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Original Article

Phyllanthin Topical Formulation with Penetration Enhancers Alleviates Imiquimod-Induced Psoriasis in Rats via Modulation of Inflammatory Biomarkers and Oxidative Stress Markers

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ABSTRACT

Background: Psoriasis is a persistent inflammatory skin condition marked by abnormal epidermal proliferation and excessive keratinization, impairing effective drug absorption. To overcome these barriers, topical formulations containing skin permeability enhancers are widely employed to improve drug delivery and therapeutic effectiveness. **Materials and Methods:** Phyllanthin isolated from *Phyllanthus acidus* leaves was formulated into an ointment alongside permeability enhancers, eucalyptus, basil, and cardamom oils. Psoriasis like symptoms were induced in Wistar rats using Imiquimod (IMQ) to assess the efficacy of phyllanthin ointments on the ear and dorsal skin. Ointment formulations containing 1% and 2% phyllanthin were applied topically once daily at 200 and 400 mg daily for ten days post-induction of psoriasis. The Psoriasis Area and Severity Index (PASI), along with the thickness of the ear, body and relative organ weight, antioxidant and anti-inflammatory markers, and histopathological changes, were all assessed in the study. **Results and Discussion:** The ointments were found to be physically stable up to 37°C. The 2% phyllanthin ointment, containing 0.5% of each essential oil, produced a profound lowering of PASI score ($p<0.01$) and ear thickness ($p<0.001$) in concern to untreated controls. No signs of acute topical toxicity were observed with phyllanthin treatment, and it did not negatively affect body weight or organ weight indices. Phyllanthin significantly increased hydroxyproline, reduced lipid peroxidation, IL-6, TNF-α, ($p<0.05$-0.001) content, keratinocyte hyperproliferation, and lymphocytic infiltration in the affected tissues. **Conclusion:** Phyllanthin ointment with essential oils effectively alleviated psoriatic symptoms and demonstrated strong antioxidant and anti-inflammatory activity, indicating its potential for once-daily use in managing psoriasis.

Keywords: Inflammation, Penetration Enhancer, Phyllanthin, *Phyllanthus acidus*, Psoriasis, Topical Formulation.

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INTRODUCTION

Psoriasis is a long-term, non-communicable autoimmune disease with a marked impact on both the skin and joints. It is most commonly manifested by the formation of erythematous, scaly plaques on the skin, referred to as psoriatic plaques. These lesions are predominantly observed on extensor surfaces such as the elbows and knees but may also develop on other areas, including the scalp and genital regions. The immune system has a pivotal impact on the pathophysiology of psoriasis, driving abnormally accelerated skin cell turnover, excess keratinocyte accumulation on the skin, and subsequent tissue damage. Psoriasis is a persistent inflammatory condition connected to metabolic syndrome with a marked increase in IL-1, IL-4, IL-6, IL-8, IL-12, and TNF inflammatory cytokines levels (Chu *et al.*, 2011). Psoriasis exhibits variability in prevalence across ethnic groups, affecting approximately 2% of the global population (Schön and Boehncke, 2005).

Phyllanthus species, a medicinal plant known for its diverse therapeutic applications, has been widely investigated for anti-inflammatory effects (Kassuya *et al.*, 2005). This species has been traditionally employed in systems like Ayurveda and Traditional Medicine for its therapeutic benefits. Amla (*Phyllanthus acidus* L.; Family: Phyllanthaceae) holds a significant status due to its diverse pharmacological properties. Amla is utilized for the management of inflammation, gastrointestinal ailments, rheumatism, bronchitis, asthma, respiratory ailments, diabetes, and hepatitis (Tan *et al.*, 2020). Among its bioactive constituents, phyllanthin and hypophyllanthin, polyphenolic

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lignan have been recognized as a key compound responsible for the pharmacological effects of amla leaves.

Phyllanthin has predominant antioxidant, anti-inflammatory, immunomodulatory, anticancer, and hepatoprotective activities (Omoruyi *et al*., 2004). Phyllanthin has attracted significant interest due to its potent biological effects, particularly in regulating inflammatory pathways (Harikumar and Kuttan, 2017). Phyllanthin has the potential to modulate inflammatory processes by influencing the release of signaling molecules associated with inflammation through the NF-κB, MAPKs, and PI3K-Akt signaling cascade. Consequently, phyllanthin has promising prospects for managing inflammation in diverse pathological conditions (Harikrishnan *et al*., 2018). Phyllanthin also demonstrated promising anti-inflammatory properties by inhibiting proinflammatory cytokines (IL-1 and TNFα) mRNA expression. Additionally, it down-regulates Th1 and Th2 cytokines, thereby modulating immune system responses effectively (Ilangkovan *et al*., 2016). Phyllanthin inhibits pro-inflammatory cytokines production and modulates immune responses by regulating NF-κB inflammatory pathways, which may contribute to a reduction in inflammation associated with psoriatic lesions (Jagtap *et al*., 2016). The ability of phyllanthin to suppress pro-inflammatory cytokine production and regulate immune responses could effectively reduce inflammation in psoriatic lesions.

Psoriasis is defined by persistent inflammation primarily mediated by TNF-α, IL-17, and IL-23. Overactive immune responses, particularly the Th1 and Th17 networks, are involved in developing psoriatic plaques and the abnormal proliferation of keratinocytes (Hu *et al*., 2021). Psoriasis leads to the formation of thickened and hyperkeratotic plaques, which impede drug penetration and diminish the effectiveness of conventional treatments. Skin penetration enhancers improve the diffusion of drugs into deeper skin layers and enhance their systemic absorption.

There has been a growing interest in utilizing herbal medicines as an alternative or complementary approach to managing psoriasis like chronic dermatological conditions in recent years. Researchers are continuously developing innovative dosage forms to enhance patient compliance and provide effective antipsoriatic responses. Among these, topical drug delivery systems have gained significant attention due to the accessibility of the target site. Effective topical antipsoriatic treatment is impacted by the barrier properties of skin along with the physicochemical properties of drug, vehicle, and the interactions between the drug, vehicle, and skin layers (Traub and Marshall, 2007). The effectiveness of psoriasis treatments is often limited by the impaired penetration of topically applied drugs due to the formation of hyperkeratotic plaques obstructing drug delivery.

In the current work, we aimed to evaluate the therapeutic effectiveness of the phyllanthin ointment, organic penetration enhancers, and its mode of action as a novel topical treatment for psoriasis. Eucalyptus, basil, and cardamom oil are included in phyllanthin ointment to increase skin permeability (Choi *et al*., 2012). Phyllanthin ameliorates Imiquimod (IMQ) induced lipid peroxidation, IL-6, and TNF-α overproduction, enhances hydroxyproline synthesis, and inhibits keratinocyte hyperproliferation. This study is the first to provide data substantiating the therapeutic potential of phyllanthin in alleviating psoriasis coapplied with penetration enhancers emphasizing antioxidant and anti-inflammatory profiles.

MATERIALS AND METHODS

Harvesting and authentication of plant material

Amla leaves were plucked from Sudumbare, Pune region, Maharashtra, India, in September 2021. The herb was identified and authenticated by botanist Dr. S. P. Giri, Research Coordinator, Department of Botany, and Dr. A.S Wabale, HOD, PVP College of Arts, Science and Commerce, Loni-Pravaranagar, Maharashtra (PVPC/Bot/2021-22/07). Leaves were thoroughly washed, air-dried for a week, and then shredded before being blended into a fine powder using a blender.

Extraction of phyllanthin

Leaves of *P. acidus* were cleaned, shade dried away from direct sunlight and ground into a coarse powder. The coarse material was extracted with absolute methanol for 6 hr at 40°C in a Soxhlet extractor. The extract was vacuum evaporated below 40°C. The methanolic extract was solubilized in chloroform and then filtered through Whatman filter paper. Phyllanthin was isolated by preparative TLC, applying hexane, acetone, and ethyl acetate (7:2:1) as the mobile phase. The methanolic solution of the extract was applied to thick pre-coated TLC plates. The plates were air-dried, developed in TLC chamber, and dipped in the mobile phase. After development, plates were dried in the open air and analyzed with a CAMAG TLC scanner at 254 nm to visualize the separated bands. The observed band with an R_f value of 0.32 corresponding to phyllanthin was then scraped off and eluted with absolute methanol (Thakur and Bigoniya, 2014). Several rounds of preparative TLC were performed to recover a sufficient quantity of separated compounds and recrystallized using methanol.

Characterization of isolated phyllanthin

Organoleptic characteristic

The organoleptic properties of the phytochemicals were assessed by observing their visual characteristics, including colour, odour, taste, and physical form.

Melting point

The melting point of phyllanthin was measured by applying the capillary method with a Thiele tube apparatus (Gokhale and Kokate, 2008).

Thin Layer Chromatography (TLC)

Separation was achieved chromatographically on a Camag TLC chamber using 10×10 cm silica gel plates (100 µm). Camag Linomat applicator was utilized to apply the sample using acetonitrile: water (80:20) as mobile phase and analyzed densitometrically at 254 nm using a UV detector (Thakur and Bigoniya, 2014).

Calibration curve

The maximum absorbance wavelength (λ_{max}) of phyllanthin was determined by applying a UV-visible spectrophotometer (Model 1800, Shimadzu, Japan). Stock solution of phyllanthin (1 mg/ 10 mL) was prepared in absolute ethanol. Stock aliquots of 20, 40, 60, and 80 mL were diluted with methanol to a final volume of 100 mL, resulting in working solutions of 20, 40, 60, and 80 µg/ mL, respectively. The absorbance of these solutions was recorded at 230 nm spectrophotometrically (Thakur and Bigoniya, 2014).

Fourier Transform Infrared Spectroscopy (FTIR)

IR spectroscopy of isolated phyllanthin was conducted to assist in their identification. The analysis was conducted utilizing the Attenuated Total Reflectance (ATR) method with a Bruker Alpha FTIR spectrophotometer (Sethiya *et al*., 2015).

High Performance Liquid Chromatography (HPLC)

Standard phyllanthin was procured from Kavya Pharma (Gujarat, India). The HPLC separation was performed on Agilent (1100, Agilent Technologies, Germany) system integrated with reversed-phase C18 column (Hypersil ODS, 4.6×250 mm i.d., 5 µm), DAD (G-13148) detector, autosampler and a G1310A ISO Pump. Precisely weighed isolated and standard phyllanthin, 1 mg each, was solubilized in methanol (1 mL) and subjected to 30 min sonication. After that, the solution was passed through 0.22 µm filter (Rankem) and subsequently utilized for HPLC analysis. The Mobile phase composed of water: methanol (34:66) was subjected to isocratic elusion at 1.0 mL/min flow rate. The sample (20 µL) was injected at 40°C and detected at 254 nm wavelength for 10 min (Annamalai and Lakshmi, 2009).

Development of phyllanthin ointment

The ointment formulation comprised 0.5 g each of wool fat, cetostearyl alcohol, hard paraffin, liquid paraffin, and 8 g of yellow soft paraffin. The process commenced with melting the grated hard paraffin on an evaporating dish placed over a water bath. After it had completely melted, the other components were incorporated and stirred gently to ensure thorough mixing and uniformity. Subsequently, the ointment was formulated by adding precisely weighed quantity of extracted phyllanthin as outlined in Table 1. In addition, eucalyptus, basil, and cardamom oils were incorporated and mixed with base to form a smooth paste. Gradually, more base was added until a consistent ointment was achieved and the final mixture was then transferred into suitable containers (Kolhe *et al*., 2018).

Evaluation of ointment

Organoleptic properties such as appearance, colour, odour, texture, phase separation, and homogeneity were examined as described by Shaikh *et al*., (2018).

pH

The ointment (1 g) was mixed with distilled water (100 mL), and kept aside for 2 hr and pH was measured with a digital pH meter (Tecnolab Instrument Services, Gujrat) (Kolhe *et al*., 2018).

Spreadability

The spreadability of the ointment was assessed by applying the "slip and drag" method, with a device consisting of two glass plates of identical dimensions and a flat wooden block propped up at one end by a pulley. To assess the drag and slip characteristics, 2 g of the ointment were applied to the lower plate, and the apparatus was suspended using a hook. To ensure release of trapped air and to achieve a uniform film between the slides 1 kg weight was positioned on top of the slides. A 50-g weight was affixed to the hook to create a pulling force, and the duration required for the top slide to traverse 9 cm was measured (Elena *et al*., 2022).

$$S=M\times L/T$$

S = Spreadability; M = Weight tide to upper slide; L = Length of glass slide; T = Time taken to separate slide.

Extrudability

The extrudability test evaluates the force needed to expel a formulation from the collapsible tube after applying a predetermined weight. A clean, lacquered aluminum collapsible tube was filled with approximately 5 g of phyllanthin ointment, which was sealed at one end, and to avoid rollback a clamp was positioned. The extrudability was assessed by measuring the quantity of ointment extruded in the form of a 0.5 cm ribbon over a duration of 5 sec under a specific load. The weight of the extruded ointment was recorded, and the percentage of ointment extruded was calculated (Shaikh *et al*., 2018).

$$\% \text{ Extrudability} = \text{Amount of ointment extruded from tube} / \text{Total amount of ointment filled in tube} \times 100.$$

Viscosity

Rheological assessments were performed utilizing a Brookfield Synchro-Lectric Viscometer (Model RVT). A 50 g sample was

allowed to acclimate for 5 min in a beaker and dial reading was measured at 10 rpm with a T-D spindle. Measurements were conducted at room temperature and repeated three times. The viscosity, expressed in centipoises, was determined by multiplying dial readings with coefficients provided in the Brookfield viscometer catalog. Averages of the three sets of measurements were calculated (Shaikh *et al*., 2018).

Loss in weight

The Loss on Drying (LOD) was evaluated by transferring ointment to a petri dish and dried in a water bath maintained at 105°C (Nalla and Chinnala, 2017).

Stability

Physical stability of the ointment was tested in accordance with ICH guideline Q1C over four-week period at 40°C, 25°C, and 37°C temperatures (Kavitha *et al*., 2013).

In vivo study

Animal

Wistar albino rats, aged 6-8 weeks and weighing 180-200 g, were obtained from the BIOCYTE Institute of Research and Development animal facility in Sangli, Maharashtra, India. Before commencing the study, animals were acclimatized for a week in controlled environmental conditions, temperature 25±1°C, relative humidity 45-55%, and 12-hr light/dark cycle. Rats were provided with unrestricted access to standard feed and purified water. Experimental procedures performed adhered to the ethical standards of the Committee for Control and Supervision of Experiments on Animals (CCSEA). Institutional Animal Ethics Committee (2114/PO/Re/5/20/CPCSEA) reviewed and approved the study protocol (IAEC/Sangli/2023-24/11, dated October 11, 2023).

Skin sensitization

The acute dermal toxicity of ointments was assessed following the OECD guidelines 402 (Acute Dermal Toxicity: Fixed Dose Procedure). Rats were randomly assigned to two groups with six in each. The dorsal area of each rat was shaved to expose a 2×2 cm section of skin. Ointments at a concentration of 2% (w/w) were prepared based on body weight and applied to the shaved skin on the first day. Starting dosages of 200, 1000, and 2000 mg/kg were administered, with two animals per dosage being monitored for acute toxicity signs. Skin allergic reactions were assessed at 6, 24, 48, and 72 hr for indicators such as redness, erythema, and edema. The rats were observed over a 14-day period for changes in fur, sleep behavior, general activity, and mortality rates (Stallard *et al*., 2004).

Treatment group

The study was conducted over a 20-day period, divided into two phases: induction of psoriasis (days 1-10) and the treatment phase (days 11-20). Group I, serving as the vehicle control, consisted of uninduced rats treated with 200 mg of the ointment base alone. Psoriasis like dermatitis was induced in Groups II to V using topical application of IMQ. Group II was disease control, and Group III (positive control) was topically treated with 0.05% tretinoin cream once daily for 10 days. Groups IV and V were treated with formulations FIII and FIII*, containing 1% phyllanthin (2 mg/day) and 2% phyllanthin (4 mg/day), respectively, through daily application of 200 mg of ointment for 10 days. Psoriasis-like dermatitis was also induced in the right ear of rats using IMQ, and the treatments were administered during the treatment phase.

Imiquimod induced psoriatic rat model

The dorsal area of each rat was shaved to remove hair, and complete cleaning of the remaining hair was done with depilatory cream (Veet, Reckitt Benckiser Pvt. Ltd., India). Psoriasis was triggered in the shaved dorsal area by applying commercially available IMQ 5% cream (Glenmark, India). Once-daily topical application of 100 mg IMQ cream was carried out for ten consecutive days (Almudaris and Gatea, 2024). The following appearance of erythema and scaling with increased thickness indicated successful induction of psoriatic lesion.

Scoring of Psoriasis Area Severity Index (PASI)

The experiment was terminated on the 20th day, and the outcome evaluation was performed on 21st day. The PASI was used to assess skin inflammation intensity by visual evaluation of scaling (desquamation), erythema (redness), and thickness (induration). Each animal was individually graded for these three characteristics separately on a scale of 0 to 4 by one researcher. On this scale, 0 = none, 1 = slight, 2 = moderate, 3 = marked, and 4 = very marked, collectively resulting in a total score between 0 to 12. The thickness of the right ear measured with a Vernier caliper was used to assess skin inflammation before induction (Day 1), after induction (Day 10th), and on 21st day (Fredriksson and Pettersson, 2009).

Body weight, thymus and spleen weight

The body weight was recorded on the first, tenth, and 21st days. The thymus and spleen were extracted from each animal, kept in cold saline, blotted dry, and weighed.

Biochemical analysis

Rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) intraperitoneally and euthanised via cardiac puncture. Tissue samples harvested from the psoriatic skin (1 cm) and right ear were promptly rinsed with Tyrode solution. Tissues were then blotted, weighed, and treated with 6N HCl in a water bath (130°C) for 4 hr in sealed Pyrex-glass tubes to facilitate hydrolysis (Dwivedi *et al*., 2016; Edwards and O'Brien, 1980). A specimen

of skin tissue was preserved in 10% neutral buffered formalin (Sigma-Aldrich, Singapore) for histopathology.

Protein

Solution A (6 mL) and distilled water (1.2 mL) were added to tissue hydrolysate (1 mL) and incubated for 10 min at room temperature. Subsequently, solution B (3 mL) was added, and incubated for 30 min at room temperature, and final absorbance was recorded at 680 nm (Lowry *et al.*, 1951).

Hydroxyproline

The hydrolysate sample (20 µL) was combined with Chloramine T solution (50 µL) prepared in citrate buffer (containing 5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid). The mixture was incubated at ambient temperature for 20 min. Following this, Ehrlich reagent (50 µL) was mixed, and the final reaction mixture was incubated at 65°C for 15 min. Hydroxyproline levels were quantified by measuring absorbance at 550 nm and referencing a standard curve with concentrations ranging from 0 - 10 µg/mL (Edwards and O'Brien, 1980).

Lipid peroxidation

A homogenate consisting of skin and ear tissue was prepared by mixing 4 mL of tissue hydrolysate (10%) with 1.5 mL each of sodium dodecyl sulfate (8.1%), acetate buffer (pH 3.5; 20%), and thiobarbituric acid (0.8%). This mixture was subjected to heating at 95°C for 60 min and subsequently set aside to cool down. After this, 5 mL of n-butanol-pyridine (15:1) was added, thoroughly vortexed, and separated into phases. A UV-visible spectrophotometer was utilized to record the absorbance of the organic phase at 532 nm (Ohkawa *et al.*, 1979; Parmar *et al.*, 2021).

Cytokines

Pro-inflammatory cytokine, IL-6, and TNF-α contents were assayed utilizing enzyme-linked immunosorbent assay kits (Krishgen Biosystem, Mumbai). The assays were conducted following the manufacturer's guidelines included with the commercially available kits, utilizing tissue homogenates for the analysis (Parmar *et al.*, 2021).

Histopathological analysis

Skin tissue samples were processed into paraffin blocks, and sectioned to 250 µm slices. These sections were then stained with hematoxylin and eosin (Sigma-Aldrich, Singapore) and examined under a light microscope (Olympus BX51, Olympus Corporation, Japan) (Bharathi *et al.*, 2020).

Statistical Analysis

The research findings were represented as Mean±Standard Error of Mean and subsequently analyzed by applying one-way analysis of variance. The Newman-Keuls multiple comparisons test was employed to evaluate the statistical differences among various groups. Statistical analysis was performed by applying Graph Pad Prism, version 5, with a significance threshold established at $p<0.05$.

RESULTS

Characterization of isolated phyllanthin

Phyllanthin was successfully isolated with a yield of 0.42%, appearing as a greenish powder with a mild pungent odour. Its melting point was recorded between 195-198°C. TLC performed using acetonitrile: water (80:20) solvent system produced an R_f value of 0.63, and UV-visible spectrophotometric analysis revealed maximum absorbance at 230 nm, as Thakur and Bigoniya (2014) reported. A calibration curve plotted for phyllanthin demonstrated linearity between absorbance and concentration, with a regression coefficient 0.989 (Figure 1).

Characterization of phyllanthin ointment

The levigation technique was employed to achieve a homogenous incorporation of the phyllanthin and permeability enhancers into the ointment base. This method ensured uniform distribution of components within the formulation while maintaining its stability during storage. Each batch exhibited a smooth and uniform consistency, with no visible traces of separation. The ointment was characterized as soft, opaque, homogenous, oleaginous, devoid of grittiness, and free from phase separation. The physical properties of the ointment were assessed, which yielded favourable pH, spreadability, extrudability, viscosity, and moisture content. The spreadability of formulations ranged from

Table 1: Formulation of phyllanthin ointment.

Ingredients	Quantity (g)										
	P1	P1a	P1b	P2	P2a	P2b	P3	P3a	P3b	FIII	FIII*
Phyllanthin	0.05	0.05	0.05	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.2
Ointment Base q. s.	10	10	10	10	10	10	10	10	10	10	10
Eucalyptus oil	0.05	----	----	0.05	----	----	0.05	----	----	0.05	0.05
Basil oil	----	0.05	----	----	0.05	----	----	0.05	----	0.05	0.05
Cardamom oil	----	----	0.05	----	----	0.05	----	----	0.05	0.05	0.05

Formulation FIII and FIII* respectively 1% (10 mg/gm) and 2% (20 mg/gm) of phyllanthin with 0.5% of each essential oil component.

Table 2: **Physical evaluation of the phyllanthin ointment formulations.**

Formulation	pH	Viscosity at 10 rpm (CPS)	Spreadability (g.cm/s)	Extrudability (%)	Loss of drying (%)
P1	6.75±0.54	2456.49±72.85	12.83±1.11	82.63±3.20	25.45
P1a	6.82±0.78	2422.13±41.71	14.52±1.54	84.38±2.41	25.21
P1b	6.70±0.42	2468.63±45.26	14.36±1.53	87.85±4.54	27.33
P2	6.81±0.68	2472.22±34.29	15.02±2.52	85.08±2.53	26.81
P2a	6.75±0.52	2497.36±60.05	13.71±1.46	83.99±3.08	27.28
P2b	6.97±0.15	2513.80±24.62	12.01±1.14	85.92±2.93	25.65
P3	6.81±0.43	2542.79±45.18	16.54±2.47	87.16±2.06	27.80
P3a	6.58±0.85	2534.69±38.55	13.83±1.36	86.78±3.65	26.73
P3b	6.64±0.21	2429.44±40.89	14.69±1.85	82.83±3.79	26.62
FIII	6.99±0.10	1984.42±46.68	20.21±2.07	90.05±2.02	22.55
FIII*	7.03±0.32	2010.19±62.76	21.76±2.53	92.12±3.32	22.67

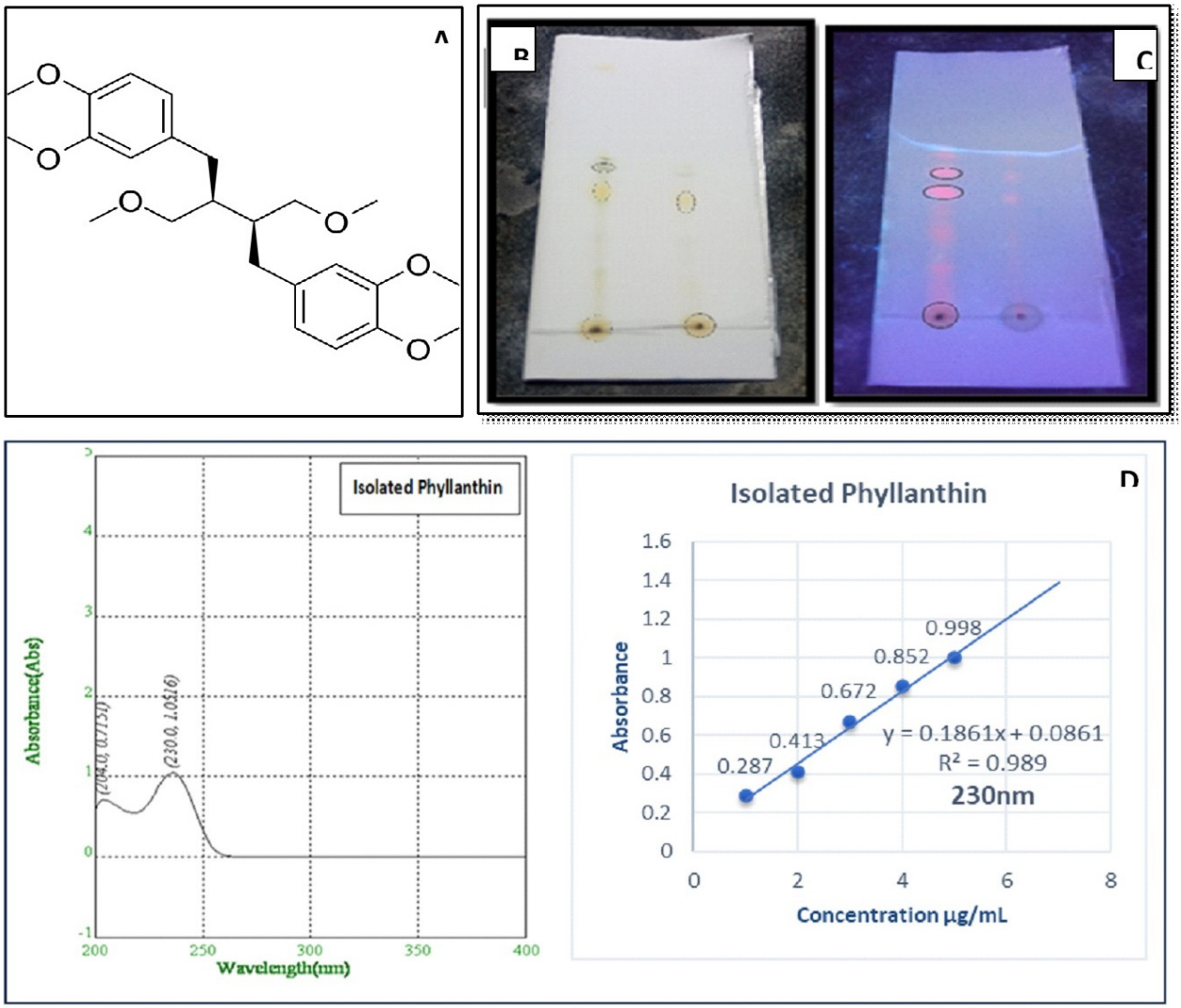

Figure 1: Characterization of isolated phyllanthin. Chemical structure (A); TLC visualized in normal light (B) and in UV Chamber (C); UV spectra in ethanol (D). The FT-IR spectrum of isolated phyllanthin showed peaks at 3078.22 cm^{-1} (C-H stretching aromatic), 1654.42 cm^{-1} and 1605.01 cm^{-1} (benzene ring), 1499.68 cm^{-1} (aromatic ring -C=C), 1355.79 cm^{-1} (-CH stretching), 1241.69 cm^{-1} and 1174.22 cm^{-1} (-CO stretch of methoxy), 905.87 cm^{-1}, 828.24 cm^{-1} and 739.34 cm^{-1} (aromatic and trans -CH). HPLC showed a Retention time (Rt) of 6.725 min for the isolated phyllanthin, matching the reported standard phyllanthin Rt of 6.702 min by Annamalai and Lakshmi (2009) (Figure 2).

12.01 to 21.76, indicating ease of application on the skin (Table 2). Stability testing was conducted over three-month period at varying temperatures (4°C, 27°C, and 37°C). Stability testing of the ointment batches demonstrated non-significant changes in pH, spreadability, and viscosity throughout the observation period (Table 3).

Acute dermal toxicity

The 2% phyllanthin ointment was found to be well-tolerated in Wistar albino rats, with no signs of skin irritation, adverse effects, or mortality. The formulation does not pose a risk of acute dermal toxicity.

PASI scoring

IMQ triggered psoriasis-like dermatitis, characterized by significant erythema, swelling, and scaling following ten days of treatment (Figure 2). The PASI score of the disease control group was 5.97 after induction, with a nonsignificant reduction to 5.50 on the 21st day.

Treatment with 0.05% tretinoin cream induced an extremely significant (p<0.001) reduction of PASI from 5.84 to 2.72. Phyllanthin ointments, 1%, and 2% commenced on day 11 after induction (p<0.5-0.001) led to a notable reduction in PASI scores on day 21 compared to respective scores after induction. The positive control (tretinoin) and 2% formulations effectively reduced erythema, scaling, and skin thickening associated with IMQ induced psoriasis (Figure 3).

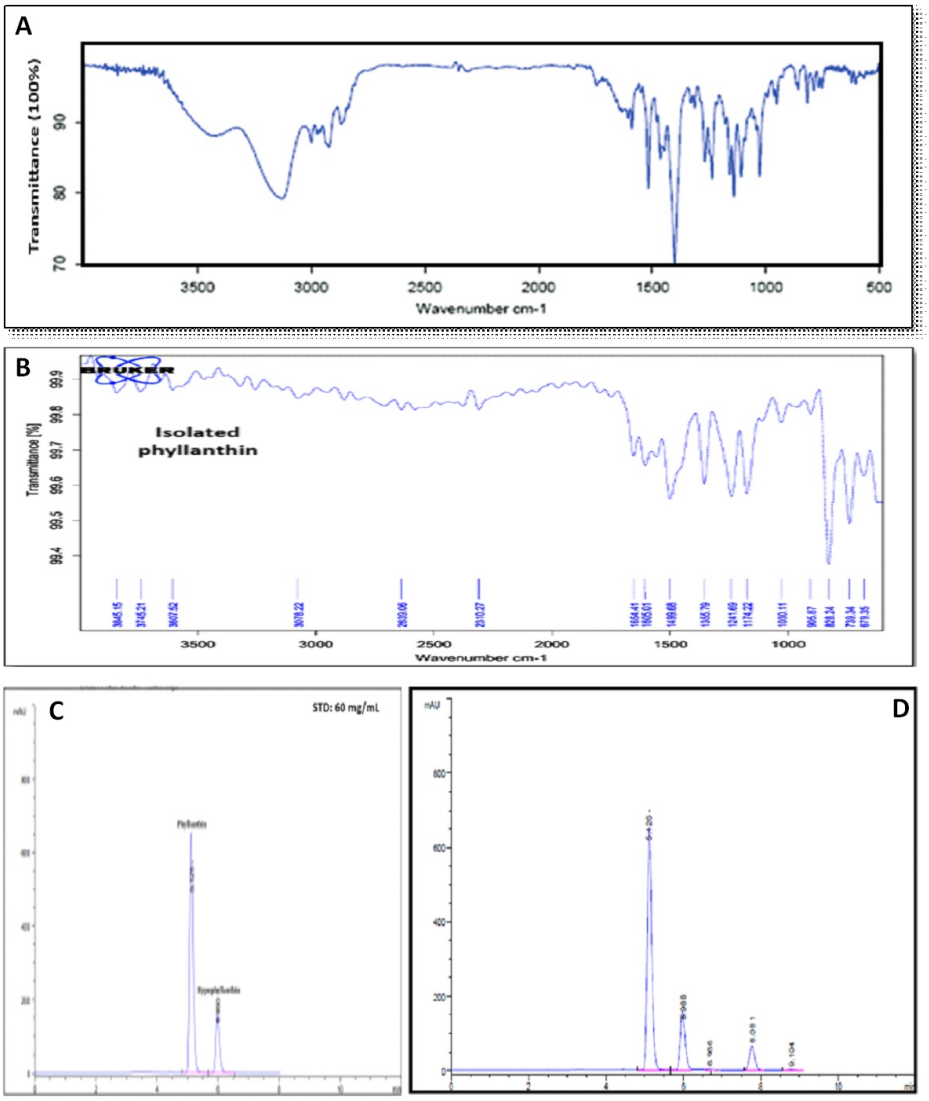

Figure 2: Spectroscopy of isolated phyllanthin. FTIR spectra of standard (A). (Sethiya *et al*., 2015) and isolated phyllanthin (B). HPLC Chromatograms showing standard phyllanthin Rt at 5.126 and hypophyllanthin Rt at 5.990 min (C), and isolated phyllanthin Rt at 5.126 and hypophyllanthin 5.988 min (D).

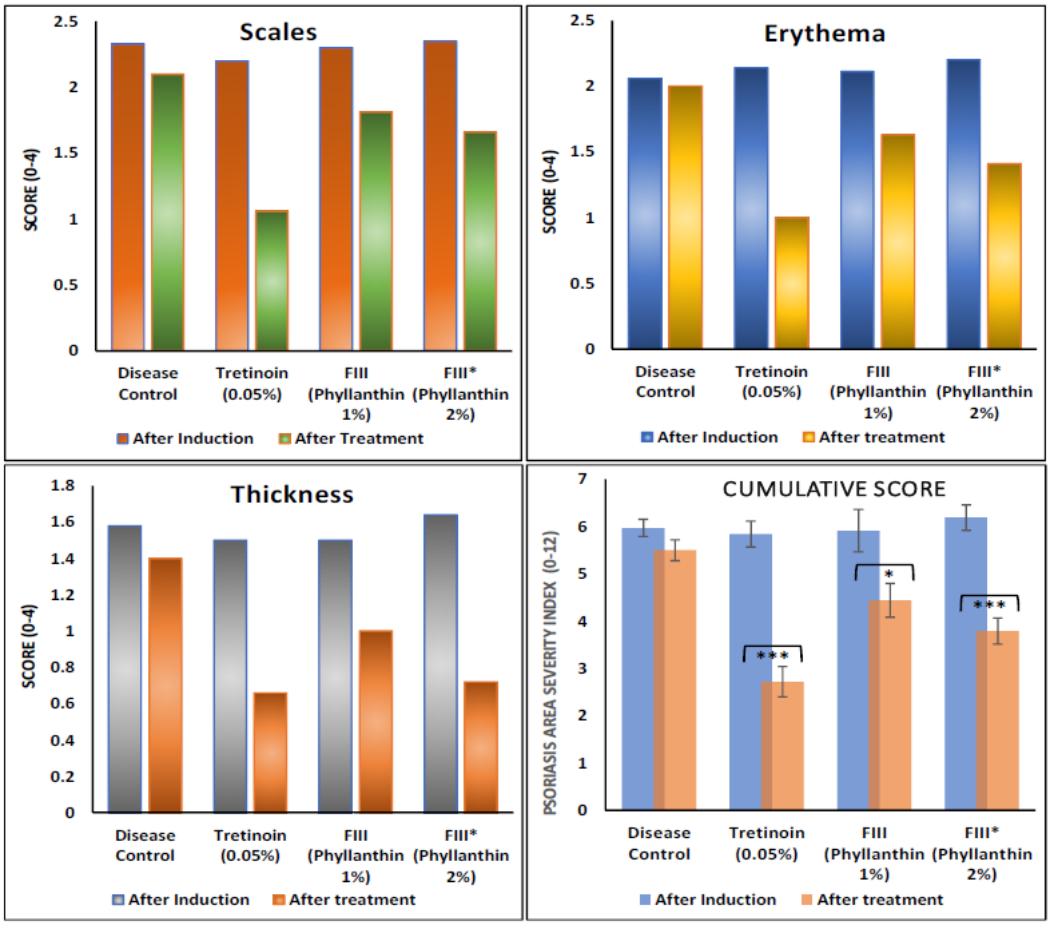

Figure 3: Antipsoriatic effect of phyllanthin ointment against inflammation induced by imiquimod in rats. Effect on parameters, i.e., erythema, scales, thickness, and cumulative score of psoriatic skin lesions on dorsal portion as indicated by psoriasis area severity index score. Values are expressed as Mean±SEM (n=6). The *p<0.01, ***p<0.001 and ns = not significant when compared to each respective value after induction.

Body weight and relative weight of thymus and spleen

Figure 4 illustrates the variations in body weight gain recorded among the experimental groups throughout various stages of the study: pre-induction, post-induction, and post-treatment. During the 21-day duration, a modest increase in body weight was evident in all groups, barring the disease control group, which experienced a slight decrease. Additionally, Table 4 indicates non-significant differences in the weights of the spleen and thymus among the groups after the induction of psoriasis and the subsequent treatment.

Ear thickness

A marked increase in ear thickness (p<0.001) was noted in all groups following the onset of psoriasis. In the disease control group, without any treatment, a minor non-significant decrease in ear thickness was observed over 10 day period. After treatment positive control as well as 2% phyllanthin ointment (Formulation FIII*) group exhibited a reduction in ear thickness, approaching baseline thickness that before induction (Figure 4).

Antioxidant and anti-inflammatory parameters

Rats induced with IMQ showed a marked increase in lipid peroxidation in both psoriatic skin (p<0.001) and ear tissues (p<0.05). Elevated levels of IL-6 and TNF-α (p<0.05-0.001) with respect to vehicle control, indicated psoriasis-like inflammatory responses. The application of tretinoin and ointments containing 1% and 2% phyllanthin significantly reduced lipid peroxidation (p<0.05), IL-6, and TNF-α levels (p<0.001) as opposed to disease control. Psoriasis induction caused a significant reduction in tissue hydroxyproline content (p<0.05). Moreover, phyllanthin ointment has significantly (p<0.05) restored hydroxyproline levels to original baseline values, whereas tretinoin was ineffective in restoring hydroxyproline levels (Table 5).

Histopathological analysis

Histological analysis demonstrated that the dermis in the vehicle control group retained its normal structure. In contrast,

Table 3: **Stability of phyllanthin ointment formulations after 3 months of storage at the specified temperature.**

Temp	Para-meters	Formulations							
		P2	P2a	P2b	P3	P3a	P3b	FIII	FIII*
Initial	Viscosity at 10 rpm (CPS)	2425.60±78.04	2476.22±73.16	2561.30±68.65	2523.88±63.08	2570.56±54.06	2418.33±58.28	2006.43±80.41	2090.49±53.24
	pH	6.64±0.47	6.50±0.68	6.28±0.52	6.55±0.26	6.37±0.72	6.87±0.28	6.77±0.40	6.88±0.51
	Spread-ability	14.85±1.73	14.33±1.50	12.54±1.68	15.97±2.63	13.69±1.78	14.56±1.70	19.03±2.14	20.90±2.60
4°C	Viscosity at 10 rpm (CPS)	2464.60±43.76^{ns}	2538.54±41.76^{ns}	2603.63±56.67^{ns}	2404.47±80.36^{ns}	2587.65±64.27^{ns}	2483.82±46.41^{ns}	2072.00±57.36^{ns}	2110.69±72.83^{ns}
	pH	6.76±0.93^{ns}	6.76±0.35^{ns}	6.60±0.57^{ns}	6.86±0.49^{ns}	6.85±0.04^{ns}	6.57±0.14^{ns}	6.81±0.93^{ns}	6.97±0.65^{ns}
	Spread-ability	14.08±2.04^{ns}	13.59±1.98^{ns}	13.75±1.56^{ns}	15.37±2.98^{ns}	14.74±1.38^{ns}	14.08±2.01^{ns}	18.24±1.93^{ns}	18.97±1.57^{ns}
27°C	Viscosity at 10 rpm (CPS)	2476.03±57.21^{ns}	2492.02±66.08^{ns}	2548.03±35.82^{ns}	2490.00±81.44^{ns}	2573.88±74.69^{ns}	2436.93±67.15^{ns}	2103.15±92.79^{ns}	21.07±84.48^{ns}
	pH	6.82±0.61^{ns}	6.73±0.74^{ns}	6.88±0.47^{ns}	6.95±0.53^{ns}	6.76±0.68^{ns}	6.85±0.85^{ns}	6.76±0.14^{ns}	7.04±0.07^{ns}
	Spread-ability	15.02±2.03^{ns}	14.97±1.76^{ns}	13.73±2.06^{ns}	15.87±2.44^{ns}	15.31±1.91^{ns}	15.03±2.03^{ns}	19.49±2.28^{ns}	19.43±2.67^{ns}
37°C	Viscosity at 10 rpm (CPS)	2412.95±50.09^{ns}	2427.36±47.46^{ns}	2576.02±83.05^{ns}	2489.77±58.56^{ns}	2591.47±72.72^{ns}	2403.01±65.03^{ns}	2046.00±80.21^{ns}	2079.00±72.37
	pH	6.54±0.48^{ns}	6.46±0.80^{ns}	6.40±0.77^{ns}	6.78±0.57^{ns}	6.98±0.03^{ns}	6.96±0.05^{ns}	6.92±0.26^{ns}	6.90±0.02^{ns}
	Spread-ability	14.95±1.98^{ns}	15.53±1.62^{ns}	14.81±2.24^{ns}	16.01±2.03^{ns}	14.52±2.87^{ns}	15.70±1.79^{ns}	20.06±2.48^{ns}	21.02±2.09^{ns}

Values are expressed as Mean±SD (n=3). The term ns = not significant, when compared to initial stability test parameter.

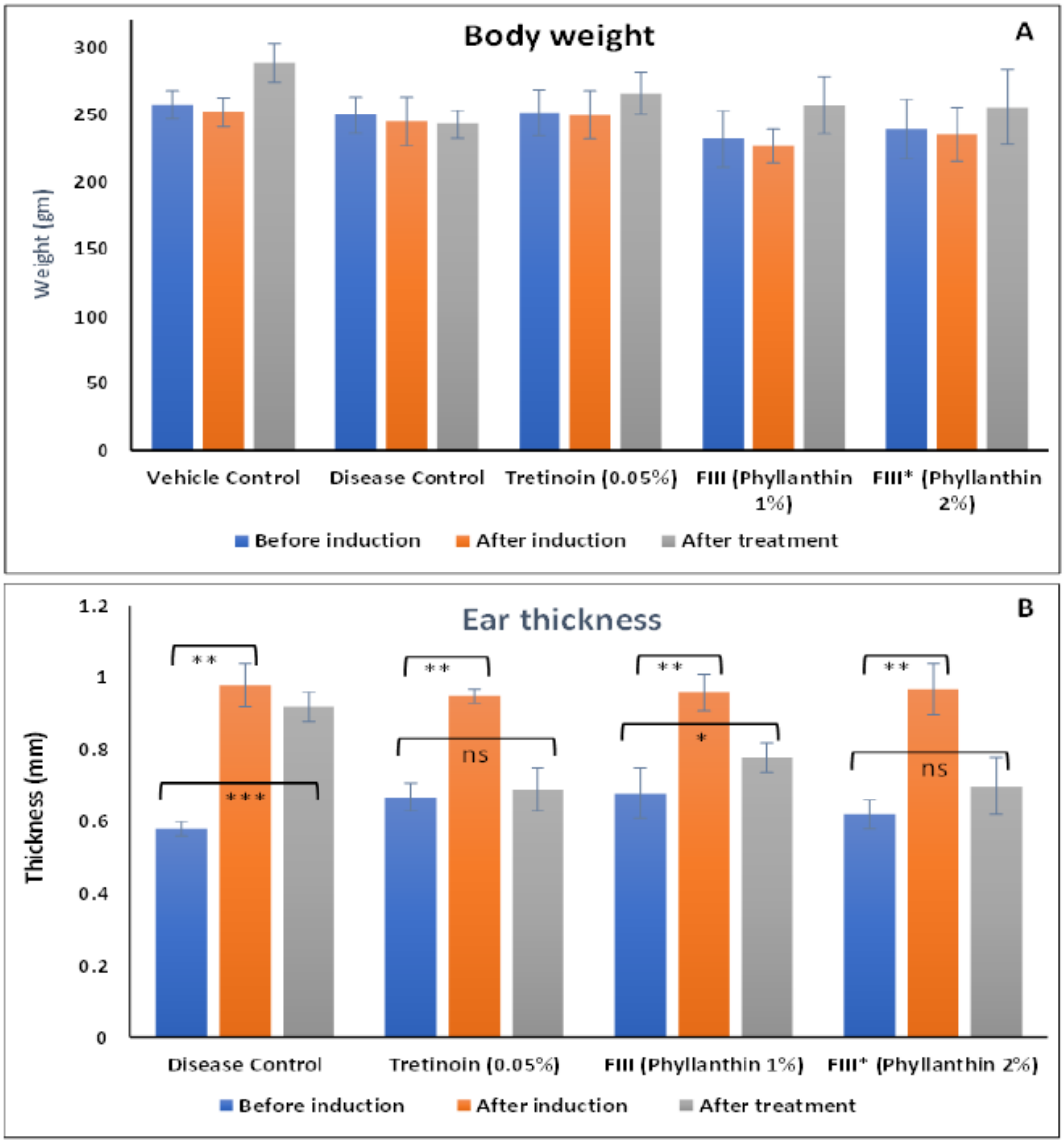

Figure 4: Effect of phyllanthin ointment application on body weight (A) and ear thickness (B) of rats with imiquimod induced psoriasis. Values are expressed as Mean±SEM (n=6). The *p<0.05, ***p<0.001 and ns = not significant when compared to each respective value before induction.

IMQ application induced significant histopathological changes, including hyperkeratosis, increased epidermal proliferation, and disrupted differentiation, leading to impaired skin structure. IMQ-treated skin displayed pronounced epidermal thickening, attributed to keratinocyte hyperproliferation and the formation of rete ridges. Additionally, the affected tissue exhibited a thickened stratum corneum and extensive infiltration of inflammatory cells into both the dermis and epidermis, contributing to erythematous plaque development.

Treatment with tretinoin markedly alleviated hyperkeratosis, keratin flaking, and lymphocytic infiltration in the upper dermis. This was accompanied by the restoration of normal skin structure, characterized by the resolution of the granular cell layer, the absence of elongated rete ridges, and no detectable polymorphonuclear cell infiltration. Similarly, treatment with 1% and 2% phyllanthin ointments reduced keratinocyte hyperproliferation, evidenced by thinning of the granular cell layer, and moderate lymphocytic infiltration, indicating their efficacy in attenuating psoriatic histopathological alterations (Figure 5).

DISCUSSION

Phyllanthin, the major bioactive compound from*Phyllanthus amarus*, alleviated psoriasis and inhibited inflammation and hyperproliferation by suppressing lipid peroxidation, IL-6, and TNF-α levels in psoriatic keratinocytes. Topical IMQ induced psoriasis in rat skin was validated through PASI scoring by assessing skin thickness, scaling, and erythema. Isolated and authenticated phyllanthin was incorporated into a stable ointment formulation containing 0.5% each of eucalyptus, basil, and

cardamom oils and applied at 2 and 4 mg/day doses. Moreover, lipid peroxidation in both psoriatic skin and ear tissues and psoriatic skin IL-6 and TNF-α were determined. The phyllanthin ointment (1% and 2%), enhanced with 0.5% each of eucalyptus, basil, and cardamom oils, offers a promising new approach for managing psoriasis.

Conventional therapies provide effective management of psoriasis, they do not cure the condition and are often associated with recurrence. This underlines the requirement for long-term, safe, and impactful treatment options. Natural products, particularly those with non-toxic and safe profiles, are increasingly being considered as promising alternatives for treating chronic diseases like psoriasis. Phyllanthin has shown effectiveness against inflammation, oxidative damage, cancer, and liver ailments. The 1% and 2% phyllanthin ointment, formulated with penetration enhancers, eucalyptus oil, basil oil, and cardamom oil, exhibited desirable rheological properties suitable for topical application. The spreadability, extrudability, and viscosity characteristics were found to be within the acceptable range for optimal formulation performance. Viscosity, spreadability, and extrudability are important indicators of the applicability of ointment and the force required to extrude the product from its container. Phyllanthin formulation showed ease of application and efficient dispensing properties. Furthermore, formulations exhibited good tolerance in animal studies, showing no signs of acute dermal toxicity.

IMQ produces psoriasis-like skin inflammation in rats, acting as a potent immune activator by acting as a ligand for Toll-Like Receptors (TLR) 7 and TLR 8. Although the IMQ induced psoriasis model has traditionally been developed for mice, Parmar *et al.*, (2021) have successfully demonstrated the induction of clinical and histopathological features of psoriasis in rats. Continuous application of IMQ over a period of 10 days led to the formation of lesions in rat skin closely resembling human psoriasis. Psoriasis was characterized by epidermal thickening, scaling, erythema, and other typical inflammatory changes. This study demonstrated commendable anti-psoriatic efficacy of phyllanthin, alleviating inflammation and hyperproliferation of keratinocytes, showing comparable efficacy to the first-line topical vitamin D3 analog, calcipotriol (Zhang *et al.*, 2023). A phyllanthin-based topical formulation with penetration enhancers was applied after the induction of psoriasis in rats after 10 days of IMQ application, with tretinoin as a positive control. The PASI scores confirmed the successful induction of the characteristic symptoms of psoriasis, including erythema, edema, and scaling. After the psoriasis was properly induced, phyllanthin ointment was administered for 10 consecutive days.

The phyllanthin ointments (1% and 2%) applied at 200 and 400 mg/day doses in combination with essential oils, demonstrated significant improvement in psoriasis symptoms, including reductions in erythema, scaling, and skin thickness, alongside enhanced antioxidant and anti-inflammatory profiles in the tissue. Psoriasis severity is often linked to an imbalance between oxidative stress and antioxidant defense systems. Elevated lipoprotein levels in psoriatic patients have been shown to

Table 4: Effect of phyllanthin ointment application on spleen and thymus weight of rats with imiquimod induced psoriasis.

Groups	Spleen weight (g)	Thymus weight (g)
Vehicle Control	0.719±0.054	0.293±0.017
Disease Control	0.926 ± 0.062^{ns}	0.387 ± 0.101^{ns}
Tretinoin (0.05%)	0.726 ± 0.049^{ns}	0.290 ± 0.030^{ns}
FIII (Phyllanthin 1%)	0.725 ± 0.017^{ns}	0.249 ± 0.015^{ns}
FIII* (Phyllanthin 2%)	0.718 ± 0.046^{ns}	0.241 ± 0.063^{ns}

Values are expressed as Mean±SEM (n=6). The term ns = not significant when compared to vehicle control value.

Table 5: Effect of phyllanthin ointment application on oxidative status of psoriatic tissues of rats with imiquimod induced psoriasis.

Groups	Lipid peroxidation (nmoles/mg of protein)		Skin (mg of protein)		
	Skin	Ear	Hydroxyproline (µg)	IL-6 (pg)	TNF-α (pg)
Vehicle Control	0.93±0.05	1.06±0.08	4.68±0.22	836.14±20.44	496.33±12.87
Disease Control	$1.72\pm0.16^{***}$	$1.65\pm0.22^{*}$	$2.64\pm0.34^{*}$	$4425.87\pm189.14^{***}$	$1126.69\pm57.14^{***}$
Tretinoin (0.05%)	$1.02\pm0.10^{ns,a}$	$1.23\pm0.12^{ns,ns}$	$3.15\pm0.76^{*,ns}$	$1154.93\pm130.07^{ns,c}$	$575.59\pm9.36^{ns,c}$
FIII (Phyllanthin 1%)	$1.06\pm0.09^{ns,a}$	$1.12\pm0.17^{ns,a}$	$3.86\pm0.73^{ns,a}$	$2854.40\pm403.43^{***,c}$	$716.86\pm42.73^{**,c}$
FIII* (Phyllanthin 2%)	$1.03\pm0.16^{ns,a}$	$1.15\pm0.24^{ns,a}$	$4.19\pm0.56^{ns,a}$	$2576.58\pm286.84^{***,c}$	$632.32\pm64.31^{*,c}$

Values are expressed as Mean±SEM (n=6). The $^{*}p<0.05$, $^{***}p<0.001$ and ns = not significant when compared to vehicle control group. The $^{a}p<0.05$, and $^{c}p<0.001$ when compared to disease control group.

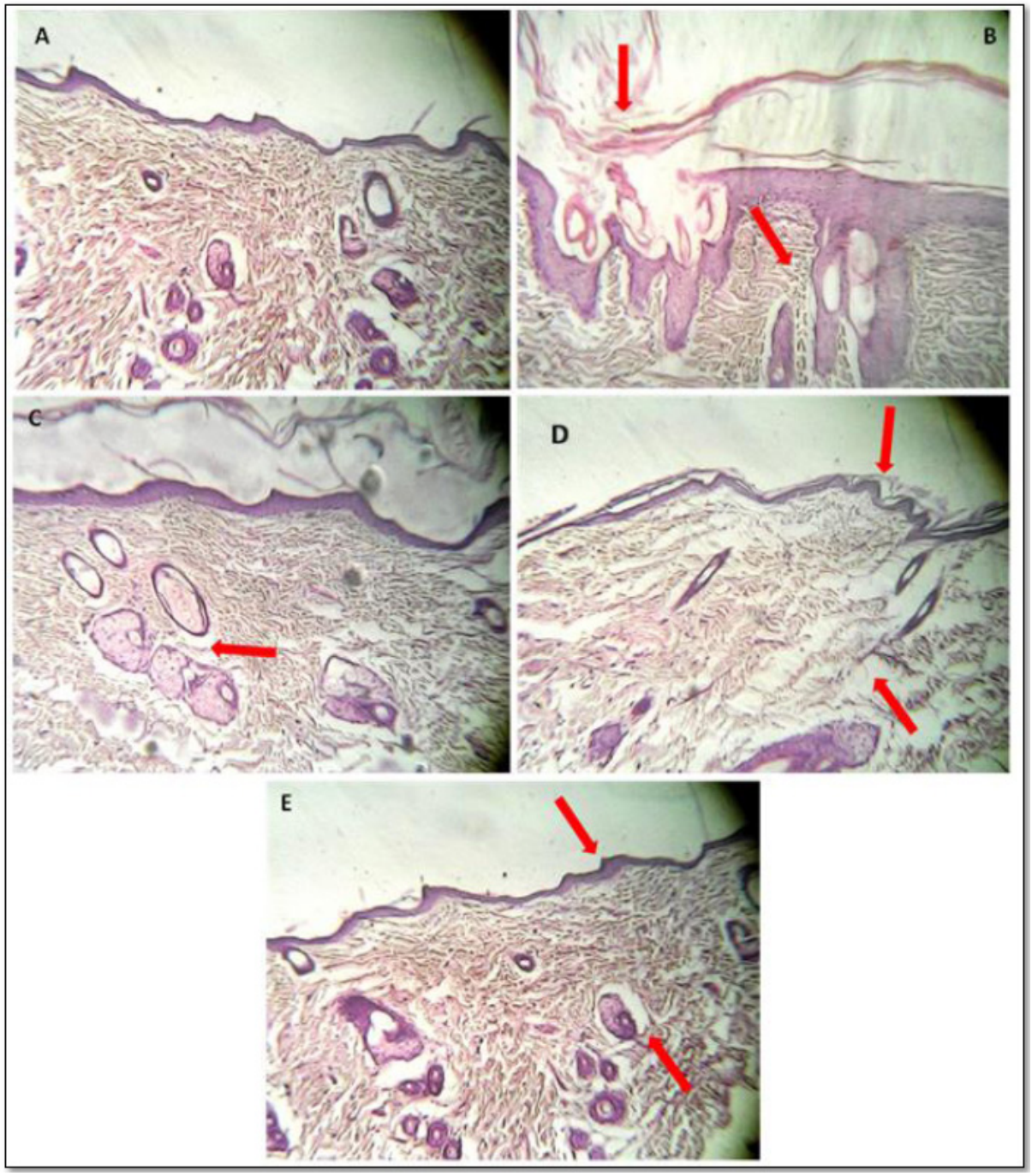

Figure 5: Antipsoriatic effect of phyllanthin ointment on histopathological changes in the skin induced by imiquimod in rats. A: Vehicle control, B: Disease control, C: Positive control, D: FIII (phyllanthin 1%) and E: FIII* (phyllanthin 2%).

correlate with increased lipid peroxidation and oxidative damage markers (Ferretti *et al*., 2012). IMQ induced negative control group exhibited significantly higher lipid peroxidation in psoriatic skin and ear tissues, along with elevated levels of IL-6 and TNF-α in contrast to normal rats. Topical application of phyllanthin formulation resulted in a transient reduction in lipid peroxidation, IL-6, and TNF-α content in psoriatic skin.

Elevated levels of lipid peroxidation, coupled with an imbalance in antioxidant defenses, have been observed in individuals with psoriasis, as reported by Parmar *et al*., (2021). Therapeutic approaches focused on reducing lipid peroxidation are vital for diminishing the expression of inflammatory mediators in psoriasis lesions, especially in keratinocytes (Kolb-Bachofen *et al*., 1994). The topical application of phyllanthin 1% and 2% ointments, in combination with essential oils such as eucalyptus, basil, and cardamom oils, resulted in a marked decrease in lipid peroxidation in psoriatic tissues. Phyllanthin is recognized for its antioxidant properties, including its capacity to neutralize free radicals, which contributes to the reduction of lipid peroxidation

(Sethiya *et al*., 2015). Phyllanthin effectively alleviated CCl_4 induced lipid peroxidation and oxidative damage *in vitro* in the HepG2 cell line and *in vivo* on rodent liver injury (Krithika *et al*., 2009).

Elevated hydroxyproline, prolidase, and matrix metalloproteinase levels are commonly observed in severe psoriasis indicating tissue collagen degradation (Guven *et al*., 2013). The metabolic disruptions of amino acids associated with psoriasis severity may be attributed to the increased demand for collagen synthesis, keratinocyte hyperproliferation, or possibly cachexia. This study found a significant reduction in skin tissue hydroxyproline content in psoriasis induced rats whereas phyllanthin treatment restored it. Tobat et al. reported enhanced hydroxyproline production by phyllanthin rich subfraction of *P. niruri* leaf in skin scar tissue following incision wound healing (Tobat *et al*., 2024). TNF-α has been shown to promote collagen degradation in various tissues, including ovine cartilage (Garvican *et al*., 2010). The elevated levels of TNF-α in IMQ induced rats are likely contributing to collagen breakdown, which, in turn, affects hydroxyproline levels. IL-6 and TNF-α are critical proinflammatory mediators involved in the pathogenesis of psoriasis in genetically predisposed individuals (Al-Awadhi *et al*., 2023). Zhang *et al*., (2023) highlighted the role of NF-κB-induced IL-17A expression and chemokine release in promoting keratinocyte proliferation in psoriasis. Psoriasis is a T-cell-mediated disorder, characterized by a complex interplay of various cytokines.

Serum levels of TNF, IFN, and IL cytokines were markedly elevated in active cases of psoriatic, correlating with the clinical severity (Arican *et al*., 2005). In the current research, phyllanthin reduced inflammatory response in psoriatic skin via inhibition of lipid peroxidation, IL-6, and TNF-α. Phyllanthin and hypophyllanthin from *P. amarus* reduced Th2 impacted ovalbumin induced airway hyperresponsiveness by modulating immune-inflammatory makers, and Th2 cytokines in a murine asthma model (Wu *et al*., 2019). Psoriasis is characterized by epidermal hyperplasia, which leads to epidermal thickening and is often accompanied by hyperkeratosis, or the thickening of the cornified layer. This condition is further characterized by inflammatory cell infiltration into both the dermis and epidermis, contributing to the formation of erythematous plaques, a defining feature of the disease (Grine *et al*., 2015). IMQ treatment on rat dorsal skin resulted in hyperkeratosis, epidermal thickening, and disruption of normal epidermal differentiation. A prominent histopathological finding was the development of acanthosis and rete ridge formation. Phyllanthin treatment combined with penetration enhancers reduced keratinocyte hyperproliferation and reduced lymphocytic infiltration. *P. emblica* fruit extract containing phyllanthin as a major bioactive component protected skin keratinocytes from UVB irradiation induced inflammation and apoptosis (Kunchana *et al*., 2021). Phyllanthin-loaded microcapsules exhibited significant antioxidant activity on fibroblast and skin keratinocytes. Additionally, they demonstrated antimicrobial efficacy against Staphylococcus aureus suggesting phyllantin potential as a multifunctional therapeutic agent for skin conditions (Lam *et al*., 2012).

Topical therapies are typically more effective during the early stages of psoriatic plaque formation. In chronic psoriasis, especially in the advanced stages of hyperkeratosis, drug delivery through the psoriatic epidermal barrier is hindered, impeding transappendageal passage (Kocsis *et al*., 2022). Phyllanthin exhibits low bioavailability, primarily due to limited aqueous solubility limiting absorption after oral administration (Murugaiyah and Chan, 2007). Phyllanthin remains stable in an aqueous solution across a pH range of 1 - 10, exhibiting a solubility pattern that is independent of pH. The log Pow value of phyllanthin measured at pH 7.48 is 3.30, indicating favourable permeability across biological membranes (Hanh *et al*., 2014). Antipsoriatic topical ointment of phyllanthin was developed in combination with essential oils to enhance the permeability of the stratum corneum to improve penetration further. Essential oils can exert synergistic effects, enhancing transdermal absorption by temporarily compromising the subcutaneous barrier without damaging viable skin cells, and are generally considered safe and non-irritating (Çalışkan and Karakuş, 2020). Topical application of phyllanthin was found to be completely safe with no adverse effects on the systemic immune system and did not influence body weight or relative organ index, confirming its safety profile.

Phyllanthin exhibits a complex anti-psoriatic effect by blocking the inflammatory activation of lipid peroxidase and cytokines in keratinocytes. Research indicates that phyllanthin not only reduces inflammation in keratinocytes (Kunchana *et al*., 2021; Lam *et al*., 2012) but also disrupts cell cycle progression (Somanabandhu *et al*., 1993), and promotes apoptosis (Paul *et al*., 2019; Abdel-Sattar *et al*., 2023). Keratinocytes are pivotal in developing and persisting psoriasis due to their production of various pro-inflammatory factors and chemokines (Zhou *et al*., 2022). In our research, combining phyllanthin with a penetration enhancer significantly diminished immune cell infiltration in skin lesions, thus reducing inflammatory damage in keratinocytes, even at a lower dosage and with a once-daily application. Phyllanthin protects against oxidative damage by inhibiting NF-κB signaling pathways in activated keratinocytes. Phyllanthin downregulates the expression of MyD88 and toll-like receptor 4, both of which are crucial for inflammatory signaling via activation of NF-κB, MAPKs, and PI3K-Akt signal transduction pathways (Harikrishnan *et al*., 2018). Additionally, phyllanthin strongly inhibits cellular and humoral immune responses (Ilangkovan *et al*., 2016). However, this study did not explore the comprehensive effects of phyllanthin on the psoriasis related systemic immune system.

CONCLUSION

This study is the first to demonstrate that phyllanthin effectively penetrates the epidermis and attenuates both cell proliferation and inflammatory response in keratinocytes. These findings suggest that topical phyllanthin could serve as a safe and effective therapeutic option for managing mild to moderate psoriatic plaques. Furthermore, it holds potential as an adjuvant treatment for patients with severe psoriasis. Phyllanthin ointments, administered once daily at 200 and 400 mg/day doses over 10 consecutive days, significantly reduced psoriatic symptoms. PASI scoring, assessments of body and organ weights, antioxidant and anti-inflammatory profiling, and histopathological examinations validated the topical antipsoriatic effectiveness of phyllanthin. Future studies should aim to refine the formulation, assess long-term effects, and explore the molecular mechanisms of action for effective psoriasis treatment.

ABBREVIATIONS

CCSEA: Committee for Control and Supervision of Experiments on Animals; **FTIR:** Fourier transform infrared spectroscopy; **HPLC:** High performance liquid chromatography; **IFNs:** Interferons; **IL:** Interleukin; **IMQ:** imiquimod; **MAPK:** Mitogen-activated protein kinase; **mRNA:** messenger Ribonucleic Acid; **MYD88:** Myeloid differentiation primary response 88; **NF-κB:** Nuclear factor kappa-light-chain-enhancer of activated B cells; **OECD:** The Organisation for Economic Co-operation and Development; **PASI:** Psoriasis area and severity index; **PI3K-Akt:** Phosphatidylinositol 3kinase/protein kinase B; **Th2:** Type 2 helper T; **TLC:** Thin layer chromatography; **TLR:** Toll-like receptors; **TNF:** Tumor necrosis factor; **UVB:** Ultraviolet B radiation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Both authors contributed equally to this work. The authors confirm contribution to the paper as follows: Methodology, Investigation, Data presentation, Statistical analysis, Writing original draft by Vanita Chature; Conceptualization, Data curation, Formal analysis, Supervision, Review, and editing by Papiya Bigoniya. All the authors reviewed the results and approved the final version of the manuscript.

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