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Phytochemical and Pharmacological Evaluation of *Bacopa monnieri* for Anti-Arthritic Activity

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Abstract

Seven Sesquiterpenes were found, out of which five were hydrocarbons i.e. calarene, vardiflorene, α- panasinsen, α-santalene, γ- himachelene, one is ketonic i.e. jatamansone and one is alcoholic i.e. epiglobulol; one Ttriterpene was observed i.e. unknown; among two others Ionol 4 (9.9%), 2,2,7,7- Tetramethyl tricyclo[6,2,1,0 (1,6)]undec-4-ene 3-one (1.7%) was observed. F-4 and F-7 extracts were found to be more stable and optimized than other formulations based on evaluation parameters. As a result, these can be investigated further for phytochemical analysis as well as *in vitro* and *in vivo* animal models against inflammation and rheumatoid arthritis.

Keywords: *Bacopa monnieri*, Rheumatoid Arthritis, Volatile Oil, HPTLC, Neutrophils.

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1. Introduction

Rheumatoid arthritis (RA) is one of the most common inflammatory disorders affecting the population worldwide [1]. It is a systemic inflammatory disease which affects not only the joints but a wide range of extra-articular organs. The disease, if not treated early, will lead to progressive joint deformity and increased morbidity and mortality [2]. RA is a potentially fatal illness, with mortality increased twofold and an average decrease in life expectancy of 7-10 years. Patients with RA have an increased prevalence of other serious illnesses. The predominant conditions leading to this increased co-morbidity and mortality include infections, renal impairment, cardiovascular disease and lymphomas [3]. The incidence of lymphoma is twofold higher than expected before considering the disease-modifying immunosuppressant drugs used in treating RA.

1.1 Epidemiology and Etiology

RA affects approximately 1% of the population worldwide. RA arises from an immunologic reaction, and there is speculation that it is in response to a genetic or infections antigen [4]. Risk factors associated with the development of RA include:

- Female gender (3:1 female to males)
- The prevalence of RA increases with age in both sexes; nearly 5% of women and 3% of men over the age of 65 years are affected by the disease.
- The peak age of incidence is about 30-50 years in women and slightly older in men.
- RA also affects young children and its classification and treatment differs slightly from adults. Current tobacco smoking. Studies have identified a direct relationship between tobacco use and RA disease severity.
- Family history of RA. Genetic studies demonstrate a strong correlation between RA and the presence of major histo-compatibility complex class II human leukocyte antigens (HLA), especially HLA-DR1 and HLA-DR4. HLA is a molecule associated with the presentation of antigens to T lymphocytes.

- Potential environmental exposures. The number of RA cases has increased during industrialization, although a specific link to environmental factors has not been determined.
- Oral contraceptive use and high ingestion of vitamin D and tea are associated with a decreased risk of RA [4-5].

1.2 Comorbidities associated with RA [6]

RA reduces a patient's average life expectancy by 3 to 10 years, but RA alone rarely causes death. Instead, specific comorbidities contribute to premature death independent of safety issues surrounding the use of immunomodulating medications. The comorbidities with the greatest impact on morbidity and mortality associated with RA are (1) cardiovascular disease, (2) infections, (3) malignancy, and (4) osteoporosis [7].

For a long period of time, ancient works of literature have recorded the use of plants for therapeutic purposes. As a result of this recording of essential traditional knowledge about medicinal plants, many significant medicines have been developed in the contemporary age [8]. *Bacopa monniera* L. (Oleaceae) is a significant medicinal plant that has been utilized for a variety of purposes throughout history. Numerous plant components have been utilized medicinally in traditional and indigenous cultures. *Bacopa monniera* is used in Ayurveda, Siddha-Ayurveda, and Yunani medicine as a laxative, diuretic, anti-venom, digestive, mild bitter tonic, and expectorant. Thus, literature review reveals that in this study decided that phytochemical and pharmacological study of stem bark of *Bacopa monniera* for Antiarthritic activity [9].

2. Materials and methods

2.1 Procurement of Plant Material

The stem-bark of *Bacopa monnieri* was procured from local market of Bhopal, Madhya Pradesh on October, 2023.

2.2 Identification and Authentication

All plant materials were air dried and was authenticated and identified by Dr. Saha Naaz, Head of Department, Department of Botany, Saifia Science College, Bhopal and the voucher specimen was submitted there for future.

2.3 Preparation of Extract

1 kg of *Bacopa monnieri* was air died and coarsely powdered and was subjected to Soxhlet extraction by using ethanol as a solvent till the complete extraction. All extracts were concentrated and was stored in air tight containers.

2.4 Isolation of volatile oil:

The air-dried stem-bark of *Bacopa monnieri* (500 g) were hydro-distilled according to the method recommended in British Pharmacopoeia, 2009. The resulting light pale-yellow oil was dried over anhydrous sodium sulphate and stored at 4℃ in the dark. The yield was 2.0 % based on fresh weight of sample.

2.5 Qualitative Phytochemical Screening

The extracts of *Bacopa monnieri* were analyzed for the presence of various phytoconstituents such as alkaloids, carbohydrates, glycosides, flavonoids, proteins, steroids, and saponins.

2.6 HPTLC Analysis

HPTLC analysis was performed in IPC, Ghaziabad by using Cammag Linomat 5 and TLC scanner 4. The solvent systems used are as follows:

- *Bacopa monnieri* Petroleum ether: Acetone (3:1)

2.7 GC Analysis

Analytical GC was carried out on a Varian 3300 gas chromatograph equipped with a silicon DB- I capillary column (30 m×0.25 m), film thickness 0.25 µm, Nitrogen carrier gas, flow rate 1.5 ml/min, split mode, temperature programmed 80-250 ℃ / min. Injector temperature and detector temperature was 250 ℃ and 300 ℃ respectively. FID was used as a Detector. For all samples, the injection volume was 0.1 µl.

***2.8 In Vivo* Antiarthritic Screening**

2.8.1 In vivo anti-arthritic animal models

2.8.1.1 Complete Freund's adjuvant (CFA) Induced Arthritis in rats

Freund's complete adjuvant induced arthritis in rats is the best and most popularly used arthritis experimental model. This model is not selective to joints, but is linked to granuloma formation in a variety of organs and tissues, including the spleen, liver, bone marrow, skin, and eyes A. Wstergren [10].

2.8.1.2 Collagen Type II induced Arthritis (CIA) in rats

CIA model is a standard animal model of RA. Collagen type II, collagen type IX, and cartilage oligomeric matrix protein are the three cartilage-derived proteins responsible for arthritis induction in rats. It is an excellent model to study mechanism of immune response to auto antigen which is generally involved in human disease [11].

2.8.1.3 Pristane induced arthritis (PIA)

PIA in rats is a chronic, symmetrical model that mimics many aspects of human disease. PIA is induced in susceptible rats with mineral oil pristine [12].

2.8.1.4 Oil-induced arthritis (OIA)

OIA appears 14 days after the induction of FCA intra-dermal injection. In this method, joint inflammation develops in the hind paw and ankle P. Emery [13].

2.8.1.5 Streptococcal cell wall-induced arthritis

In rats, a single intraperitoneal injection of cell wall peptidoglycan polysaccharide fragments from *streptococci* and other bacteria causes severe arthritis [14].

2.8.1.6 Aureus-induced arthritis

To induce arthritis, each mouse receives an intravenous injection of live bacteria (*S. aureus*) suspension [15].

2.8.1.7 Skin Irritation Studies

Skin Irritation studies Protocol Kumar *et al*. [16]

The study was conducted in compliance with the modification "OECD Guidelines for Testing of Chemicals" (No. 404 Acute Dermal Irritation/Corrosion, Section-4: Health Effects (Adopted: 28th July, 2015) and "The Pesticide Manual" (thirteenth edition ISBN 19013996 13 4. Page no. 237-239).

3. Results and discussion

3.2 Identification and authentication

Bacopa monnieri were identified and authenticated successfully at Saifia Science College, Bhopal with Ref. No 24/1525.

3.3 Preparation of Extracts

The % yields of *Bacopa monnieri* are summarized in **Table 1.**

Table 1 Summary of % yields of *Bacopa monnieri* (Extracts)

S. No.	Extract	Part used	% Yield	Colour
1.	*Bacopa monnieri* (ethanolic)	Stem Bark	20.5	Dark brown

3.4 Phytochemical screening

Based on the various phytochemical tests, *Bacopa monnieri* showed the presence of Alkaloids, Carbohydrates, Flavonoids, Proteins and Saponins which were tabulated in **Table 2**.

Table 2 Phytochemical analysis of extracts of *Bacopa monnieri*

S. No.	Chemical Constituents	Tests	*Bacopa monnieri*
1.	Alkaloids	Dragendorff's Test	+
		Hager's Test	+
		Wagner's Test	+
2.	Carbohydrates	Fehling's Test	+
		Molish's Test	+
		Benedict's Test	+
3.	Flavonoids	Shinoda Test	+
		Zn. HCl Test	+
4.	Tannins	$FeCl_3$ Test	+
		Lead acetate Test	+
5.	Proteins	Biuret Test	+
6.	Steroids	Salkowski Test	–
7.	Glycosides	NaOH Test	–
8.	Saponins	Frothing with $NaHCO_3$	+

3.5 Identification of Phytoconstituents of ethanolic extracts

In *Bacopa monnieri* ethanolic extract, four phytoconstituents were observed by HPTLC at 254 nm and 365 nm.

3.6 Skin irritation studies

Skin reaction

No skin reaction was observed for skin reaction at the site of application in intact as well as abraded skin.

Clinical signs

No Clinical signs were observed at the site of application for both formulations.

Evaluation of dermal skin irritation index

'0' dermal irritation index was found at the site of application for intact as well as abraded skin.

3.7 Paw Volume and Paw edema

There was a significant increase in paw volume from 0.8 to 1.3 ml in the Arthritic Control Group compared with the Control Group of 0.19 to 0.21 ml from 0 to 21 days respectively. The Standard Drug, Diclofenac sodium, showed decreased paw volume from 0.78 to 0.63 ml from 0 to 21 days respectively. On the 7th day, F-4 treated group produced a significant reduction in paw volume of 0.64 to 0.52 ml on comparing with the arthritic control group. Formulation F-7 displayed a significant reduction in paw volume of 0.61 to 0.48 ml on comparing with the Control and arthritic control groups. F-7 exhibited higher inhibition of paw edema even more than Std. drug diclofenac sodium and F-4. This % inhibition of paw edema increased with the number of days.

Table 3: Changes in Paw volume of animals at different time intervals

Groups	Paw Volume (ml)			
	0 day	7 day	14 day	21 day
Control	0.19±0.01****	0.2± 0.01****	0.2±0.01****	0.21±0.01****
Arthritic Control	0.8±0.012	1.07±0.03	1.18 ± 0.06	1.3± 0.02
Standard	0.78±0.01***	0.71± 0.01***	0.68±0.03***	0.63±0.04***
F-4	0.72±0.012***	0.64± 0.08***	0.59±0.03***	0.52±0.09***
F-7	0.69±0.015****	0.61±0.07****	0.56±0.03****	0.48±0.03****

All values were taken as Mean ± SD with n=6. Symbol** represents P value <0.00001, *** represents P value <0.0001, ** represents P value <0.001, * represents P value <0.01and ns denotes P value non-significant respectively compared to Arthritic control (Group-II) using one way ANOVA followed by multiple comparison Turkey's Test.*

Table 4: % Inhibition of Paw edema of Animals at Different Time Intervals

Groups	Inhibition of Paw edema (%)		
	7 day	14 day	21 day
Standard	35	50	71.4286
F-4	40	65	95.2381
F-7	40	65	100

3.8 Body weight

Body weight was normal in the control group (280 to 295 g) while it decreased in the range of 280 to 265 g in the arthritic group from 0 to 21 days**.** The formulation F-4 and F-7 showed a significant body weight increase of 284 to 301 g and 295 to 302 g respectively on comparing with the arthritic control group. This increase in body weight was even more than the Std. of 282 to 298 g**.**

Table 5: Changes in Body weight (g) of animals at different time intervals

Groups	Body weight (g)			
	0 day	7 day	14 day	21 day
Control	285±12.00***	289±16.00***	295 ±17.00***	297±18.00***
Arthritic Control	280±15.00	275±18	270± 21	265± 18
Standard	282± 12.00**	294± 19.00**	296± 21.00**	298± 24.00**
F-4	284± 18.00***	295± 22.00***	298± 18.00***	301± 21.00***
F-7	295±22.00***	297±19.00****	299±26.00****	302±22.00****

All values were taken as Mean±SD with n=6. Symbol****** indicates P value <0.00001, *******indicates P value <0.0001, ******indicates P value <0.001, ***** indicates P value <0.01and ns- P value non-significant respectively compared to Arthritic control (Group-II) using one way ANOVA followed by multiple comparison Turkey's Test.*

3.9 Haematological Parameters

3.9.1 WBC

In the control group, WBC was found to be 8.99 to 9.55 while it increased significantly in the arthritic group 18.24 -19.11 103/µl from 7 to 21 days, respectively, due to inflammation in the joint which caused the production of antibodies called WBC. On the 7th day, the treatment group F-4 and F-7 showed a significant decrease in WBC count of 17.18 and 16.43 103/µl respectively compared with the19.11 103/µl of arthritic control group. After 21 days of treatment, F-7 displayed significant decreased WBC count of 12.11 103/µl compared to 18.24 103/µl of the arthritic control group. This decrease in WBC count was even more than the Std. of 15.02 103/µl and F-4 of 14.05 103/µl**.**

3.9.2 RBC

In the control group, RBC was found to be 7.52 to 7.92 106/µl while it decreases significantly in the arthritic group in of 4.11-3.41 106/µl from 7 to 21 days, respectively due to abnormal or insufficient amount of haemoglobin. On the 7th day, the treatment group F-4 and F-7 exhibited a small increase in RBC count of 3.99 and 4.14 106/µl respectively compared with 3.41 106/µl of the arthritic control group. After 21 days of treatment, F-7 produced a slightly increased RBC count of 5.90 106/µl compared to 4.11 106/µl of arthritic control group. This increase in RBC count was even more than the Std. of 4.78 106/µl and F-4 of 4.98 106/µl, however the increase in RBC Count was found to be non-significant with respect to Arthritic Control group.

3.9.3 Haemoglobin Content

Haemoglobin (Hb) was found to be 12.55 to 12.86 g/dl in the control group while it decreased significantly in the arthritic group of 3.41-8.82 g/dl from 7 to 21 days, respectively due to chronic inflammation which led to lower production of RBC in the bone marrow or due to iron deficiency. On the 7th day, the treatment group F-4 and F-7 presented a small increase in Hb content of 8.75 and 9.18 g/dl respectively compared with 8.57 g/dl of the arthritic control group. After 21 days of treatment, F-7 revealed a slightly increased Hb content of 9.97 g/dl compared to 8.82 g/dl of the arthritic control group. This increase in Hb content was even more than the Std. of 9.01 g/dl and F-4 of 9.34 g/dl, however the increase in Hb Count was found to be non-significant with respect to Arthritic Control group.

3.9.4 PCV (Haematocrit)

PCV or haematocrit is the percent of total blood volume that is made up of RBC. In the control group, PCV was found to be 40.97 to 41.87 % while it decreased significantly in the arthritic group of 35.15-35.74 % from 7 to 21 days, respectively due to chronic inflammation which led to lower production of RBC and haemoglobin content. On the 7th day, the treatment group F-4 displayed no significant increase in PCV % while F-7 and Std. showed a significant increase in PCV% of 36.15% compared with 35.15% of the arthritic control group. After 21 days of treatment, F-7 elicited a significantly increased PCV % of 37.52 % compared to 35.74 % of the arthritic control group. This increase in PCV% was even more than the Std. of 36.95 % and F-4 of 36.25 %**.**

3.9.5 Platelet Count

Platelet count was found to be 1048.19 to 1055.42 105/mm3 in the control group while it decreased significantly in the arthritic group of 895.11-899.12 105/mm3 from 7 to 21 days, respectively due to autoimmune reaction in Rheumatoid arthritis which led to the destruction of platelets. On the 7th day, the treatment group F-4 and F-7 presented a small increase in Platelet count of 898.11 and 901.17 105/mm3 respectively compared with 895.11 105/mm3 of the arthritic control group. After 21 days of treatment, F-7 produced slightly increased Platelets count of 955.24 105/mm3 compared to 892.12 105/mm3 of the arthritic control group. This increase in Platelet count was even more than the Std. of 919.57 105/mm3 and F-4 of 938.24 105/mm3, however the increase in Platelet Count was found to be non-significant with respect to Arthritic Control group.

3.9.6 Neutrophils

Neutrophils are the most abundant leucocytes present in the inflamed joints and play a key role in the progression of rheumatoid arthritis. Neutrophils form the major component of synovial fluid and pannus, due to its trafficking from blood to the synovial cavity, a lower count of neutrophils (Neutropenia) is observed in rheumatoid arthritis. Neutrophils count was found to be 26 to 26.1 % in the control group while it decreased significantly in the arthritic group of 21.7-22.5 % from 7 to 21 days, respectively. On the 7th day, the treatment group F-4 and Std. elicited a small increase in Neutrophils of 22.7 % respectively compared with 21.7% of the arthritic control group. After 21 days of treatment, F-7 presented slightly increased Neutrophils of 24.2 % compared to 22.5 % of the arthritic control group. This increase in Neutrophils was even more than the Std. of 22.8% and F-4 of 23.2%, however the increase in Neutrophils was found to be non-significant with respect to Arthritic Control group.

3.9.7 Lymphocytes

Lymphocytes are a type of White Blood Cell (WBC). They play an important role in the body's immune system. In the presence of inflammation, the lymphocytes count increases in the blood (Lymphocytosis), which leads to lymphocyte infiltration in the synovial fluid. Lymphocyte count was found to be 71.4 to 71.6 % in the control group, while it increased significantly in the arthritic group of 75.1 - 75.7 % from 7 to 21 days, respectively. On the 7th day, the treatment group F-4 and Std. produced a small decrease in Lymphocytes of 74.6 % respectively, compared with 75.7 % of the arthritic control group. After 21 days of treatment, F-7 displayed slightly decreased Lymphocytes of 73.4 % compared to 75.1 % of the arthritic control group. This decrease in Lymphocytes was even more than the Std. of 74.6 and F-4 of 74.1 % respectively, however the Std. showed non-significant decrease in Lymphocytes with respect to Arthritic Control group.

3.9.8 Monocytes

Monocytes are the type of Lymphocytes. In Rheumatoid arthritis lymphocytosis occurs which causes an increase in monocytes to count in the blood which leads to a higher number of macrophages in the synovium which results in inflammation and bone erosion. Monocytes count was found to be 1.26 to 1.3 % in the control group while it increased slightly in the arthritic group of 1.3- 1.38 % from 7 to 21 days, respectively. On the 7th day, the treatment group F-4 and F-7 presented no decrease in Monocytes compared with the arthritic control group. After 21 days of treatment, F-7 produced slightly decreased Monocytes of 1.24 % compared to 1.3 % of the arthritic control group. This decrease in Monocytes was even more than the Std. and F-4 of 1.37 % respectively, however the decrease in Monocytes was found to be non-significant with respect to Arthritic Control group.

3.9.9 Eosinophils

Eosinophils are the type of White Blood Cells, due to increased WBC content in Rheumatoid arthritis eosinophils count increases in the blood (Eosinophilia). Eosinophils count was found to be 1.01 to 1.20 % in the control group while it increased significantly in the arthritic group of 1.1-1.26 % from 7 to 21 days, respectively. On the 7th day, the treatment group F-4 and Std. displayed no decrease in Eosinophils compared with the arthritic control group, while F-7 revealed slightly decreased Eosinophils of 1.1 %. After 21 days of treatment, Std., F-4 and F-7 exerted no significant decrease in Eosinophils compared to 1 .1% of the arthritic control group**.**

4. Conclusion

The various qualitative phytochemical tests indicated the presence of Alkaloids, Carbohydrates, Flavonoids, Proteins and Saponins in *Bacopa monnieri*. The various phytoconstituents were also observed in different extracts of *Bacopa monnieri* by using HPTLC which further requires isolation and characterization.

Many volatile oils have recently been discovered to be beneficial in the management of various illnesses. The GC and GC-MS analytical techniques were very useful technique in qualitative and quantitative estimation of volatile constituents.

Therefore further *in vitro* anti- inflammatory and *in vivo* antiarthritic evaluation is required. As per the *in vitro* anti-inflammatory studies the F-4 and F-7 extracts were found to possessed good synergistic effect on comparing with Std. drug Diclofenac Sodium. All three extracts of *Bacopa monnieri* consists of bioactive molecules which helps in inhibition of inflammation by producing synergistic effects. When compared to the reference drug, Diclofenac sodium, these extracts proved to have extremely potent anti-arthritis activity. This was supported by IC_{50} values. It inhibits the synthesis of inflammatory mediators by inhibiting the protein denaturation. This indicated higher efficacy of extracts than Diclofenac Sodium. Since these extracts are of natural origin, so these were found to be safer also. Thus, these can be further evaluated for *in vivo* animal model against Rheumatoid arthritis.

When compared with Standard drug, Diclofenac Sodium, all extracts F-4 and F-7 possessed good anti-arthritic activity based upon various Physical, Hematological, Antioxidant and Histopathological parameters. The irritation index was calculated for intact and abraded skin is 0.0 and 0.0 respectively. The result obtained

from the present study concludes that the "F4, F7 & F4+F7" was non-irritating on intact skin as well as abraded skin. Formulations F-4 containing extracts of *Bacopa monnieri* produced good synergistic effects and inhibited paw edema even more than the Standard drug. F-4 and F-7 also normalized the body weight of arthritic animals.

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