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RESEARCH ARTICLE

Phytochemical Profiling of *Phyllanthus niruri* Leaf Ethanolic Extract and its Potential in Rheumatoid Arthritis in Modulation of Inflammatory Pathways

Asheesh Kumar Gupta[1]*, Prem Saini[2], Vidhan Chand Bala[1], Jay Kumar Singh[1],
[1]School of Pharmaceutical Sciences, Faculty of Pharmacy,
IFTM University, Moradabad 244102, Uttar Pradesh, India.
[2] Dev Bhoomi Uttarakhand University, Dehradun, Uttarakhand, India- 248007
*Corresponding Author E-mail: **guptaasheesh01@gmail.com**

ABSTRACT:

Background: *Phyllanthus niruri*, also known as Bhumi Amla and belonging to the Euphorbiaceae family, has been traditionally used for its liver-protective and anti-inflammatory properties. This study explored the potential of its ethanolic leaf extracts to combat rheumatoid arthritis through various in vitro and in vivo approaches. **Methods:** The ethanolic extract of *Phyllanthus niruri* leaves was prepared using Soxhlet extraction and then underwent phytochemical analysis. Its anti-inflammatory properties were evaluated through in vitro tests that involved stabilizing human red blood cell (HRBC) membranes and preventing protein denaturation. To assess its anti-rheumatoid arthritis efficacy in vivo, arthritis was induced in rats using Complete Freund's adjuvant (CFA), and the extract was administered at doses of 200 and 400 mg/kg, with Leflunomide as a reference. Hematological and histopathological evaluations were conducted to verify effectiveness. **Results:** The extract showed notable stabilization of HRBC membranes (80.3% at 125 µg/mL) and inhibition of protein denaturation (70.2% at 500 µg/mL). In vivo, oral administration led to a significant reduction in paw swelling (92.8%) and joint diameter (66.9%) by day 15. Hematological parameters, including hemoglobin and RBC counts, improved significantly, while ESR and CRP levels decreased. Histopathological analysis revealed protection of cartilage and synovial structures compared to arthritic controls. **Conclusion:** The findings suggest that the ethanolic extract of *Phyllanthus niruri* exhibits strong anti-arthritic effects, likely through the suppression of inflammatory mediators and oxidative stress. These results support its potential as a natural treatment for rheumatoid arthritis.

KEYWORDS: *Phyllanthus niruri*, Bhumi Amla, inflammation, autoimmune disease, and Rheumatoid arthritis.

INTRODUCTION:

A chronic inflammatory disease, rheumatoid arthritis (RA) is express as inflammation, deformity, cartilage deterioration, and discomfort in the joints and synovial membranes. It can lead to limited limb motions and loss of joint function[1,2]. The proximal interphalangeal, ankle, wrist, metacarpophalangeal, and metatarsophalangeal joints are all affected by the regular progression of RA[3,4]. About 1% to 2% of the global population suffer with this immune-inflammatory arthritis[5]. It is linked to both a low quality of life and much greater lifetime costs. Researchers found that synovial inflammation is caused by the release of pro-inflammatory markers into joints, too the entry of immune-inflammatory cells[6,7]. In addition to activating primary nociceptive fibres, the manufacture of inflammatory cytokines counting

prostaglandins and leukotrienes causes joint increased pain response and painful joint[8]. Furthermore, cyclooxygenase (cox) enzymes are essential for immune cell activation and inflammatory cytokine production[9,10]. Therefore, it is hypothesized that one of the key processes in the pathophysiology of RA is the production of free radicals[11]. Many efforts have been made over the last few years to make innovative therapeutic constituents for the treatment of RA based on these crucial mechanisms[12].

The perfect medication for this devastating inflammatory disease has not yet been discovered by medical science. Numerous medication classes are available, but they have many undesirable side effects and little effectiveness[13]. They are also contraindicated in a number of conditions. Therefore, scientists have been searching for more recent medications that could manage the unchecked, abnormal inflammation without having negative side effects. In recent years, naturopathy has grown in popularity because people think that these medications are sufficiently efficient and don't cause any negative side effects[14]. They also have the added benefit of being affordable and easily accessible, which is crucial in developing nations. To address a variety of health issues, people have begun utilizing nutraceuticals, phytonutrients, and herbal medicines[15, 16]. Around the world, it has caused a rise in people's interest in natural medicines. For rheumatoid arthritis (RA), however, allopathic therapy has been examined in greater detail than herbs and herbal medicine[17]. There is a huge discrepancy between their widespread use and people's perceptions of their effectiveness and what science has shown. Practitioners of alternative medicine, primarily Ayurveda and Unani, have utilized a variety of botanical extracts as anti-rheumatic medicines[18]. The anti-rheumatic phytoconstituents in these herbal remedies serve as the basis for their use[19]. Therefore, prior to their biological examination, these chosen plants underwent pharmacognocal and phytochemical analysis[20,21].

Phyllanthus niruri, commonly known as “Iyin Olobe” among the Yoruba people of Nigeria, “Dukung anak” in Malaysia and “Bhumi Amla” in India, be the member of *Euphorbiaceae* family[22]. This herbaceous annual plant is measured a common tropical weed that originally appeared from tropical America and later spread across other tropical and subtropical regions. It succeeds mostly in moist and humid wastelands, where it grows as a hardy weed[23]. The genus *Phyllanthus* contains over 500 species distributed across temperate and tropical climates, between which *Phyllanthus niruri* is one of the most distinguished[24, 25]. The plant is a small, erect herb, typically attaining a height of 30-40 cm, and is categorised by its tiny, alternate leaves, yellowish-green flowers, and smooth, capsule-shaped green fruits (**Figure 1**). The flowers possess five white sepals and apically pointed anthers, while the seeds are longitudinally rugose with smooth, fruiting pedicels[26]. Traditionally, it has been utilized in various indigenous medical systems, particularly Ayurveda, where it has been prescribed for centuries for the treatment of urolithiasis, hepatitis, asthma, and jaundice. Due to its extensive ethnomedicinal applications, *Phyllanthus niruri* is regarded as the most significant traditional therapeutic plants in India[27, 28].

Figure 1: Life of *Phyllanthus niruri*

MATERIAL AND METHODS:

Reagents and chemicals

All chemical and reagent utilized in this study was of analytical quality. Chloroform, petroleum ether, hydrochloric acid, sodium hydroxide, copper sulfate, acetic anhydride, glacial acetic acid, sulfuric acid, n-butanol, α-naphthol, N-hexane, ethyl acetate, and silica gel-G were utilized as organic solvents for the extraction of plant material. All of these were supplied by SD Fine Chemicals, Mumbai, India.

Collection and authentication plant material

From March to April of 2025, fresh plants were gathered from Moradabad. The freshness and leaves quality of the samples were taken into consideration when choosing them. Dr. Ashok Kumar, PhD, Head of the Botany Department at IFTM University Moradabad, Uttar Pradesh, verified the authenticity of the sample (ref. no.2025/SOS/BOT/191).

The extraction technique

Petroleum ether was used to extract the dried leaves of *Phyllanthus niruri* for up to 72 hours at 60–80 degrees Celsius. Following the extraction technique, the solvent was extracted and dark redcoll oil was extracted. Following petroleum ether extraction, the marc was dried and subsequently extracted using 99% v/v ethanol for up to 72 hours in a Soxhlet system. To get an average yield, the extract was evaporated at lower pressure. Its ability to recognize learning and memory activities was determined by removing the solvent under

reduced pressure, which left behind a sticky residue with a brownish black appearance. CMC (1% w/v) was used as a suspending agent to create a specific concentration in the test solution of *Phyllanthus niruri* leaves extract[29,30].

Photochemistry screening

The extracts of *Phyllanthus niruri* leaves extract were subjected to a phytochemical parameter test. Flavonoids, saponins, alkaloids, tannins, sterols, proteins, carbohydrates, amino acids, and lipids were identified in the extracts[31, 32].

Animals

Wister albino rats weighing 150-200g of either sex were used in the experiments. The animals were purchased from IFTM University's animal house in Moradabad and kept on a normal diurnal cycle (12 h. light, 12 h. dark) at a room temperature of roughly 24 to 26°C. They were also agreed unrestricted access to water and regular food pellets. Prior to being exposed to behavioral tests, animals will acclimate for a minimum of 10 days[32].

Acute oral toxicity study

A male Westar albino rat was used in an acute oral toxicity investigation in accordance with OECD (organization for economic co-operation and development) guideline 423. Following an overnight fast, the scheduled animals were split up into 4 experimental groups, each containing of 3 animals. After feeding, the animals were observed every hour for seven days, and then every four hours on occasion[29, 32, 33].

***In vitro* HRBC membrane stabilization activity**

The anti-inflammatory activity was measured by calculating the stabilization of human red blood cell (HRBC) membranes. Fresh human blood (10 mL) was collected from a healthy volunteer who had abstained from NSAID use for at least two weeks prior to the experiment. The blood was transferred into heparinized centrifuge tubes and centrifuged at 3000 rpm for 10 minutes. The obtained cells were washed three times with equal volumes of saline solution to obtain a 10% v/v HRBC suspension.

Different concentrations (100, 200, 300, 400, and 500 µg/mL) of the ethanolic extract of *Phyllanthus niruri* were prepared. For each concentration, 1 mL of phosphate buffer, 2 mL of hypotonic saline, and 0.5 mL of HRBC suspension were mixed. The mixtures were incubated at 37°C for 30 minutes, followed by centrifugation at 3000 rpm. The absorbance of the supernatant was slow at 560 nm using a spectrophotometer to determine hemoglobin release. Normal saline helped as the control, while Leflunomide (10 µg/mL) was used as the reference standard[34]. The subsequent formula was used to calculate the % of HRBC membrane stabilization (assuming that 100% of the haemolysis generated in hyposaline):

% Inhibition of hemolysis =
(1-[(TS2-TS1)]/[(TS3-TS1))] ×100

Where, TS1 = Test sample in isotonic solution; TS2 = Test sample in hypotonic solution and TS3 = Control sample in hypotonic solution.

***In vitro* Proteins denaturation activity**

The in vitro anti-inflammatory activity was assessed using the protein denaturation method. The reaction mixture (5 mL total volume) consisted of 0.2 mL of fresh egg albumin, 2.8 mL of phosphate-buffered saline (PBS, pH 6.4), and 2 mL of the ethanolic extract of *Phyllanthus niruri* at varying concentrations (100, 200, 300, 400, and 500 µg/mL). Double-distilled water was used as the control, while Leflunomide (10 µg/mL) served as the reference standard.

The mixtures were incubated at (37 ± 2) °C for 15 minutes in a biological oxygen demand (BOD) incubator, followed by heating at 70 °C for 5 minutes. After cooling, the absorbance of each sample was measured at 660 nm using the respective vehicle as a blank[35,36]. The percentage inhibition of protein denaturation was calculated using the following formula:

% Inhibition of proten denaturation =
(1-[(TS2-TS1)]/[(TS3-TS1))] ×100

Where, TS1 = Test sample before heated; TS2 = Test sample after heated and TS3 = Control sample after heated.

***In vivo* anti-arthritic activity**

Adjuvant-induced arthritis (AIA)

Arthritis was caused in rats via intradermal injection of 0.1 mL CFA near the tail's base. Animals were categorized into 5 groups (n = 6):
Grp I: Control group (vehicle)
Grp II: Arthritic control (CFA only)
Grp III: Standard (Leflunomide 10 mg/kg)
Grp IV: *Phyllanthus niruri* leaves extract (200 mg/kg)
Grp V: *Phyllanthus niruri* leaves extract (400 mg/kg)
Treatment was given by orally for 21 days. Paw volume and joint diameter were measured periodically using a plethysmometer and vernier caliper[37].

Hematological and biochemical parameters

Upon conclusion of the study, blood samples were obtained for the assessment of haemoglobin (Hb), red and white blood cell (RBC) counts and erythrocyte sedimentation rate (ESR) were evaluated utilizing commercial kits[38].

Histopathological Examination
For histological analysis, the ankle joints of three rats from each group were isolated, cleansed, and preserved in cold physiological saline prior to being kept in a 10% formaldehyde solution. During the staining process, tibiotarsal joint sections were sliced to a thickness of 5 µm using a microtome, deparaffinised, and subsequently stained with hematoxylin and eosin stain. Mounting the specimens on slides was done using Distrene Phthalate Xylene (DPX). A light microscope (Olympus DP71, Australia) was employed to examine specimen slices to get a fundamental understanding of its histological characteristics and cellular infiltration in the epithelium and sub-epithelium[39,40].

Statistical analysis
Data were presented as mean ± SEM (n = 6). Statistical significance was calculated using one-way ANOVA, succeeded by Tukey's post hoc test. Changes were deemed significant at $p < 0.05$.

RESULTS AND DISCUSSION:

Qualitative phytochemical screening
Phyllanthus niruri leaves ethanolic extract were screened contents using a variety of phytochemical tests. The phytochemical screening confirmed the existence of amino acid, proteins, flavonoid, tannins, poly phenols and sterols in the ethanolic extract of *Phyllanthus niruri* leaves.

HRBC membrane stabilization activity
Ethanol extracts shown notable membrane stabilizing action in the current investigation. The ethanolic extract demonstrated 80.29% membrane stabilizing action at 125µg/ml, while the standard medication Leflunomide demonstrated 93.56% membrane stabilizing activity at the same concentration (**Figure 2**).

Proteins denaturation activity
Protein denaturation was significantly inhibited by the ethanolic extract. At 500µg/mL, the ethanolic extracts' percentage suppression of protein denaturation was 70.20%. At the same concentration, Leflunomide exhibited 86.25% membrane stabilizing efficacy (**Figure 3**).

Acute oral toxicity studies
The oral toxicity of *Phyllanthus niruri* leaves extract at dosages of 2000 mg/kg was investigated. During the acute toxicity investigation, the extract did not result in any toxicity or death in the treated animals. Consequently, 200 and 400 mg/kg were chosen for experimental research.

Freund's adjuvant-induced paw volume
A mixture of heat-killed *Mycobacterium tuberculosis* in paraffin oil, CFA produces extremely painful reactions in administration site. In this work, CFA injection significantly increases paw volume ***(P<0.05) when compared to normal control. When related to the harmful control, leaves extract significantly **(P<0.05) reduced paw volume starting on day 15. On day 15, leaves extract at 400 mg/kg demonstrated the highest inhibition of paw volume (92.76%), whereas on the same day, 200 mg/kg demonstrated 91.73% inhibition (**Table 1**).

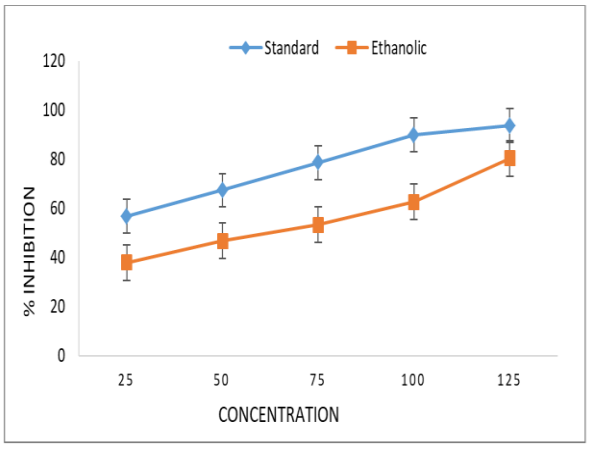

Figure 2: Displays the *Phyllanthus niruri* leaves extract in vitro membrane stabilizing activity.

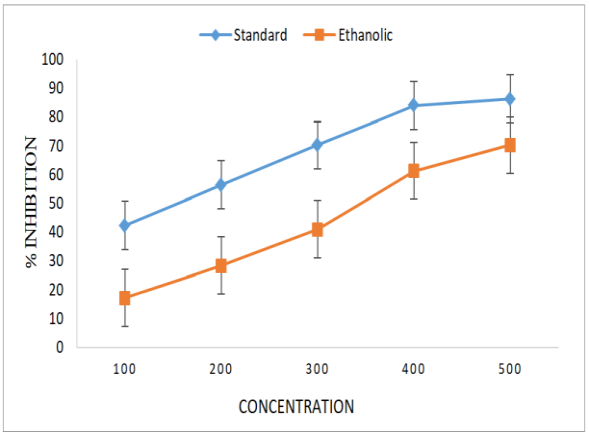

Figure 3: Display the *Phyllanthus niruri* leaves extract in vitro protein denaturation activity.

Table 1: Effect of *Phyllanthus niruri* leaves extract on paw volume caused by Complete Freund's adjuvant.

Day	Groups				
	I	II	III	IV	V
3	0.68±1.22	3.56±0.51	2.08±0.46	2.82±0.04	2.46±0.51
5	0.86±0.95	6.12±1.41***	4.1±0.45**	5.66±0.64	4.22±0.53**
7	0.86±0.95	8.42±1.88***	4.9±1.19**	7.78±0.53	5.8±0.51b*
9	0.86±0.95	10.96±1.67***	9.42±1.97	10.06±0.43	9.78±0.64
11	0.876±0.8	13.4±1.22***	10.3±1.93**	12.62±0.48	11.14±0.51**
13	0.87±0.95	13.9±0.77***	9.52±2.11**	11.96±0.43	11.82±0.64**
15	0.87±0.95	14.48±1***	9.74±2.84**	11.06±0.43**	10.52±1.38**

The findings, which are given as mean ± SEM for n = 6 mice, were examined by two-way ANOVA and post-hoc testing for (***P<0.005) vs control and (**P<0.05) vs disease control.

Table 2: *Phyllanthus niruri* ethanolic extract's impact on joint diameter.

Day	Groups				
	I	II	III	IV	V
3	0.10±0.03	0.64±0.05***	0.40±0.07	0.44±0.05	0.48±0.06
5	0.10±0.03	1.10±0.07***	0.56±0.05**	0.84±0.50	1.00±0.05
7	0.10±0.03	1.36±0.09***	0.80±0.07	1.12±0.05	1.44±0.05
9	0.10±0.03	2.04±0.10***	0.96±0.09**	1.56±0.05	1.88±0.03
11	0.10±0.03	2.44±0.09***	1.06±0.06**	1.38±0.05**	2.16±0.06
13	0.10±0.03	3.22±0.09***	0.78±0.05**	1.28±0.05**	1.94±0.05**
15	0.10±0.03	3.50±0.13***	0.70±0.07**	1.16±0.05**	1.74±0.05**

The findings, which are given as mean ± SEM for n = 6 mice, were examined by two-way ANOVA and post-hoc testing for (***P<0.005) vs control and (**P<0.05) vs disease control.

Freund's adjuvant-induced joint diameter

The CFA injection caused a progressive increase in joint width in toxic control compared to normal control (***p<0.05). On day 15th, Leflunomide (10 mg/kg) significantly **P<0.05 reduced joint diameter by 95.10% in comparison to toxic control. Joint diameter decreased significantly (**P<0.05) in the animals treated with the *Phyllanthus niruri* leaves extract at 400 and 200 mg/kg on day 15, with 66.85% and 50.28%, respectively (**Table 2**).

Effect of haemoglobin in arthritis rats

When experimental animals were given 0.1 mL CFA, their haemoglobin content significantly (***P<0.5) decreased in differentiation to the control group, which had a haemoglobin content of 6.43±0.45 mg/dL. Comparing the ethanolic extract (400 and 200 mg/kg) to the disease control, the haemoglobin content rose by 38.29% and 27.42%, respectively, considerably (**P<0.001) (**Table 3**).

Table 3: Effect of *Phyllanthus niruri* ethanolic extracts on haemoglobin.

Groups	Hb (mg/dL)
I	13.42±0.10
II	6.43±0.45*
III	12.04±0.08***
IV	10.42±0.13***
V	8.86±0.28***

The findings are given as Mean ± SEM (n=6), and ANOVA and the Tukey test are used to compare the results (***P<0.001) to the control and the disease control respectively.

Effect of RBC count in arthritis rats

When 0.1 mL CFA was administered to experimental animals, the RBC count was considerably (***P<0.001) decreased in differentiation to the control group, which was 4.14±0.17 million/mm^3. In comparison to toxic control, Leflunomide 10 mg/kg significantly (**P<0.001) raised the RBC count by 47.79% under comparable experimental settings. Comparing the RBC count to the disease control, the ethanolic extract (400 and 200 mg/kg) considerably (***P<0.001) raised it by 34.80% and 24.03%, respectively (**Table 4**).

Table 4: Effect of *Phyllanthus niruri* ethanolic extracts' on RBC counts.

Groups	RBC (mg/dL)
I	8.62±0.11
II	4.14±0.17***
III	7.93±0.02***
IV	6.35±0.05***
V	5.45±0.23ns

The findings are given as Mean ± SEM (n=6), and ANOVA and the Tukey test are used to compare the results (***P<0.001) to the control and the disease control, respectively.

Effect of ESR in arthritis rats

When experimental animals were given 0.1 mL CFA, their ESR increased significantly (***P<0.001) in differentiation to the control group, which had an ESR of 23.2±0.66 mm/h. In comparison to the disease control, ethanolic extract (400 and 200 mg/kg) suggestively (***P<0.001) decreased ESR by 51.29% and 36.47%, respectively (**Table 5**).

Table 5: Effect of *Phyllanthus niruri* ethanolic extracts on the rate ESR labels.

Groups	ESR (mm/h)
I	12.60±0.40
II	23.2±0.66 ***
III	13.8±0.37 ***
IV	15.4±0.50 ***
V	17±0.31 ***

The findings are presented as Mean ± SEM (n=6), and ANOVA and the Tukey test are used to compare the results (***P<0.001) to the normal control and the toxic control, respectively.

Analysis of joint histopathology

Figure A shows the normal synovial membrane, articular cartilage, and epiphyseal plate in the histopathology of the normal ankle joint stained with hematoxylin and eosin. Massive punus development and chondrocyte loss were seen in the disease control group (**Figure 4b**). Rats given *Phyllanthus niruri* (ethanolic extracts at 400 mg/kg) (**Figure 4e**) and Leflunomide (10 mg/kg) (**Figure 4c**) demonstrated notable protection against inflammatory cells, cartilage degradation, cell necrosis, and moderately irregular synovial space. Rats given 200 mg/kg of *Phyllanthus niruri* ethanolic extracts (**Figure 4d**) displayed abnormal cartilage degradation, cell necrosis, and synovial destruction in contrast to those given 400 mg/kg.

Figure 4: Hematoxylin and eosin stained ankle joint histopathological study (a) control, (b) disease control, (c) standard drug treatment (10 mg/kg), (d) *Phyllanthus niruri* 200 mg/kg, and (e) *Phyllanthus niruri* 400 mg/kg ethanolic extract.

CONCLUSION:

Significant anti-arthritic proparty was shown by the ethanolic extract of *Phyllanthus niruri* leaves in both in vitro and in vivo models. The extract contained flavonoids, saponins, alkaloids, steroids, amino acids, carbohydrates, proteins, and tannins, according to phytochemical examination. According to *in vitro* tests, the extract's effects on membrane stability and protein denaturation inhibition were on par with those of the common medication leflunomide. At 400 mg/kg, the extract dramatically decreased paw edema and joint diameter in rats with arthritis caused by Complete Freund's Adjuvant. It also improved hematological indicators such as hemoglobin and RBC count. According to these results, *Phyllanthus niruri* leaves extract has encouraging anti-arthritic qualities that call for more research in order to determine whether it may be used therapeutically to treat arthritic disorders.

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CONFLICT OF INTEREST:

No conflict of interest.

ETHICAL APPROVAL:

Reg. no. 837/PO/ReBiBt/S04CPCSEA

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