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Phytochemical Profiling and Pharmacological Evaluation of Cocculus Hirsutus Extract for the Management of Chronic Inflammatory Skin Disorders

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ABSTRACT

Background: Chronic inflammatory skin disorders such as atopic dermatitis and psoriasis are managed largely with corticosteroids and calcineurin inhibitors, which are limited by relapse and adverse effects with prolonged use. Cocculus hirsutus (L.) W. Theob. (Menispermaceae) is a climbing herb used in Indian traditional medicine, but its activity against inflammatory skin disease has received little systematic study. Objective: To profile the phytochemical constituents of successive extracts of the whole plant, compare their antioxidant capacity, and assess the safety and anti-inflammatory potential of the most promising extract in a chronic dermatitis model. Methods: Powdered whole plant was extracted successively in a Soxhlet apparatus with petroleum ether, chloroform, methanol and water. Extracts were screened for major secondary metabolite classes, and total phenolic (TPC) and flavonoid (TFC) contents were measured spectrophotometrically. Radical scavenging was assessed by the DPPH assay (20–100 µg/mL) against ascorbic acid. The methanolic extract (MECH) was tested for acute oral toxicity (OECD 423) and for activity in oxazolone-induced chronic dermatitis in Wistar rats at 100, 200 and 400 mg/kg, with 0.1% tacrolimus ointment as reference. Results: Methanol gave the highest yield (10.87%), TPC (86.35 ± 1.24 mg GAE/g) and TFC (47.83 ± 0.92 mg QE/g). MECH was also the most active plant extract in the DPPH assay (85.72% at 100 µg/mL; estimated IC_{50} 48.5 µg/mL, against 37.7 µg/mL for ascorbic acid). No mortality or toxic signs occurred at 2000 mg/kg. In oxazolone-induced dermatitis, MECH reduced the dermatitis score by 29.2%, 49.4% and 66.9% at 100, 200 and 400 mg/kg (tacrolimus 75.3%), and lowered skin thickness, serum TNF-α, IL-6 and IL-1β and the histological severity score in a dose-dependent manner ($p < 0.001$ versus disease control). Conclusion: The methanolic extract of C. hirsutus is rich in phenolic and flavonoid constituents, shows strong in-vitro antioxidant activity and was well tolerated at the limit dose, which supports its further development as a candidate for chronic inflammatory skin conditions

Keywords: Cocculus hirsutus; phytochemical screening; total phenolic content; DPPH; oxazolone-induced dermatitis; anti-inflammatory; tacrolimus; Wistar rats,

1.INTRODUCTION

Atopic dermatitis and psoriasis are the two chronic inflammatory skin diseases that account for most of the clinical burden in dermatology. Both follow a relapsing course, both disturb sleep and quality of life, and both involve a sustained immune response in which keratinocytes, T cells and a long list of cytokines keep the lesion going [1,2,3,4,5]. Topical corticosteroids and calcineurin inhibitors such as tacrolimus remain the mainstays of treatment. They work, but skin atrophy, burning sensation, tachyphylaxis and the cost of newer biologics leave many patients looking for alternatives they can use over long periods.

Oxidative stress is now regarded as more than a bystander in these conditions. Reactive oxygen species generated in inflamed skin activate redox-sensitive transcription factors, among them NF-κB, which in

turn raises the output of TNF-α, IL-6 and IL-1β and feeds back into further radical production [6,7]. Plant polyphenols and flavonoids can interrupt this loop at more than one point [8]. They scavenge radicals directly, and in keratinocyte models compounds such as luteolin have been shown to blunt TNF-induced NF-κB activation and the release of IL-6 and IL-8 [9]. This is the rationale for screening medicinal plants that are rich in such compounds [10, 11]

Cocculus hirsutus (L.) W.Theob., known in India as Patalagarudi or Vasuka, is a twining shrub of tropical Asia and Africa. The leaves and roots feature in folk preparations for fever, skin eruptions, rheumatic pain and wounds. Phytochemical work on the species has described alkaloids such as jasminitine, hirsutine and cohirsitine, the flavonoids rutin and quercetin, and steroids and triterpenes including β-sitosterol. Antioxidant activity has been reported for leaf extracts [12]. Anti-inflammatory effects have also been described: aerial-part extracts inhibited carrageenan paw oedema in rats [13], an ethanol extract suppressed NF-κB signalling and Src/Syk phosphorylation in macrophages and protected mice from gastric and lung injury [14], and an aqueous stem extract lowered TNF-α and IL-6 in a dengue mouse model [15]. The same review points out, however, that most chemical work on the plant is decades old and that very few studies have used animal models [12]. To our knowledge, the whole plant has not been examined in a model that reproduces the chronic, T-cell driven character of human dermatitis.

Oxazolone, a haptenating agent, is widely used to induce dermatitis in rodents. Repeated topical challenge after sensitisation gives a lesion with epidermal thickening, mast-cell and lymphocyte infiltration and scratching, which resembles chronic atopic-type disease more closely than a single irritant exposure does [16,17,18]. Tacrolimus is an established topical treatment for atopic dermatitis [19] and serves as a suitable reference drug.

The present work therefore had three aims: (i) to prepare four successive extracts of whole-plant C. hirsutus and compare their phytochemical profile, phenolic and flavonoid content; (ii) to rank their antioxidant capacity by the DPPH method; and (iii) to determine the acute oral safety of the best extract, the methanolic extract (MECH), and evaluate it in oxazolone-induced chronic dermatitis in Wistar rats against tacrolimus 0.1%.

2.MATERIALS AND METHODS MATERIALS

The whole plant of Cocculus hirsutus (L.) W.Theob. was collected from the Satpura region of Maharashtra, India, and authenticated before use. Petroleum ether, chloroform, methanol, distilled water and other analytical-grade chemicals were used for successive extraction and phytochemical investigations. Gallic acid, quercetin, Folin–Ciocalteu reagent and DPPH were used for quantitative phytochemical and antioxidant assays. Oxazolone and tacrolimus 0.1% ointment were used for chronic dermatitis evaluation. Commercial ELISA kits for TNF-α, IL-6 and IL-1β, along with haematoxylin, eosin and formalin, were used for biochemical and histopathological studies. The chemicals and reagents were sourced from reputed suppliers, including Merck Life Science, SRL, Loba Chemie, HiMedia Laboratories and Sigma-Aldrich.

Collection and Authentication of Plant Material

The whole plant of Cocculus hirsutus (L.) W.Theob. (Family: Menispermaceae) was collected from the Satpura region of Maharashtra, India. The collected material was thoroughly washed with running water, followed by distilled water, to remove adhering soil and impurities. The plant was authenticated by Prof. Dr. S. V. Patil, Acting Principal and Head of the Department of Botany, Bhusawal Arts, Science and P. O. Nahata Commerce College, Bhusawal, Maharashtra. The authenticated plant material was shade-dried under well-ventilated conditions, protected from direct sunlight, and subsequently pulverized using a mechanical grinder. The coarse powder obtained was stored in an airtight container under dry conditions until further extraction.

Morphological and Organoleptic Evaluation

The whole plant of Cocculus hirsutus was examined for morphological characteristics, including root, stem, leaves, flowers and fruits. Organoleptic properties such as colour, odour, taste, texture and appearance were recorded through visual and sensory observation.

Preparation of plant extracts by successive Soxhlet extraction

Successive solvent extraction of the powdered whole plant of Cocculus hirsutus was performed using a Soxhlet apparatus with solvents of increasing polarity, namely petroleum ether (60–80°C), chloroform, methanol and distilled water. Approximately 100 g of powdered plant material was placed in an extraction thimble, and 1000 mL of the respective solvent will be used for extraction, maintaining a drug-to-solvent ratio of 1:10 (w/v). Each extraction was carried out for approximately 6–8 hours under controlled reflux conditions. Following each extraction, the solvent was filtered through Whatman No. 1 filter paper, and the residual plant marc was used for subsequent extraction with the next solvent.[20] The collected extracts was concentrated separately using a rotary vacuum evaporator under reduced pressure. The aqueous extract was concentrated using an appropriate low-temperature concentration or freeze-drying technique. The dried extracts was weighed to determine percentage extraction yield, transferred into properly labelled amber-coloured containers and stored at 2–8°C until further phytochemical and pharmacological investigations. Percentage extraction yield will be calculated using the following formula:

$$\textbf{Extract yield } (\%) = \frac{\textbf{Weight of dried extract}}{\textbf{Weight of powdered plant material}} \times \textbf{100}$$

Preliminary Phytochemical Screening

Preliminary phytochemical screening of petroleum ether, chloroform, methanol and aqueous extracts of Cocculus hirsutus was carried out to identify the major classes of secondary metabolites. The extracts were evaluated for the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, terpenoids, steroids, glycosides and carbohydrates using standard qualitative chemical tests. Each dried extract was dissolved in a suitable solvent to prepare a stock solution of 10 mg/mL. Appropriate reagent blanks were maintained during the analysis to avoid interference.[21]

Test for Alkaloids

The presence of alkaloids was examined using Dragendorff's, Mayer's and Wagner's tests. For Dragendorff's test, 2 mL of extract solution was mixed with 1 mL of Dragendorff's reagent. The formation of an orange or reddish-brown precipitate indicated the presence of alkaloids. In Mayer's test, 2 mL of extract was treated with a few drops of Mayer's reagent, and the appearance of a cream-coloured precipitate indicated a positive reaction. For Wagner's test, the addition of Wagner's reagent to 2 mL of extract solution produced a reddish-brown precipitate in the presence of alkaloidal constituents.

Test for Flavonoids

Flavonoids were detected using the Shinoda and alkaline reagent tests. In the Shinoda test, 2 mL of extract solution was treated with a small quantity of magnesium turnings, followed by the gradual addition of concentrated hydrochloric acid. The development of pink, crimson or reddish coloration indicated the presence of flavonoids. In the alkaline reagent test, 2 mL of extract solution was mixed with a few drops of 10% sodium hydroxide solution. The appearance of an intense yellow colour, which disappeared after the addition of dilute hydrochloric acid, indicated a positive reaction.

Test for Phenolic Compounds

For the detection of phenolic compounds, 2 mL of extract solution was treated with 2–3 drops of 5% ferric chloride solution. The development of blue, green or violet coloration indicated the presence of phenolic constituents.

Test for Tannins

Approximately 2 mL of extract solution was mixed with 2 mL of 5% ferric chloride solution. The formation of blue-black or greenish-black coloration indicated the presence of tannins.

Test for Saponins

The presence of saponins was evaluated using the foam test. Approximately 2 mL of extract solution was mixed with 5 mL of distilled water and shaken vigorously for 2 minutes. The formation of persistent froth that remained stable for approximately 10–15 minutes indicated the presence of saponins.

Test for Terpenoids (Salkowski Test)

Approximately 2 mL of extract solution was mixed with 2 mL of chloroform, followed by the careful addition of concentrated sulphuric acid along the inner wall of the test tube. The development of reddish-brown coloration at the interface indicated the presence of terpenoid constituents.

Test for Steroids (Liebermann–Burchard Test)

Approximately 2 mL of extract solution was dissolved in chloroform and treated with acetic anhydride, followed by the careful addition of concentrated sulphuric acid. The development of blue-green coloration indicated the possible presence of steroidal constituents.

Test for Cardiac Glycosides (Keller–Killiani Test)

Approximately 2 mL of extract solution was mixed with glacial acetic acid containing a trace amount of ferric chloride. Concentrated sulphuric acid was carefully added along the inner wall of the test tube. The formation of a brownish ring at the interface indicated the possible presence of deoxy-sugar-containing cardiac glycosides.

Test for Carbohydrates (Molisch's Test)

For carbohydrate detection, 2 mL of extract solution was treated with 2–3 drops of Molisch's reagent, followed by the careful addition of concentrated sulphuric acid along the inner wall of the test tube. The formation of a violet-coloured ring at the interface indicated the presence of carbohydrates.

Quantitative Estimation of Phytochemical Constituents

Quantitative phytochemical estimation of the petroleum ether, chloroform, methanol and aqueous extracts of Cocculus hirsutus was performed to determine the total phenolic and flavonoid contents.

Determination of Total Phenolic Content (TPC)

The total phenolic content of the different extracts was determined using the Folin–Ciocalteu colorimetric method. Gallic acid was used as the reference standard, and standard solutions of different concentrations (20–100 µg/mL) were prepared. Approximately 0.3 mL of appropriately diluted extract solution was mixed with 2.25 mL of Folin–Ciocalteu reagent (diluted 1:10 with distilled water) and allowed to stand for 5 minutes. Subsequently, 2.25 mL of sodium carbonate solution (60 g/L) was added, and the reaction mixture was incubated for 90 minutes at room temperature. The absorbance was measured at 725 nm using a UV–Visible spectrophotometer against a reagent blank. The total phenolic content was calculated from the gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per gram of dried extract (mg GAE/g).[22]

Determination of Total Flavonoid Content (TFC)

The total flavonoid content was estimated using the aluminium chloride colorimetric method, with quercetin as the reference standard. Standard quercetin solutions (20–100 µg/mL) were prepared in methanol. Approximately 0.5 mL of appropriately diluted extract solution was mixed with 2.25 mL of distilled water and 0.15 mL of 5% sodium nitrite solution. After 6 minutes, 0.3 mL of 10% aluminium chloride solution was added. The mixture was allowed to stand for 5 minutes, followed by the addition of 1 mL of 1 M sodium hydroxide solution. The final reaction mixture was mixed thoroughly, and absorbance was measured at 510 nm using a UV–Visible spectrophotometer. The flavonoid content was calculated using the quercetin calibration curve and expressed as milligrams of quercetin equivalents per gram of dried extract (mg QE/g).[23]

In-vitro Antioxidant Activity

DPPH Free Radical Scavenging Assay

The antioxidant activity of petroleum ether, chloroform, methanol and aqueous extracts of Cocculus hirsutus was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Ascorbic acid was used as the reference standard. Different concentrations of each plant extract (20, 40, 60, 80 and 100 µg/mL) were prepared using a suitable solvent. A fresh DPPH solution (0.1 mM) was prepared in methanol and protected from light. Approximately 1 mL of each extract concentration was mixed with 1 mL of DPPH solution and incubated in the dark at room temperature for 30 minutes.[24] The absorbance of the reaction mixture was measured at 517 nm using a UV–Visible spectrophotometer against a suitable solvent blank. A control containing DPPH solution without plant extract was maintained under identical experimental conditions. Similarly, different concentrations of ascorbic acid were treated using the same procedure. The reduction in DPPH absorbance indicated the free radical scavenging potential of the plant extracts. The percentage inhibition of DPPH radicals was calculated using the following equation:

$$\textbf{DPPH Inhibition } (\%) = \frac{\textbf{Ac} - \textbf{As}}{\textbf{Ac}} \times \textbf{100}$$

Where:

Ac= Absorbance of the control.

As= Absorbance of the sample.

The percentage inhibition values were plotted against the corresponding extract concentrations, and the half-maximal inhibitory concentration (IC_{50}) was determined using an appropriate concentration-response regression model. A lower IC_{50} value indicated greater free radical scavenging activity.

In-vivo Pharmacological Evaluation

The in-vivo pharmacological study was designed to investigate the safety and anti-inflammatory potential of the methanolic extract of Cocculus hirsutus against experimentally induced chronic inflammatory skin disorders. The methanolic extract was selected based on its proposed higher phytochemical content and antioxidant activity. The investigation comprised acute oral toxicity assessment and evaluation of anti-inflammatory activity using an experimental dermatitis model.

Procurement and Maintenance of Experimental Animals

Healthy adult Wistar albino rats of either sex, weighing approximately 150–200 g, were procured from a CPCSEA-registered animal breeding facility (National Institute of Biosciences, GAT No. 69, At. Dhan Ganga Wadi, Niga Dewada Road, Pune–Bengaluru Highway, Taluka Bhor, District Pune, Maharashtra–412205). The animals were housed in the institutional animal house of TVES's Hon. Loksevak Madhukarrao Chaudhari College of Pharmacy, Faizpur, Jalgaon, Maharashtra–425503, under standard laboratory conditions. They were maintained in clean polypropylene cages with suitable bedding at a temperature of 22 ± 3°C, relative humidity of 50–60%, and a 12-hour light/dark cycle. Standard laboratory pellet diet and filtered drinking water were provided ad libitum. The animals were acclimatized to the laboratory conditions for at least seven days before the commencement of the study. All animal handling and experimental procedures were conducted in

accordance with CPCSEA guidelines after obtaining prior approval from the Institutional Animal Ethics Committee (IAEC Approval No.: IAEC/04/25/Proposal No. 02). Animal health, welfare and humane endpoints were monitored throughout the experimental period. [25]

Acute oral toxicity study of methanolic extract of cocculus hirsutus

The acute oral toxicity study of the methanolic extract of Cocculus hirsutus was designed according to OECD Guideline 423 (Acute Toxic Class Method). Healthy adult rodents were acclimatized under standard laboratory conditions, and the extract was administered orally as a single dose using a saline solution as vehicle, following the guideline's stepwise dose-selection procedure. The animals were closely monitored for behavioural changes, locomotor activity, respiration, tremors, convulsions, signs of toxicity and mortality during the initial hours and throughout the 14-day observation period. Body weight, food and water consumption, and general health conditions were recorded. Gross necropsy was included at the end of the study. The toxicity assessment was intended to establish the preliminary safety profile and guide dose selection for subsequent pharmacological investigations. [26]

Evaluation of anti-inflammatory activity in experimental chronic skin inflammation

The anti-inflammatory and dermato-protective activity of the methanolic extract of Cocculus hirsutus (MECH) was planned for evaluation using an oxazolone-induced chronic dermatitis model. The study was designed to investigate the effects of MECH on skin inflammation, clinical dermatitis manifestations and inflammatory changes. Tacrolimus 0.1% ointment was selected as the standard reference treatment based on its reported efficacy in experimental chronic oxazolone-induced dermatitis.

Experimental design and grouping

The experimental animals were divided randomly into six groups, consisting of six animals each (n = 6). The experimental groups included normal control, disease control, standard treatment and three MECH-treated groups receiving different dose levels.(Table 1)

Table 1. Experimental design and grouping for evaluation of anti-inflammatory activity of MECH

Group	Experimental condition	Treatment
I	Normal control	Vehicle (Normal Saline) without oxazolone induction
II	Disease control	Oxazolone induction + vehicle (Normal Saline)
III	Standard control	Oxazolone induction + Tacrolimus 0.1% topical ointment
IV	MECH low dose	Oxazolone induction + MECH 100 mg/kg
V	MECH medium dose	Oxazolone induction + MECH 200 mg/kg
VI	MECH high dose	Oxazolone induction + MECH 400 mg/kg

Oxazolone-Induced Chronic Dermatitis Model

The anti-inflammatory activity of the methanolic extract of Cocculus hirsutus (MECH) was evaluated using an oxazolone-induced chronic dermatitis model in rats, with tacrolimus 0.1% ointment as the standard treatment. Animals were divided into six groups (n = 6), including normal control, disease control, standard control and three MECH-treated groups receiving 100, 200 and 400 mg/kg orally. Dermatitis was induced by initial sensitization with 1% oxazolone, followed by repeated topical challenges with 0.3% oxazolone. Dermatitis severity was assessed by scoring erythema, oedema, scaling and dryness on a 0–3 scale.[27] Skin thickness and scratching behaviour were also evaluated. At the end of the study, blood and skin samples were collected for estimation of TNF-α, IL-6 and IL-1β using ELISA and histopathological examination using haematoxylin and eosin staining. Histological changes were assessed for epidermal hyperplasia, hyperkeratosis and inflammatory-cell infiltration. **Statistical analysis**

Data are shown as mean ± SD (n = 3 for in-vitro assays; n = 6 for in-vivo groups). Group differences were tested by one-way ANOVA followed by Dunnett's multiple comparison test against the disease control [28]; repeated measurements over time were analysed by two-way ANOVA with a suitable post-test. Differences were taken as significant at $p < 0.05$. Analyses and graphs were prepared in GraphPad Prism version 10.4.1. Pearson's correlation was used to relate TPC and TFC to DPPH IC_{50}. For the in-vivo data, group means were compared by one-way ANOVA with Dunnett's test (versus disease control) and Tukey's test (between treated groups).

3.RESULTS

Morphological and Organoleptic Evaluation

C. hirsutus was a woody climber with cylindrical, hairy stems, alternate ovate hairy leaves, small yellowish-green flowers and small dark-purple fruits. The leaves were green with a characteristic odour and taste. These features agree with the botanical description of the species and are useful as simple identity and quality-control markers for the raw drug.

Table 2. Morphological and Organoleptic Characteristics of Cocculus hirsutus

Parameter	Characteristics
Habit	Woody climber
Stem	Cylindrical, hairy
Leaves	Alternate, ovate, hairy
Flowers	Small, yellowish-green
Fruits	Small, dark purple
Colour	Green leaves
Odour	Characteristic
Taste	Characteristic
Texture	Hairy leaves



Figure 1. Botanical features of Cocculus hirsutus

Extraction yield

Successive extraction gave yields of 6.80% (petroleum ether), 8.30% (chloroform), 10.87% (methanol) and 7.90% (water) from 100 g of powder (Table 3). Methanol therefore recovered the largest share of extractable matter. Because the solvents were applied in order of rising polarity, each one worked on a marc already stripped of less polar material, and the high methanol yield suggests that much of the plant's extractable mass is moderately to highly polar, which is consistent with the abundance of phenolics and glycosides found below. Yield alone does not signify activity, but together with the data in the next sections it was the reason MECH was taken forward.

Table 3. Percentage yield of successive solvent extracts of Cocculus hirsutus

Sr. No.	Solvent extract	Weight of dried extract (g)	Extraction yield (%)
1	Petroleum ether	6.80	6.80
2	Chloroform	8.30	8.30
3	Methanol	10.87	10.87
4	Distilled water	7.90	7.90

Preliminary phytochemical screening

Preliminary phytochemical screening was performed to identify the major classes of phytoconstituents present in different solvent extracts of Cocculus hirsutus. The methanolic extract (MECH) was expected to show a broader phytochemical profile, particularly for phenolic compounds, flavonoids, tannins and alkaloids, owing to the extraction capacity of methanol.

Table 4. Preliminary Phytochemical Screening of Different Extracts of Cocculus hirsutus

Phytochemical constituents	Petroleum ether	Chloroform	Methanol	Aqueous
Alkaloids	−	+	+++	++
Flavonoids	−	+	+++	++
Phenolic compounds	−	+	+++	++
Tannins	−	−	+++	++
Saponins	−	−	++	+++
Terpenoids	++	+++	++	+
Steroids	++	++	+	−
Glycosides	−	+	++	++
Carbohydrates	−	−	++	+++

Interpretation: − = absent; + = weak presence; ++ = moderate presence; +++ = strong presence.

The anticipated phytochemical profile indicates that the methanolic extract may contain a broader range of secondary metabolites, particularly phenolics, flavonoids, tannins and alkaloids. These phytoconstituents are of interest because of their reported antioxidant and anti-inflammatory properties. Therefore, MECH may be considered for further quantitative phytochemical and pharmacological evaluation.

Quantitative estimation of phytochemical constituents

The total phenolic content (TPC) and total flavonoid content (TFC) of the four solvent extracts of Cocculus hirsutus were evaluated using Folin–Ciocalteu and aluminium chloride colorimetric methods, respectively. Results are expressed as mean ± SD (n = 3).

Determination of Total Phenolic Content (TPC)

Table 5. Total Phenolic Content of Different Extracts of Cocculus hirsutus

Sr. No.	Extract	TPC (mg GAE/g)
1	Petroleum ether	28.46 ± 0.72
2	Chloroform	42.18 ± 0.91
3	Methanol	86.35 ± 1.24
4	Aqueous	61.72 ± 1.08

The methanolic extract exhibited the highest total phenolic content (86.35 ± 1.24 mg GAE/g), followed by the aqueous extract (61.72 ± 1.08 mg GAE/g). This higher phenolic content may be attributed to the ability of methanol to extract a wide range of phenolic compounds. The findings support further investigation of MECH for its potential antioxidant and anti-inflammatory properties. (Table 5, Fig. 2)

Determination of Total Flavonoid Content (TFC)

Table 6. Total Flavonoid Content of Different Extracts of Cocculus hirsutus

Sr. No.	Extract	TFC (mg QE/g)
1	Petroleum ether	12.35 ± 0.41
2	Chloroform	18.62 ± 0.56
3	Methanol	47.83 ± 0.92
4	Aqueous	32.16 ± 0.71

The methanolic extract showed the highest flavonoid content (47.83 ± 0.92 mg QE/g), whereas the petroleum ether extract showed the lowest value (12.35 ± 0.41 mg QE/g). The higher flavonoid content of MECH suggests that methanol was more effective in recovering flavonoid constituents from the plant material. (Table 6, Fig. 2)

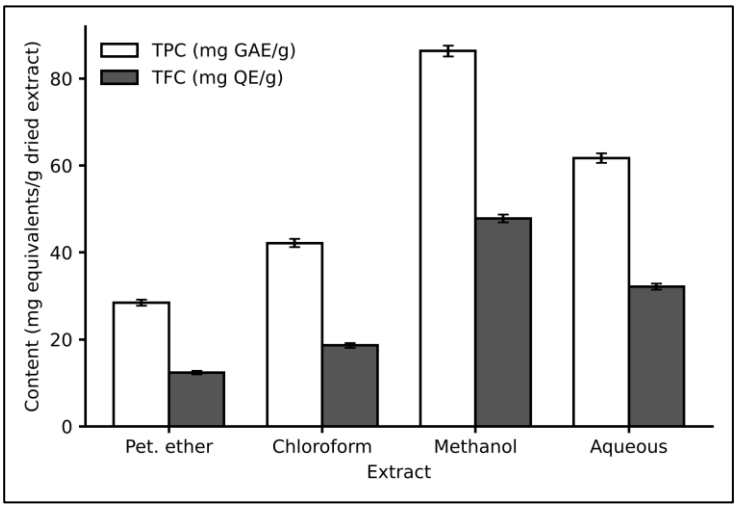


Figure 2. Total phenolic (TPC) and total flavonoid (TFC) contents of the four extracts. Bars show mean ± SD (n = 3).

In-vitro Antioxidant Activity

DPPH Free Radical Scavenging Assay

All extracts scavenged DPPH in a concentration-dependent way (Table 7, Fig. 3). At 100 µg/mL the inhibition was 54.82% (petroleum ether), 66.45% (chloroform), 72.34% (aqueous) and 85.72% (methanol), against 94.58% for ascorbic acid. Over the range tested the response was close to linear ($R^2 \geq 0.997$ for every sample), so IC_{50} values were interpolated from the regression line (Fig. 4). MECH had the lowest IC_{50} of the plant extracts (48.5 µg/mL), roughly 1.3 times that of ascorbic acid (37.7 µg/mL), whereas the petroleum ether extract needed about 92 µg/mL. A crude, multi-component extract reaching an IC_{50} of the same order as a pure reference antioxidant is a notable result. The IC_{50} values should be read as estimates, since 50% inhibition lies inside the tested range only for the more active samples and a five-point linear fit does not substitute for a full sigmoidal dose–response curve. The DPPH assay is only one of several in-vitro tests of antioxidant capacity [29].

Table 7. DPPH Free Radical Scavenging Activity of Different Extracts of Cocculus hirsutus (mean ± SD, n = 3)

Concentration (µg/mL)	Petroleum ether (%)	Chloroform (%)	Methanol (%)	Aqueous (%)	Ascorbic acid (%)
20	12.45 ± 0.62	18.36 ± 0.71	28.52 ± 0.84	21.43 ± 0.75	35.62 ± 0.91
40	21.38 ± 0.75	29.64 ± 0.83	43.76 ± 0.92	34.28 ± 0.81	51.84 ± 0.86
60	32.56 ± 0.82	41.72 ± 0.91	59.83 ± 1.05	47.65 ± 0.94	68.42 ± 1.02
80	43.27 ± 0.91	54.38 ± 1.12	73.65 ± 1.14	60.82 ± 1.08	82.36 ± 1.15
100	54.82 ± 1.03	66.45 ± 1.18	85.72 ± 1.26	72.34 ± 1.16	94.58 ± 1.21

All extracts showed a concentration-dependent increase in DPPH radical scavenging activity. Among the plant extracts, the methanolic extract exhibited the highest activity, reaching 85.72% inhibition at 100 µg/mL. This activity may be associated with its higher phenolic and flavonoid contents, which can contribute to free-radical neutralization. Ascorbic acid showed the highest overall scavenging activity and served as the reference standard. These findings, confirmed experimentally that the MECH give the better potential antioxidant properties.

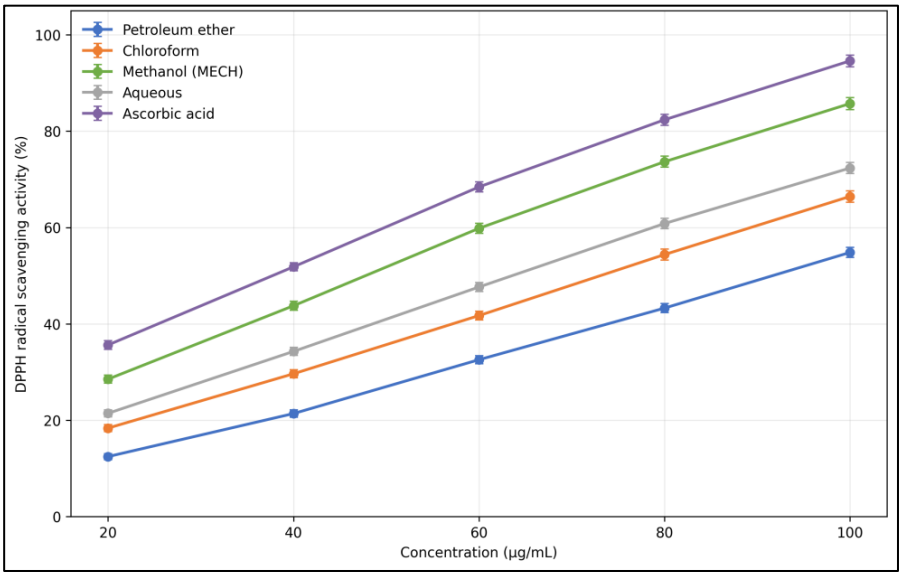


Figure 3. DPPH free radical scavenging activity of different solvent extracts of Cocculus hirsutus and ascorbic acid

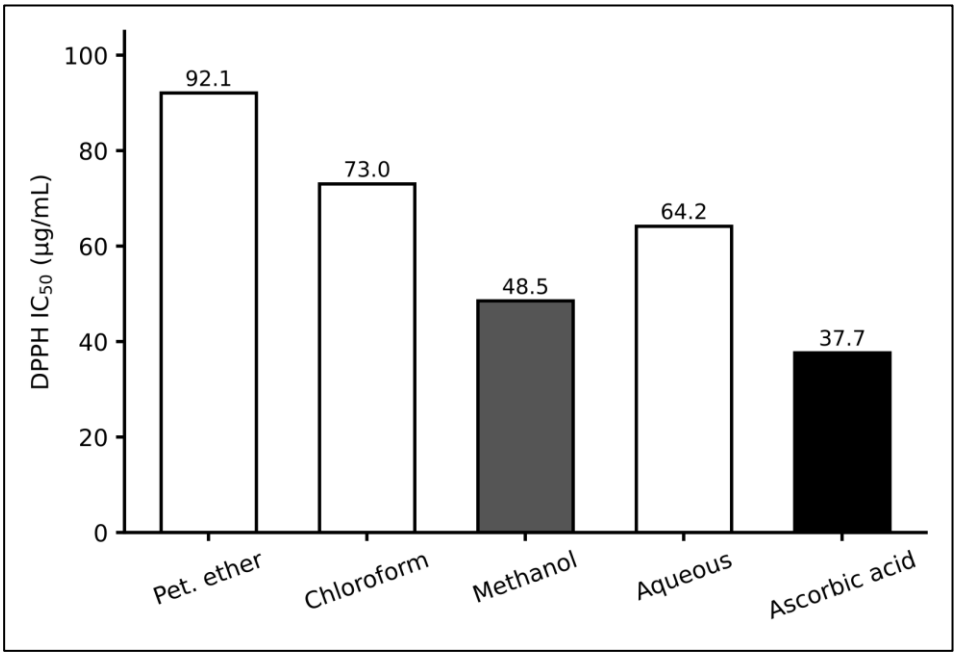


Figure 4. DPPH IC_{50} of the extracts and ascorbic acid (lower value = stronger activity).

Acute oral toxicity study

A single oral dose of 2000 mg/kg MECH caused no mortality in the 14-day observation period. There were no behavioural changes, tremors or convulsions; locomotion, respiration and food and water intake were normal; body weight was unchanged; and gross necropsy showed no abnormality (Table 9). The oral LD_{50} cut-off value of MECH is therefore above 2000 mg/kg under OECD 423, so the extract falls into the lowest-concern category of the guideline [26]. The tested doses of

100, 200 and 400 mg/kg lie well inside this margin. Acute data say nothing about repeated-dose or organ-specific toxicity, which would need a sub-acute study before any clinical suggestion is made. The alkaloid content of the genus is another reason to look at chronic toxicity.

Table 8. Acute Oral Toxicity Results of Methanolic Extract of Cocculus hirsutus at 2000 mg/kg

Observation parameter	Result
Test substance	Methanolic Extract of Cocculus hirsutus
Dose administered	2000 mg/kg body weight
Observation period	14 days
Behavioural changes	No changes
Locomotor activity	Normal
Respiration	Normal
Tremors and convulsions	Absent
Food and water consumption	Normal
Body weight	Unchanged
Mortality	No mortality
Gross necropsy	No apparent abnormalities
Overall toxicity	No observable signs of acute toxicity

The methanolic extract of Cocculus hirsutus did not produce observable acute toxic effects at the administered dose of 2000 mg/kg body weight. The absence of mortality, behavioural abnormalities and gross pathological changes during the observation period suggests a favourable preliminary acute oral toxicity profile. Based on these findings, the oral LD_{50} may be reported as greater than 2000 mg/kg body weight, subject to the complete OECD 423 decision sequence and appropriate reporting of animal numbers and dose progression.

Anti-inflammatory activity in oxazolone-induced chronic dermatitis

Data are mean ± SD (n = 6). Group differences were assessed by one-way ANOVA with Dunnett's test against the disease control; comparisons among treated groups used Tukey's test. Significance marks in the figures refer to the disease control: ** $p < 0.01$, *** $p < 0.001$.

Dermatitis severity

Oxazolone raised the dermatitis score from 0.42 ± 0.18 in normal animals to 8.65 ± 0.72 in the disease control group (Table 9, Fig. 5A), which confirms that the model produced marked erythema, oedema, scaling and dryness. Treatment lowered the score in every group (ANOVA $F = 240.8$, $p < 0.001$). Tacrolimus reduced it by 75.3%, to 2.14 ± 0.36. MECH reduced it dose-dependently, to 6.12 ± 0.54 at 100 mg/kg, 4.38 ± 0.43 at 200 mg/kg and 2.86 ± 0.39 at 400 mg/kg, which represent reductions of 29.2%, 49.4% and 66.9% (all $p < 0.001$ against the disease control; each dose differed from the next, $p < 0.001$). The 400 mg/kg score did not differ significantly from that of tacrolimus ($p = 0.11$).

Table 9. Effect of MECH on dermatitis severity score in oxazolone-induced chronic dermatitis (mean ± SD, n = 6)

Group	Treatment	Score	Reduction vs disease control (%)
I	Normal control	0.42 ± 0.18	—
II	Disease control	8.65 ± 0.72	—
III	Tacrolimus 0.1%	2.14 ± 0.36	75.3
IV	MECH 100 mg/kg	6.12 ± 0.54	29.2
V	MECH 200 mg/kg	4.38 ± 0.43	49.4
VI	MECH 400 mg/kg	2.86 ± 0.39	66.9

Skin thickness

Skin thickness increased from 0.42 ± 0.03 mm in normal rats to 1.28 ± 0.08 mm after oxazolone, a threefold rise reflecting oedema and epidermal hyperplasia (Table 10, Fig. 5B). MECH brought it down to 1.02 ± 0.07, 0.81 ± 0.06 and 0.65 ± 0.05 mm at 100, 200 and 400 mg/kg (20.3%, 36.7% and 49.2% reduction; $p < 0.001$ for all), and tacrolimus gave 0.55 ± 0.04 mm (57.0%). Skin thickness at 400 mg/kg was close to the tacrolimus value, and the two did not differ at the 5% level ($p = 0.054$).

Table 10. Effect of MECH on skin thickness (mean ± SD, n = 6)

Group	Treatment	Skin thickness (mm)	Reduction vs disease control (%)
I	Normal control	0.42 ± 0.03	—
II	Disease control	1.28 ± 0.08	—
III	Tacrolimus 0.1%	0.55 ± 0.04	57.0
IV	MECH 100 mg/kg	1.02 ± 0.07	20.3
V	MECH 200 mg/kg	0.81 ± 0.06	36.7
VI	MECH 400 mg/kg	0.65 ± 0.05	49.2

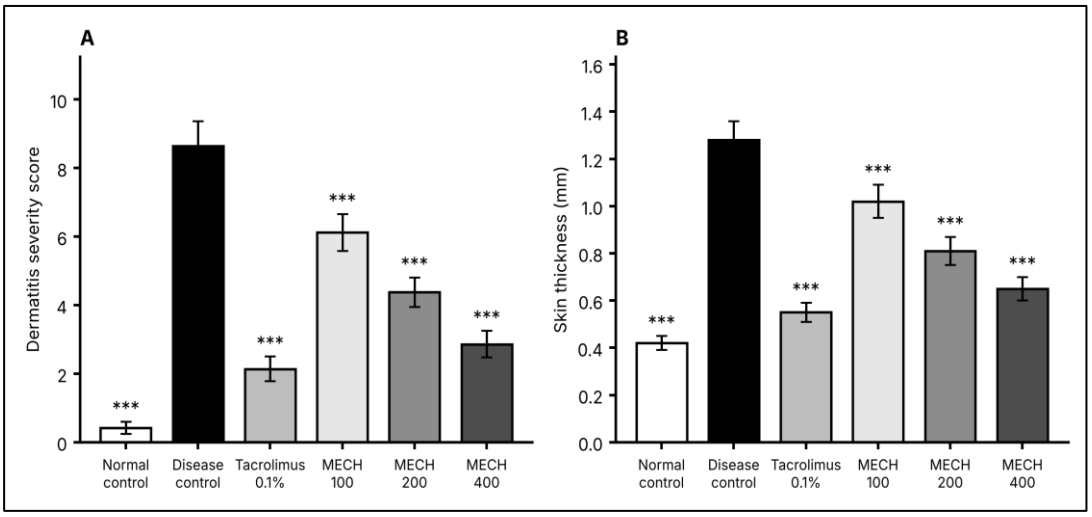


Figure 5. Effect of MECH on (A) dermatitis severity score and (B) skin thickness. Bars are mean ± SD (n = 6). ** p < 0.01, *** p < 0.001 versus disease control (one-way ANOVA, Dunnett). MECH doses are in mg/kg.

Inflammatory cytokines

Serum TNF-α, IL-6 and IL-1β rose three- to fivefold in the disease control group (Table 11, Fig. 6). TNF-α went from 28.45 ± 2.16 to 96.72 ± 5.43, IL-6 from 19.62 ± 1.84 to 82.46 ± 4.72 and IL-1β from 15.38 ± 1.52 to 71.35 ± 4.18 pg/mL. MECH lowered all three in a dose-related fashion. At 400 mg/kg the reductions were 54.8% for TNF-α, 57.8% for IL-6 and 58.5% for IL-1β ($p < 0.001$), against 63.2%, 66.8% and 68.5% for tacrolimus. Unlike the clinical scores, the cytokine levels at 400 mg/kg remained higher than those of the tacrolimus group ($p = 0.013$, 0.007 and 0.002), so the extract at its top dose approached, but did not match, the reference drug on these biomarkers.

Table 11. Effect of MECH on serum inflammatory cytokines (pg/mL; mean ± SD, n = 6)

Group	Treatment	TNF-α	IL-6	IL-1β
I	Normal control	28.45 ± 2.16	19.62 ± 1.84	15.38 ± 1.52
II	Disease control	96.72 ± 5.43	82.46 ± 4.72	71.35 ± 4.18
III	Tacrolimus 0.1%	35.64 ± 2.83	27.38 ± 2.14	22.46 ± 1.76
IV	MECH 100 mg/kg	78.53 ± 4.86	66.72 ± 4.25	58.62 ± 3.74

V	MECH 200 mg/kg	61.28 ± 3.92	49.56 ± 3.48	42.35 ± 2.96
VI	MECH 400 mg/kg	43.76 ± 3.15	34.82 ± 2.68	29.64 ± 2.23

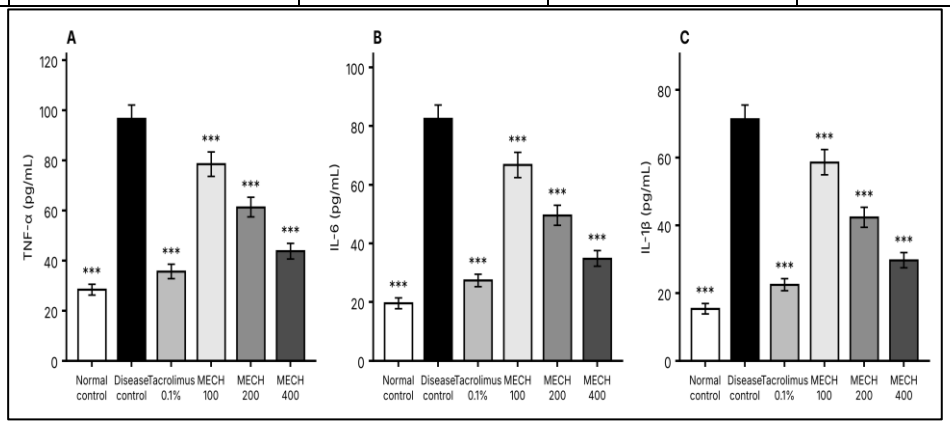


Figure 6. Effect of MECH on serum (A) TNF-α, (B) IL-6 and (C) IL-1β. Bars are mean ± SD (n = 6); *** p < 0.001 versus disease control (one-way ANOVA, Dunnett).

Histopathology studies:

Normal skin showed an intact epidermis and dermis with no inflammatory infiltrate. Disease control skin showed marked epidermal hyperplasia, hyperkeratosis and heavy inflammatory cell infiltration (Table 12, Fig. 7). Tacrolimus restored a near-normal architecture with minimal infiltration. MECH improved the tissue picture step by step: moderate epidermal thickening and infiltration at 100 mg/kg, reduced thickening and a moderate fall in infiltration at 200 mg/kg, and clear recovery of epidermal structure with only mild infiltration at 400 mg/kg. Semi-quantitative scoring (0 = normal to 4 = severe) agreed with these descriptions (Table 13, Fig. 8): the disease control scored 3.83 ± 0.41, and MECH lowered this to 2.83 ± 0.41, 2.00 ± 0.63 and 1.17 ± 0.41 ($p = 0.002$, < 0.001 and < 0.001), against 0.67 ± 0.52 for tacrolimus. The 400 mg/kg score was not significantly different from tacrolimus ($p = 0.39$).

Table 12. Histopathological findings of skin tissue (H&E)

Group	Treatment	Observations
I	Normal control	Normal epidermal architecture, intact dermis, no inflammatory infiltration
II	Disease control	Marked epidermal hyperplasia, hyperkeratosis, severe inflammatory cell infiltration
III	Tacrolimus 0.1%	Near-normal epidermal structure, minimal inflammatory infiltration
IV	MECH 100 mg/kg	Mild improvement; moderate epidermal thickening and infiltration
V	MECH 200 mg/kg	Reduced epidermal thickening; moderate reduction in infiltration
VI	MECH 400 mg/kg	Marked improvement in epidermal architecture; mild infiltration

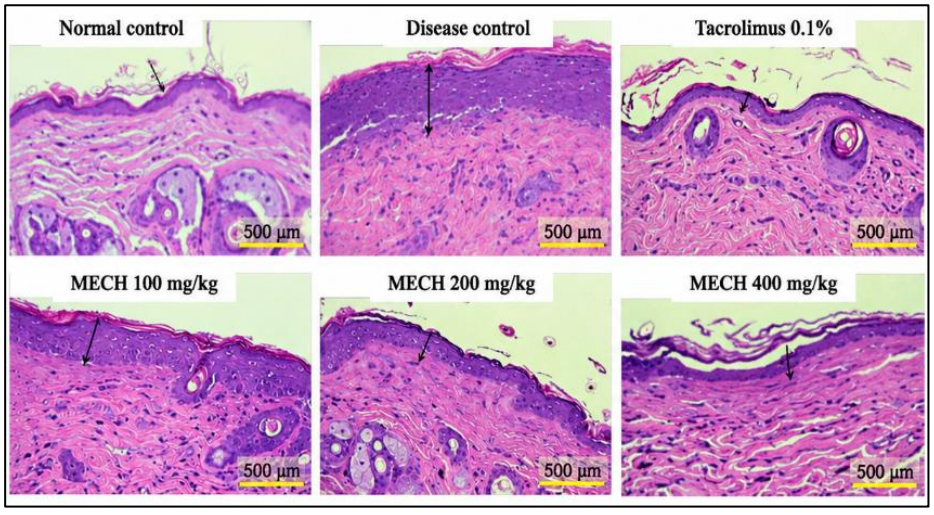


Figure 7. Representative H&E-stained skin sections. (A) Normal control, (B) disease control, (C) tacrolimus 0.1%, (D) MECH 100 mg/kg, (E) MECH 200 mg/kg, (F) MECH 400 mg/kg.

Table 13. Histopathological severity scores (0 = normal, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe; mean ± SD, n = 6)

Group	Treatment	Score	Reduction vs disease control (%)
I	Normal control	0.00 ± 0.00	—
II	Disease control	3.83 ± 0.41	—
III	Tacrolimus 0.1%	0.67 ± 0.52	82.5
IV	MECH 100 mg/kg	2.83 ± 0.41	26.1
V	MECH 200 mg/kg	2.00 ± 0.63	47.8
VI	MECH 400 mg/kg	1.17 ± 0.41	69.5

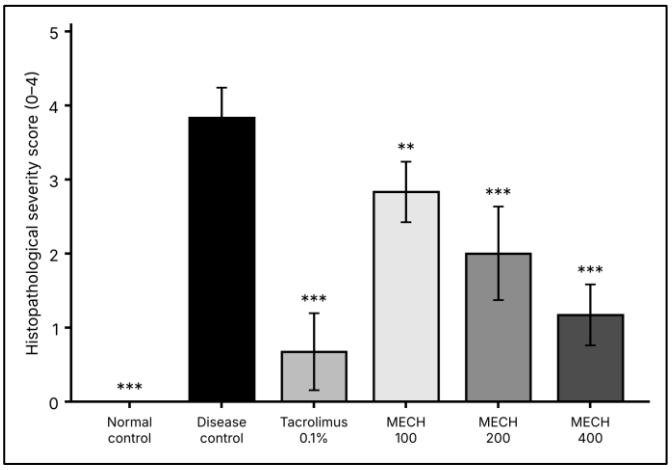


Figure 8. Histopathological severity score of skin sections. Bars are mean ± SD (n = 6); ** p < 0.01, *** p < 0.001 versus disease control.

4.DISCUSSION

Oxazolone sensitisation followed by repeated challenge reproduced the features that define chronic dermatitis in rodents: a threefold increase in skin thickness, epidermal hyperplasia, hyperkeratosis, dense infiltrate and a sharp rise in pro-inflammatory cytokines [16,17]. Against this background MECH acted on every endpoint we measured, and it did so in a dose-ordered way, with each dose differing from the next. That the clinical score, skin thickness, cytokine levels and tissue damage all moved together makes it less likely that the effect is an artefact of one assay.

The size of the effect deserves a measured reading. At 400 mg/kg MECH matched tacrolimus statistically on dermatitis score, skin thickness and histology, but not on cytokines, where tacrolimus still gave the lower values. Tacrolimus is a calcineurin inhibitor that blocks T-cell activation directly and is an established treatment for atopic dermatitis [19], so equivalence on clinical measures should not be over-read, particularly because the two agents were given by different routes. A reasonable summary is that MECH showed substantial, dose-dependent activity, with the top dose approaching the reference drug.

Several lines of evidence suggest why a polyphenol-rich methanolic extract could behave this way. TNF-α, IL-6 and IL-1β are controlled to a large extent by NF-κB, whose activation is promoted by reactive oxygen species [7, 30]. The extract had the highest phenolic and flavonoid content and the strongest radical-scavenging activity of the four fractions, and flavonoids such as luteolin suppress NF-κB induction and IL-6 release in keratinocytes [9]. Other work on the same plant points in this direction: an ethanol extract of C. hirsutus reduced NF-κB promoter activity and the phosphorylation of Src, Syk, IκBα and p65 in macrophages, and protected mice against gastric and lung inflammation [14]; an aqueous stem extract lowered TNF-α and IL-6 in a dengue mouse model [15]; and aerial-part extracts inhibited carrageenan paw oedema [13]. Our results extend this picture to a T-cell-driven skin model. Equally, the antioxidant data and the cytokine data run in parallel but we did not test whether one causes the other. The mechanism therefore remains an inference, and the phenolic fraction may not be the only active component, since alkaloids were also abundant in MECH.

The reduction in skin thickness and epidermal hyperplasia is clinically relevant because epidermal thickening, scaling and

barrier disruption are what patients experience as dryness and itch. Plant extracts have shown comparable histological benefits in oxazolone dermatitis in mice, for example in a study of Daphnopsis costaricensis [18], although that work used different species and endpoints, so the magnitudes are not directly comparable. Dosing at 100–400 mg/kg is within one-fifth of the limit dose tolerated in our acute study, and conversion to a human-equivalent dose can be done by body-surface-area scaling [27], though a topical formulation may be the more practical route for skin disease.

5.CONCLUSION

The present study demonstrated the phytochemical richness and promising pharmacological potential of Cocculus hirsutus (L.) W.Theob. Among the four successive solvent extracts, the methanolic extract (MECH) exhibited the highest extraction yield (10.87%), total phenolic content (86.35 ± 1.24 mg GAE/g) and total flavonoid content (47.83 ± 0.92 mg QE/g). It also showed the strongest DPPH radical-scavenging activity among the plant extracts, with 85.72% inhibition at 100 µg/mL and an estimated IC_{50} of 48.5 µg/mL. The acute oral toxicity assessment indicated no observable signs of toxicity or mortality at 2000 mg/kg body weight under the reported experimental conditions. Furthermore, MECH produced dose-dependent improvements in oxazolone-induced chronic dermatitis, reducing dermatitis severity, skin thickness, serum TNF-α, IL-6 and IL-1β levels, and histopathological alterations. The highest dose (400 mg/kg) demonstrated the greatest activity among the extract-treated groups and approached the effect of tacrolimus 0.1% on clinical and histological parameters. Overall, these findings suggest that MECH has promising antioxidant and anti-inflammatory potential and may be considered for further investigation in chronic inflammatory skin disorders. Further studies involving bioactive compound identification, repeated-dose toxicity, mechanistic evaluation and topical formulation development are necessary to establish its therapeutic potential.

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AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTEREST

The authors declare no conflict of interest..

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