta* leaves, *Feronia limonia* leaves and *Abelmoschus esculentus* fruits were collected from Chhatarpur and Indore district of Madhya Pradesh. The plant specimen was verified to be of the correct species by Dr. S.N. Dwivedi, Head of Department of Botany Janata PG College, A.P.S. University, Rewa, M.P., India. Voucher specimen no. 141, 131, 146, and 143.

Drugs and Chemicals

The diclofenac sodium, egg albumin and bovine serum obtained from Sigma-Aldrich and All other chemicals and solvents were used of analytical grade available commercially.

Preparation of the Plant Extracts

Selected parts of *Imperata cylindrica*, *Abroma augusta*, *Feronia limonia*, and *Abelmoschus esculentus* were collected and dried. Then the material was blended to form a fine powder and extracted with petroleum ether, ethyl acetate and ethanol using Soxhlet apparatus for 7 hrs at 55ºC and with water by maceration. All four solvents were absolutely removed by rotary evaporator.

Preliminary Phytochemical Analysis

In phytochemical evaluation, the petroleum ether, ethyl acetate, ethanol and aqueous extract of *Imperata cylindrica* roots, *Abroma augusta* leaves, *Feronia limonia* leaves and *Abelmoschus esculentus* fruits were powdered and subjected to phytochemical screening for the detection of various plant constituents mainly carbohydrates, phenols, tannins, alkaloids, flavonoids, glycosides and saponins which majorly responsible for the desired activity.

Estimation of Phytoconstituents

The phytoconstituents present in dried coarsely powdered parts of *Imperata cylindrica*, *Abroma augusta*, *Feronia limonia* and *Abelmoschus esculentus* were estimated using standard procedures. The content of total phenolics and total flavonoid content in the powdered drug was determined by using standard methods.[15-17]

Table 1: Different proportion of ethanolic extract of selected plants of *Imperata cylindrica*, *Abroma augusta*, *Feronia limonia* and *Abelmoschus esculentus.*

Polyherbal Fraction	*Imperata cylindrica*	*Abroma augusta*	*Feronia limonia*	*Abelmoschus esculentus*
PHF1	1	1	1	1
PHF2	1	1	1	2
PHF3	1	1	2	1
PHF4	1	2	1	1
PHF5	2	1	1	1

In-Vitro Anti-Arthritic Activity of Polyherbal Fractions [18-21]

Inhibition of Protein Denaturation

The study includes the albumin denaturation which is performed by using Bovine serum albumin (BSA). When Bovine serum albumin is heated it undergoes denaturation and express antigens combined with type-III hypersensitivity responses and that is related to diseases such as lupus erythromotosus, rheumatoid arthritis, serum sickness and glomerulonephritis.

Preparation of the standard solution: The reaction mixture (0.5 ml) were prepared using 0.45 ml of Bovine serum albumin (5 % w/v aqueous solution) and 0.05 ml of Diclofenac sodium solution in different concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml).

Preparation of the test solution: The reaction mixture (0.5 ml) were prepared using 0.45 ml of Bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of test solution in various concentration (10, 50, 100, 200, 400, 800 and 1000 µg/ml).

Preparation of the test control solution: This reaction mixture (0.5 ml) was prepared using of 0.45 ml of bovine serum albumin (5% w/v aqueous solution) and 0.05 ml of distilled water.

Experimental procedure: All the above solutions were incubated at 37°C for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube and the turbidity was measured spectrophotometrically at 660 nm for control test 0.05 ml distilled water was used instead of extracts while product control test lacked bovine serum albumin.

The % inhibition of protein denaturation was calculated using the following formula:

Percentage inhibition = [Abs control–Abs test sample/Abs Test Control] × 100

Abs = Absorbance.

The control represents 100% protein denaturation.

Inhibition of Albumin Denaturation

Preparation of the standard solution: The standard solutions 5 ml were prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4) and 2 mL of Diclofenac sodium solution in various concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml).

Preparation of the test solution: - The test solutions 5 ml were prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4) and 2 mL of different concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml).

Preparation of the test control solution: This solution prepared using 0.2 mL of egg albumin (from fresh hen's egg), 2.8 mL of phosphate buffered saline (pH 6.4) and 2 mL of distilled water.

Experimental procedure: - All reaction mixtures were incubated at 37°C in incubator for 15 min and after that heated at 70°C for 5 min. once cooling their absorbance were determined at 660 nm using vehicle as a blank.

The % inhibition of protein denaturation was determined using the following formula**:**

Percentage inhibition = [Abs control–Abs test sample/Abs Test Control] × 100

Abs = Absorbance.

HRBC Membrane Stabilization Method

Preparation of the standard solution: The standard solution containing of 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of 10%w/ v human red blood cells in isotonic saline 0.5 mL of Diclofenac sodium solution in various concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml) and 2 ml of distilled water.

Preparation of the test solution: The test solution containing of 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of 10%w/ v human RBC in isotonic saline 0.5 mL of polyherbal extract solution in different concentrations (10, 50, 100, 200, 400, 800 and 1000 µg/ml) and 2 ml of distilled water

Preparation of test control: The test control solution containing of 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of 10%w/ v human RBC in isotonic saline, 2.5 mL of distilled water.

Experimental procedure: All the reaction mixtures were incubated at 37ºC for 30 min and centrifuged at the rate of 2500 rpm. The supernatant liquid was transferred and the hemoglobin content was determined through UV spectrophotometer at 560 nm.

The % of HRBC membrane stabilization against hypotonicity stimulated hemolysis was determined by the formula follows:

Percentage inhibition = [Abs control–Abs test sample/Abs Test Control] × 100

Abs = Absorbance.

Pharmacological Investigation [21-26]

The poly herbal fractions (PHF1-PHF5) containing different proportion of ethanolic extract of *Imperata cylindrica, Abroma augusta, Feronia limonia, Abelmoschus esculentus* were screened for anti arthritic activity by in-vitro models. Among the tested poly herbal fractions, the poly herbal fraction PHF3 and PHF4 shows significant effect. Hence these two poly herbal fractions PHF3 and PHF4 were subjected to further pharmacological activity.

Acute Toxicity Studies

Materials

3% CMC was used to formulate poly herbal fraction PHF3 and PHF4 to make a standard solution of 4000 mg/kg and is managed to the animal (1 ml/100g b.w).

Experimental animals

Healthy *Wistar* albino rats of either sex, and of approximately the same age, weighing about 250-200 g were procured from Animal House. The whole procedure was approved by the Institutional Animal Ethical Committee (IAEC) which is certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the IAEC of SVCP, protocol number **IAEC/SVCP/2022/12.** The *Wistar* rats were kept in clean and dry cages and maintained in a well ventilated, temperature controlled 22 º C (±3ºC) animal house with 12 hrs light and 12 hrs dark cycles. The all group of rats were fed with standard pellet diet and water was provided as libitum. For experimental purpose, the rats were fasted overnight but allowed access to water.

Procedure

Overnight fasted animals were treated with oral doses of 4000 mg/kg b.w. of PHF3 and PHF4 suspended in3% CMC. Individually, animals were examined carefully for every 30 min for the first four hour and then the next 24 hour to access mortality and to detect any changes in the autonomical or behavioural responses viz. alertness, irritability, aggressiveness, tremor, spontaneous activity, corneal reflex, urination, convulsion, respiration and salvation etc.

The animals were observed regularly for 14 days to note the mortality or toxic symptoms. Since there was no death as per the guidelines, the study was repeated with the same dose to confirm the results.

IN-VIVO ANTI ARTHRITIC ACTIVITY

Models of human diseases reproduced in animals have long been a requisite for discovering new modalities in managing diseases therapeutically. Rodents being the animals of choice for modern medical researchers with their short life span (2-3 years) which allows scientists to observe within a short time span, the prognosis and pathogenesis of a disease. Pharmacological screening is essential to evaluate the efficacy and potency of the plant drug.

Animals Grouping

Animals were divided into 7 groups of 6 animals each and each group was given a dose schedule as follows:

Group I: Normal control (1% (w/v) CMC in normal saline, 3 mL/kg, i.p.)

Group II: Arthritic control (0.02 ml of turpentine oil into the synovial cavity)

Group III: Polyherbal fraction 3 (300 mg/kg b.wt orally)

Group IV: Polyherbal fraction 3 (600 mg/kg b.wt orally)

Group V: Polyherbal fraction 4 (300 mg/kg b.wt orally)

Group VI: Polyherbal fraction 4 (600 mg/kg b.wt orally)

Group VII: Diclofenac sodium (10 mg/kg b.wt orally).

Turpentine Oil-Induced Joint Edema in Rats

Animals were divided into seven groups of six animals each as above.

Group I served as control and received normal saline (3 mL/kg, i.p.), Group II served as Arthritic control and received 0.02 mL of turpentine oil into the synovial cavity of the right knee joint. Group III-IV received PHF3 (300-600 mg/kg b.wt orally.), respectively, and group V-VI received PHF4 (300 -600 mg/kg b.wt orally.).

Group VII served as standard control and received Diclofenac sodium(10 mg/kg b.wt orally) Acute non-immunological inflammatory joint edema was produced by injecting 0.02 mL of turpentine oil into the synovial cavity of the right knee joint, 30 min following the drug administration. Diameter of the animal joint was recorded using a micrometer screw gauge at hourly intervals for 6 hrs.

Percentage inhibition of knee joint edema of arthritis control, test extract and standard groups was computed by following formula.

Percentage inhibition = VC−VT ÷VC×100

VC = Joint edema of arthritis control group; VT = Joint edema of the test group.

Formaldehyde-Induced Arthritis in Rats

The male *Wistar* rats were divided into seven different groups of six animals each as above. Baseline recording of the paw volume was made using plethysmometer.

Group I served as control and received normal saline (3 mL/kg, i.p.),Group II served as Arthritic control and received sub plantar injection of 0.1mL formaldehyde (2% v/v) group III- IV received PHF3 (300 and 600 mg/kg b.wt orally.), respectively, and group V-VI received PHF4 (300 - 600 mg/kg b.wt, orally.), Group VII served as standard control and received Diclofenac sodium(10 mg/kg b.wt orally) for 10 days. On day 1, 30min after the drug administration, acute non immunological arthritis was induced by sub plantar injection of 0.1mL formaldehyde (2% v/v) into the right hind paw of all the animals except group I animals and repeated on day 3. Arthritis was assessed by measuring the mean increase in paw volume over a period of 10 days.

Percentage inhibition of paw edema of rats was computed out as explained previously.

Complete Freund's Adjuvant Induced Arthritis in Rats

The male *wistar* rats were partitioned into seven unique groupings of six animals each as above. Preceding the trial, paw volume of every animal at multi day was estimated.

In complete Freund's adjuvant (5 mg of warmth executed, powdered mycobacterium tuberculosis cell was suspended with fluid paraffin to get a 5 mg/ml suspension) was utilized to prompt joint inflammation in rats. The rats were anesthetized with intra peritoneal infusion of 40 mg/kg thiopentone sodium. Mineral oil was infused in right lower leg joint of typical gathering of animals. Adjuvant joint pain was incited by subcutaneous infusion of FCA (0.1 ml) into sub plantar tissue of the correct rear paw of each rodent. The test groupings comprised of FCA infused rats tested with the separate portions of the test medications regulated orally 24 h before FCA infusion while, the vehicle control rats were infused with 0.1 ml of fluid paraffin (Fragmented Freund's adjuvant) as it were. The medication medicines were preceded with once every day on a similar time after the test for 20 more days. The swelling in the infused and contralateral rear paws of the rats were checked every day utilizing fluid uprooting plethysmometer. Increment in the degree of erythema and edema of the tissues demonstrates the seriousness of the irritation. The adjustment in body weight and paw edema were recorded at

wanted regular intervals. At the finish of the investigation, blood tests were pulled back from all groupings through retro-orbital plexus cut, and entire blood was utilized for hematological examination and serum was utilized for biochemical analysis.

Statistical analysis

The values were expressed as mean ±SEM and statistically analyzed using one way ANOVA followed by Dunnett's test. The results were considered statistically significant when $p < 0.05$.

RESULTS

***In - vitro* bioassay**

The present study summarizes the *in vitro* bioassay of anti-rheumatoid arthritis effect against HRBC membrane stabilization method, protein denaturation method using egg albumin, and protein denaturation method using bovine serum, the results as follows.[Tables 2-4 and Figures 1-3].

Acute Toxicity Study

During the acute toxicity study, the poly herbal fractions PHF3 and PHF4 was administered orally and animals were observed for mortality and behavioral responses. There was no mortality observed even at 4000 mg/Kg for the tested polyherbal fractions PHF3 and PHF4.[Tables 5-6].

***In - vivo* study**

Overall these *in vitro* results complement well with the *in vivo* study findings and supplement the indication that the polyherbal fraction is indeed a potent anti-inflammatory and anti-rheumatoid arthritis effect. It is evaluated by different methods turpentine oil-induced joint edema in rats, formaldehyde-induced arthritis in rats and complete freund's adjuvant induced arthritis in rats. [Tables 7-10and Figures 4-6].

Table 2: Inhibition of Protein Denaturation by Poly herbal fractions (PHF1-PHF5)

Conc. µg/ml	Standard	POLY HERBAL FRACTIONS				
		PHF1	PHF 2	PHF 3	PHF 4	PHF 5
10	52.82±0.2	33.02±0.5	32.25±0.2	42.82±0.24	40.36±0.14	32.29±0.2
50	56.71±0.6	41.24±0.3	41.81±0.4	52.91±0.3	46.18±0.4	40.92±0.4
100	66.04±0.8	48.39±0.8	46.62±0.15	56.64±0.6	57.26±0.9	44.19±1.9
200	78.61±0.5	54.72±2.5	53.64±0.9	69.24±4.3	62.92±2.4	51.28±2.5
400	84.81±0.2	64.60±0.8	65.24±0.25	78.36±2.6	68.72±0.8	62.42±1.2
800	92.16±0.9	72.0.7±0.5	73.72±0.8	85.29±1.2	77.20±0.9	69.26±0.8
1000	95.19±0.2	80.56±0.2	78.82±0.24	89.17±0.8	82.28±0.27	78.28±2.5

Table 3: Inhibition of Albumin Denaturation by Poly herbal fractions (PHF1-PHF5)

Conc. µg/ml	Standard	POLY HERBAL FRACTIONS				
		PHF1	PHF 2	PHF 3	PHF 4	PHF 5
10	52.32±0.2	46.12±0.25	42.12±0.5	46.89±0.5	49.42±0.24	42.32±0.36
50	58.24±0.8	48.24±0.62	44.24±0.2	49.12±0.32	53.91±0.3	44.28±0.46
100	65.04±1.7	51.42±0.24	48.42±0.4	52.22±0.8	57.64±0.6	47.15±0.20
200	71.24±1.4	56.12±2.5	51.12±1.5	58.25±2.15	69.24±1.3	53.24±0.7
400	75.08±1.18	63.46±0.4	59.46±1.4	64.14±0.6	74.36±0.6	61.38±0.8
800	82.16±1.5	72.24±0.2	68.24±1.2	73.43±0.4	76.29±0.9	69.74±0.45
1000	89.14±1.2	79.26±0.5	74.26±0.5	81.12±0.15	83.16±0.6	74.84±0.9

Table 4: HRBC membrane stabilization by Poly herbal fractions (PHF1-PHF5)

Conc. µg/ml	Standard	POLY HERBAL FRACTIONS				
		PHF1	PHF 2	PHF 3	PHF 4	PHF 5
10	56.82±0.4	41.56±0.6	39.96±0.4	40.56±0.4	48.56±1.2	38.56±02
50	62.81±0.5	49.21±0.2	50.21±1.3	45.21±0.6	61.01±0.2	43.21±06
100	89.14±0.9	62.13±1.3	70.13±1.6	57.13±0.5	78.13±0.9	64.13±04
200	91.59±1.5	75.14±0.2	80.14±1.2	78.14±1.2	89.14±1.4	72.14±12
400	94.26±0.8	81.46±1.4	86.46±0.9	84.46±0.6	92.46±0.4	81.46±16
800	96.59±0.9	88.49±1.2	90.49±0.6	89.49±1.3	94.49±0.5	88.49±13
1000	98.98±2.0	90.98±0.6	92.88±0.7	93.98±0.4	96.98±0.6	90.78±16

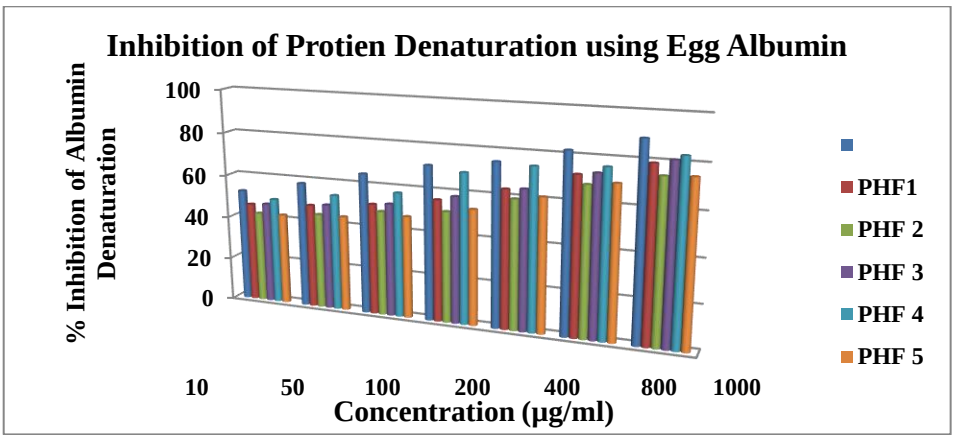

Figure 1: Inhibition of Protein Denaturation by Poly herbal fractions (PHF1-PHF5)

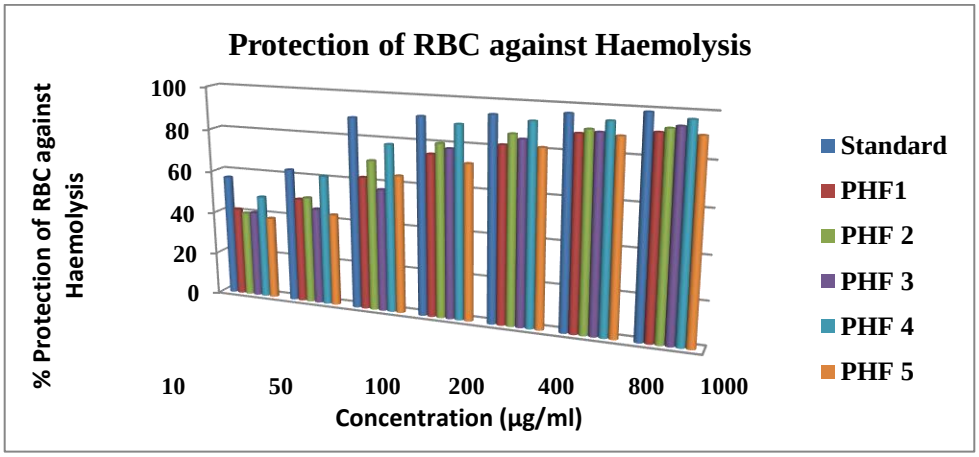

Figure2: Inhibition of Albumin Denaturation by Poly herbal fractions (PHF1-PHF5)

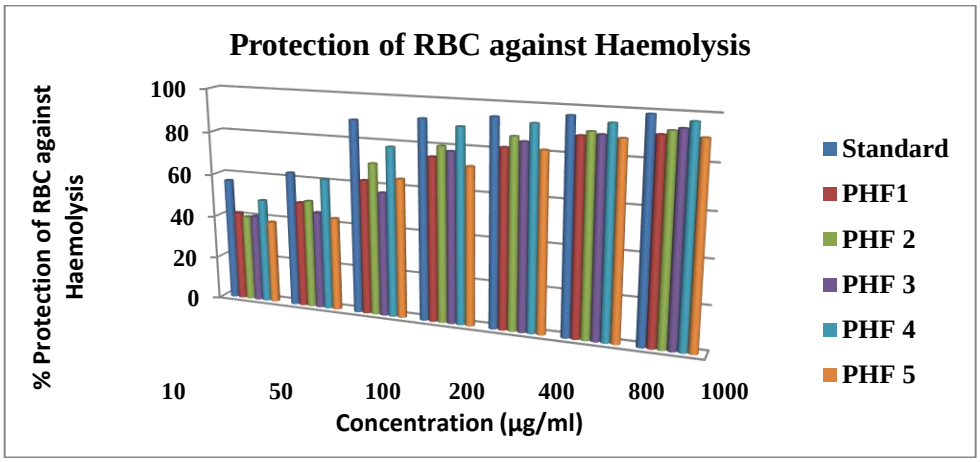

Figure 3: HRBC membrane stabilization by Poly herbal fractions (PHF1-PHF5)

Table 5: Effect of Poly Herbal Fractions PHF3and PHF4 on the Behavioral Patterns of Animals

SIGNS (Observations)	PHF3			PHF4		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
Skin and Fur	Normal	Normal	Normal	Normal	Normal	Normal
Eyes and mucous membranes	Normal	Normal	Normal	Normal	Normal	Normal
Behavior	Normal	Normal	Normal	Normal	Normal	Normal
Somatomotor activity	Normal	Normal	Normal	Normal	Normal	Normal
Convulsions	Absent	Absent	Absent	Absent	Absent	Absent
Salivation	Absent	Absent	Absent	Absent	Absent	Absent
Diarrhoea	Absent	Absent	Absent	Absent	Absent	Absent
Death	No	No	No	No	No	No
Other symptoms	Nil	Nil	Nil	Nil	Nil	Nil

Table 6: Effect of the Polyherbal Fractions PHF3 and PHF4 on the body weight

Animal Grouping (Rats)	Weight in grams for PHF3			Weight in grams for PHF4		
	Day 1	Day 7	Day14	Day 1	Day 7	Day 14
1	230	240	230	260	240	260
2	260	280	290	270	300	300
3	250	270	280	250	270	280

Table 7: Effect of the Polyherbal Fractions PHF3and PHF4 in Turpentine Oil-Induced Joint Edema.

ANIMAL GROUP	TREATMENT	DIAMETER OF THE JOINT EDEMA (MM)						% of Inhibition
		1hr	2hr	3hr	4hr	5hr	6hr	
GROUP I	Normal control	4.2±0.0a	4.2±0.0a	4.2±0.0a	4.1±0.0a	4.1±0.0a	4.3±0.0a	0
GROUPII	Arthritic control	9.33 ± 0.0a	10.25± 0.1a	13..34± 0.1a	15.98± 0.0a	18.38± 0.0a	21.96± 0.0a	-
GROUP II	PHF 3 (300mg/kg b.wt orally)	8.89 ± 0.0a	8.78± 0.0a	8.50±0.0a	8.3±0.1a	8.22±0.0a	7.8±0.1a	59.3
GROUP IV	PHF3 (600mg/kg b.wt orally)	8.65± 0.0a	8.4±0.1a	8.12±0.0a	7.80±0.0a	7.53±0.0a	7.00±0.0a	70.0
GROUP V	PHF4 (300mg/kg b.wt orally)	8.75 ± 0.0a	8.58± 0.1a	8.30±0.0a	8.0±0.0a	7.82±0.0a	7.5±0.1a	61.2
GROUP VI	PHF4 (600 mg/kg b.wt orally)	8.35± 0.0a	8.00±0.0a	7.82±0.1a	7.54±0.1a	7.03±0.0a	6.32±0.0a	76.6
GROUP VII	Diclofenac sodium (10 mg/kg b.wt orally).	7.02±0.1a	6.78±0.0a	6.20±0.0a	6.00±0.1a	5.58± 0.0a	5.20±0.0a	88.0

Values are expressed as mean ± SEM; n=3; One-way ANOVA followed by Dunnett's test used and *p<0.05* was considered as significant when compared with arthritic control group where b = p<0.01, a = p<0.001

Table 8: Effect of the Polyherbal Fractions PHF3 and PHF4 on Formaldehyde-Induced Arthritis

ANIMAL GROUP	TREATMENT	INCREASEDJOINTDIAMETERIN (MM)						% Inhibition
		1day	2 day	4 day	6 day	8 day	10 day	
GROUPI	Normal control	4.2±0.2a	4.3±0.1a	4.3±0.2a	4.3±0.2a	4.2±0.1a	4.3±0.1a	0
GROUPII	Arthritic control	8.7 ±0.2a	11.0±0.1a	15.6±.1a	17.2±0.1a	18.3±0.2a	19.5±.2a	-
GROUP III	PHF 3(300mg/kg b.wt orally)	8.4 ±0.1a	8.2 ±0.0a	7.9 ±0.0a	7.0 ±0.0a	6..5 ±0.0a	5.2 ±0.1a	69.8
GROUPIV	PHF 3(600mg/kg b.wt orally)	8.0±0.1a	7.8±0.0a	7.2 ±0.0	6.25±0.0	5.9±0.0a	4.9 ±0.1a	75.6
GROUP V	PHF 4(300mg/kg b.wt orally)	8.1±0.0a	7.9±0.1a	7.3 ±0.0a	6.4 ±0.1a	6.0 ±0.1a	5.0 ±0.0a	71.2
GROUPVI	PHF 4(600mg/kg b.wt orally)	7.8 ±0.0a	7.5± 1.0a	6.32±0.0a	5.9 ±0.0a	5.5±0.0a	4.8 ±0.0a	78.0

Values are expressed as mean ± SEM; n=3; One-way ANOVA followed by Dunnett's test used and p<0.05 was considered as significant when compared with arthritic control group where b = p<0.01, a = p<0.001.

Table 9: Effect of the Polyherbal Fractions PHF3and PHF4 on Complete Freund's Adjuvant Induced Arthritis.

ANIMAL GROUP	TREATMENT	Volume of Paw Edema (mm)			
		1st Day	5th Day	10th Day	15th Day
GROUPI	Normal control	4.3±0.0a	4.3±0.1a	4.3±0.1a	4.3±0.2a
GROUPII	Arthritic control	7.2±0.0a	16.2 ±0.0a	20.5 ±0.2a	23.7 ±0.0a
GROUPIII	PHF 3 (300mg/kg b.wt orally)	5.8±0.2a	8.2±0.0a	6.9±0.0a	6.0±0.0a
GROUPIV	PHF 3 (600mg/kg b.wt orally)	5.5±0.2a	7.6±0.3a	6.4±0.2a	5.5± 0.0a
GROUPV	PHF4 (300mg/kg b.wt orally)	5.9±0.0a	7.2±0.1a	6.3±0.0a	5.8±0.2a
GROUPVI	PHF4 (600mg/kg b.wt orally)	5.3±0.0a	6.9±0.3a	5.7±0.2a	5.1±0.0a
GROUPVII	Diclofenac sodium (10mg/kg b.wt orally).	5.1±0.2a	6.6±0.0a	5.6±0.0a	4.5±0.0a

Values are expressed as mean ± SEM; n=3; One-way ANOVA followed by Dunnett's test used and p<0.05 was considered as significant when compared with arthritic control group where b = p<0.01, a = p<0.001.

Table 10: Effect of the Polyherbal Fractions PHF3and PHF4 on Hematological Parameters

ANIMAL GROUP	TREATMENT	HEMATOLOGICALPARAMETERS					
		Hb(g/dL)	RBCs 106/µL	WBCs 103/µL	Platelets103/µL	ESR mm/1sthr	RF IU/mL
GROUPI	Normal control	14.3±0.2a	7.4±0.2a	5.2±0.1a	311 ±3.2a	3.0± 0.5a	14±0.0a
GROUPII	Arthritic control	9.3±0.1a	4.9±0.0a	9.4±0.2a	1225±105.3a	20.3±0.8a	48.3±2.0a
GROUPIII	PHF 3 (300mg/kg b.wt orally)	12.9±0.1a	5.8±0.1a	6.2±0.2a	395.3±3.7a	9.3±0.5a	20.0±1.1a
GROUPIV	PHF 3 (600mg/kg b.wt orally)	13.0±0.0a	6.3±0.1a	7.2±0.2a	592.3±2.7a	11.3±0.8a	28.0±2.3a
GROUPV	PHF4 (300mg/kg b.wt orally)	13.3±0.0a	6.5±0.0a	6.9±0.2a	498.3±4.1a	9.8± 0.5a	21.3±0.8a
GROUPVI	PHF4 (600mg/kg b.wt orally)	14.0±0.1a	6.8±0.0a	7.5±0.2a	692.00±3.1a	12.0± 0.5a	25.6±0.7a
GROUPVII	Diclofenac sodium(10mg/kg b.wt orally).	12.6±0.0a	7.0±0.1a	7.9±0.2a	732.3±4.6a	12.3±1.2a	25.3±2.3a

Values are expressed as mean ± SEM; n=3; One-way ANOVA followed by Dunnett's test used and $p<0.05$ was considered as significant when compared with arthritic control group where b = $p<0.01$, a = $p<0.001$.

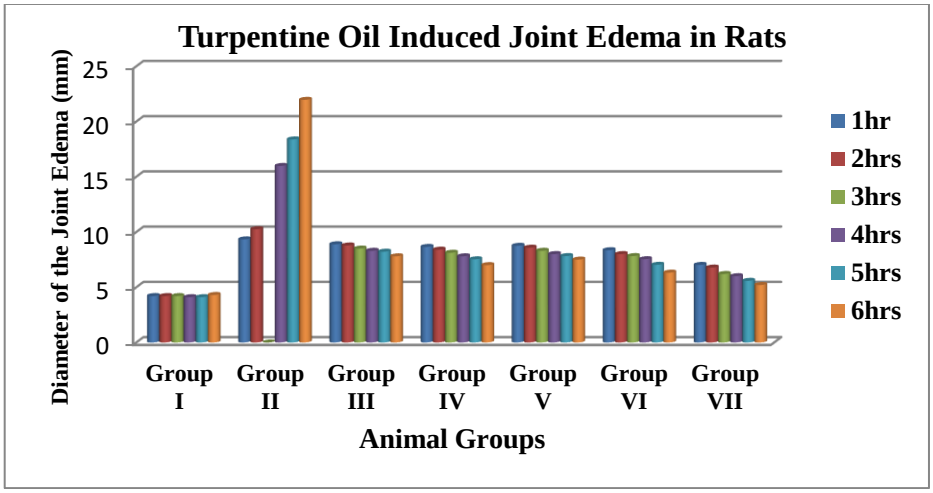

Figure 4: Effect of the Polyherbal Fractions PHF3 and PHF4 on Turpentine Oil-Induced Joint

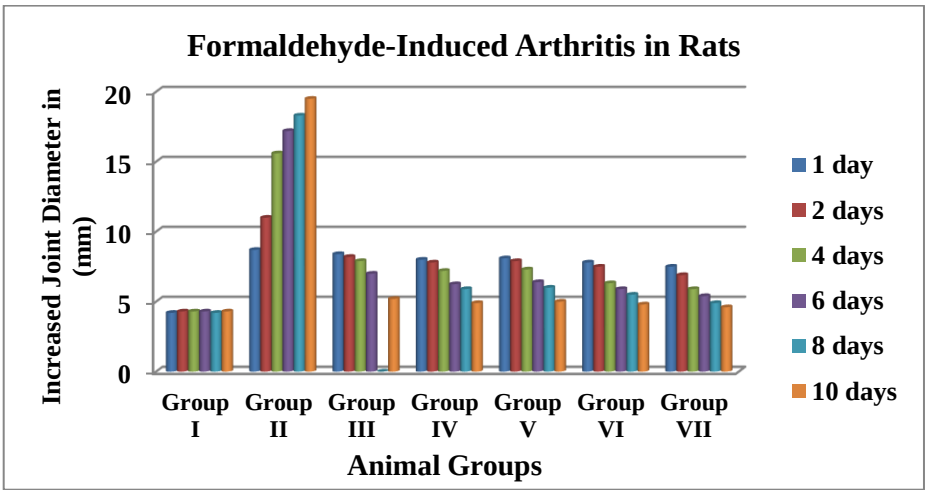

Figure 5: Effect of the Polyherbal Fractions PHF3 and PHF4 on Formaldehyde-Induced Arthritis

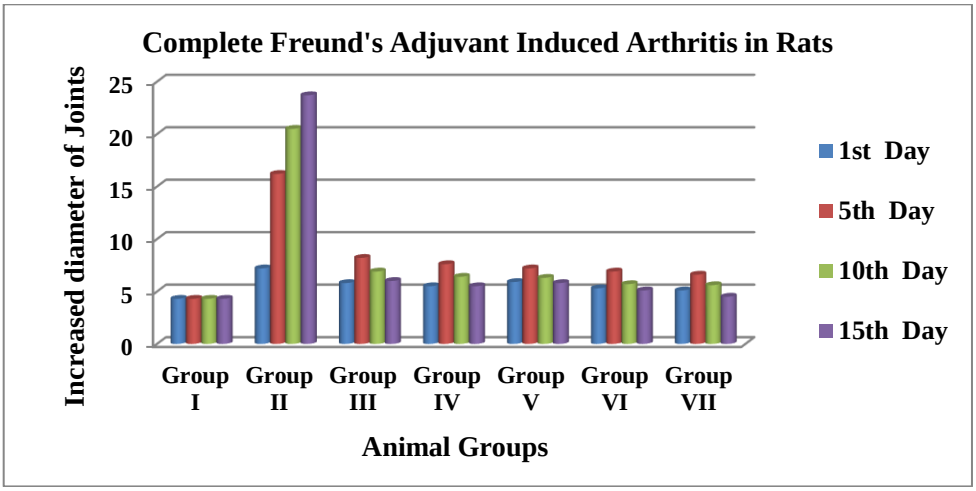

Figure 6: Effect of the Polyherbal Fractions PHF3 and PHF4 on Complete Freund's Adjuvant Induced Arthritis

DISCUSSION

World Health Organization (WHO) estimates that approximately 80 % of the developing world's population is using traditional medicine for primary healthcare. The poly herbal fractions (PHF 1-PHF5) containing different proportion of ethanol extract of *Imperata cylindrica, Abroma augusta, Feronia limonia, Abelmoschus esculentus* at different concentrations (dose levels) provided significant protection against denaturation of proteins. The maximum percentage inhibition was observed in the poly herbal fractions PHF3 (89%) and PHF4 (82%) at higher concentration. They possess significant activity comparable to that of Diclofenac sodium.

The poly herbal fractions of selected plants extract at different concentrations (dose levels) provided significant protection against denaturation of albumin. The maximum percentage inhibition was observed in the poly herbal fractions PHF3 (81%) and PHF4 (83%) at higher concentration. They possess significant activity comparable to that of Diclofenac sodium.

The maximum percentage stabilization was observed in the poly herbal fractions PHF3 (93%) and PHF4 (96%) respectively at higher concentration. They possess significant activity comparable to that of Diclofenac sodium. The above result gives a conclusion that the poly herbal ethanolic fractions with the different concentration have the anti- inflammatory and anti- arthritic activity and the PHF3 and PHF4 having significantly more potential. Hence these two poly herbal fractions PHF3 and PHF4 are evaluated for anti-arthritic effect by *in-vivo* methods.

No sign of toxic symptom or mortality was observed throughout the experimental period. During evaluation of toxicity, No significant changes were detected in wellness parameters. mucous membrane, Skin irritation, fur, eyes, behavioral pattern, Somatomotor activity, salivation, sleep pattern parameters and diarrhoea of the treated animals were found to be normal (Table 5). No mortality was observed in all tested animals after the administration of PHF3 and PHF4.

Rats treated with various doses of poly herbal fractions PHF3 and PHF4 had a progressive weight gained (Table 6).

The poly herbal fractions (PHF3 and PHF4) were containing different proportion of ethanolic extract of *Imperata cylindrica, Abroma augusta, Feronia limonia* and *Abelmoschus esculentus* were evaluated for its anti- arthritic activity using *in-vivo* animal models.

Turpentine oil is one which can induce the arthritis by entailing a chronological release of the inflammatory mediators such as histamine and serotonin in early phase, kinin-like substances in intermediate phase, and prostaglandins in late phase. The acute inflammatory reactions were stimulated by turpentine oil in the knee joint of animals, acute inflammation was reduced in a dose-dependent approach via oral administration of the poly herbal fractions PHF3 and PHF4 (300 and 600 mg/kg b.w)and the results were showed in Table 7 and Figure 6.

The formaldehyde-induced arthritis is also one of most commonly used acute non-immunological arthritis model for evaluating of the anti-arthritic activity of selected plant extract. Studies have been reported that swelling of around the ankle joint and paw of arthritic animals following the injection of formaldehyde may be due to edema of particular tissues such as ligament and capsule.

In the present study, the poly herbal fractions PHF3 and PHF4 (300 and 600 mg/kg b.w) corroborated perceptible anti-arthritic activity by

reducing the paw swelling and soft tissue thickening at the depth side throughout the observation period and results showed in Table 8 and Figure 7.

Alternatively, an increase leukocyte and platelet counts in blood due to the stimulation of immune system against the attacking pathogenic microorganism, it is evident by the influx of inflammatory mononuclear cells in the joints of arthritic rats which was shown in the Table 9.

The paw diameter reached maximum up to 5thday of adjuvant injection and after that it was slightly decreased. There was a significant increase in rat paw volume in FCA injected control rats when compared to the standard and poly herbal fraction PHF3 and PHF4 treated rats. The poly herbal fraction PHF4 was found to be most effective compared to poly herbal fraction PHF3.

CONCLUSION

The above result gives a conclusion that the polyherbal formulations with the different concentration have the anti-arthritic activity and the PHF3 and PHF4 having significantly more potential. Further investigation is required to use the two formulations in the treatment of rheumatoid arthritis. The above polyherbal extract with different portionis a medicinally valuable plant and its anti-arthritic effect might be due to its anti-inflammatory, antioxidant, and immunosuppressant actions, although, actual mechanism is not known.

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