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PHYTOCHEMICAL STANDARDIZATION AND ISOLATION OF SAPONIN FROM TRIBULUS TERRESTRIS AND EVALUATION OF IMMUNOSTIMULATORY EFFECTS ON WISTAR RATS

Pharma

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ABSTRACT

The present study aimed to standardize and isolate saponins from and evaluate their immunostimulatory effects using Wistar albino rat models. Tribulus terrestris, a plant traditionally used in Ayurvedic and folk medicine, is rich in bioactive compounds, especially steroidal saponins known for their immune-enhancing properties. The aerial parts of the plant were collected, authenticated, and subjected to Soxhlet extraction using 70% methanol, yielding a crude extract that was further purified through solvent partitioning and column chromatography. Phytochemical screening confirmed the presence of saponins, which were identified and semi-purified using TLC techniques.The saponin extract showed a dose-dependent enhancement in both humoral and cellular immune responses, with significant increases in antibody titers, DTH reaction, and cytokine levels at 400 mg/kg, comparable to the standard drug. These findings validate the traditional use of Tribulus terrestris as an immunostimulant and suggest its potential as a natural immunomodulatory agent.

KEYWORDS

INTRODUCTION

Immunostimulation refers to the use of biological agents or synthetic compounds to enhance the body's immune defenses. [1]. This immune activation can be achieved through various methods, including the use of cytokines, adjuvants, vaccines, herbal extracts, and immunomodulatory drugs [2].Immunostimulatory therapies are particularly beneficial in cases of immunodeficiency, infections, cancer, or chronic inflammatory diseases where the immune system is weakened or underactive [3]. For instance, plant-based compounds such as polysaccharides from Echinacea purpurea, Bacopa monnieri, Withania somnifera, and Tinospora cordifolia have demonstrated significant immunostimulatory effects by enhancing phagocytosis, cytokine production, and antibody formation [4, 5].Adjuvants are used to boost the body's immune response to an antigen, improving immunological memory and protection [6]. Immunostimulation is also essential in vaccine development. Cancer vaccines and immune checkpoint inhibitors are examples of cutting-edge immunostimulatory techniques used in oncology that aid the immune system in identifying and eliminating tumor cells [7].

MATERIAL AND METHOD

Collection of Plant Material,Authentication and Extraction The fruits of Tribulus terrestris were sourced from a local market in Kanpur, selected for their expected high phytochemical content. The botanical identity of the plant was verified and authenticated by Dr. Navin K. Ambasht, Head of the Botany Department, Christ Church College, Kanpur, Uttar Pradesh.Collected fruits were thoroughly washed with distilled water to remove dust and soil particles. They were then dried in a hot air oven at 40°C until they reached a constant weight. Once dried, the fruits were finely powdered using a mechanical grinder, passed through a 40-mesh sieve, and stored in airtight containers at room temperature for subsequent use.[8]

SAPONIN ISOLATION PROCEDURE

After obtaining the crude saponin-rich extract with 70% ethanol, further purification steps were carried out to isolate the saponin fraction. The isolation process involved both liquid-liquid partitioning and column chromatography to effectively enrich and purify the steroidal saponins.[9]

PHYTOCHEMICAL SCREENING

Phytochemical analysis plays a crucial role in detecting various groups of bioactive constituents present in the pumpkin pulp extract. The following qualitative tests were conducted to identify major phytochemical classes[10]

ALKALOID DETECTION

Wagner's Test: A few drops of Wagner's reagent along with 2–3 drops of concentrated hydrochloric acid were added to 2 mL of the extract. The appearance of a brown precipitate was indicative of alkaloid content[11]

SAPONIN DETECTION

Foam Test: To detect saponins, 2 mL of the extract was vigorously shaken with 5 mL of distilled water. The formation of stable, persistent foam indicated the presence of saponins.[12]

GLYCOSIDE DETECTION

Keller-Killiani Test (for Cardiac Glycosides): The alcoholic extract was diluted with water and treated with 0.5 mL lead acetate, followed by filtration and extraction with chloroform. The chloroform layer was mixed with ferric chloride and glacial acetic acid, then layered carefully over concentrated sulfuric acid. A reddish-brown ring at the interface that transitions to bluish-green confirmed the presence of cardiac glycosides.[13]

CHROMATOGRAPHIC STUDIES

Chromatography is a commonly employed analytical technique that separates components within a mixture based on their varying affinities toward a mobile phase (which flows through the system) and a stationary phase (which remains fixed).During the operation of adsorption chromatography, it depends on choosing the adsorption elements on a solid base [14]

Rf = (Distance traveled by the compound) / (Distance traveled by the solvent front)

ACUTE TOXICITY STUDIES

Acute Oral Toxicity Study of Saponin-Rich Extract of Tribulus terrestris Post-administration, animals were observed continuously for 4 hours to monitor any immediate toxic effects. They were then observed daily for 14 days for any delayed toxicity or behavioral abnormalities, including changes in:

- Spontaneous movement
- Irritability
- Corneal reflex
- Grooming habits
- Salivation
- Urination and defecation

At the conclusion of the 14-day observation period, all animals were humanely euthanized, and key organs—specifically the liver and stomach—were collected for histopathological examination. Throughout the study, no mortality or signs of acute toxicity were observed at any of the tested dose levels. Microscopic evaluation of the excised tissues revealed no pathological alterations in hepatic or gastric structures. These findings indicate that the saponin-enriched extract of Tribulus terrestris is non-toxic up to a dose of 2000 mg/kg body weight and can be regarded as safe for subsequent pharmacological evaluation.[6]

Pharmacological Screening of Saponin-Rich Extract from Tribulus terrestris

EXPERIMENTAL ANIMALS

Healthy Wistar albino rats of either sex, weighing between 150–200 g, were used for the study. The animals were housed under standardized laboratory conditions, with a controlled temperature of 22 ± 2°C, relative humidity of 60 ± 5%, and a 12-hour light/dark cycle. Throughout the experimental period, they were provided with standard pellet feed and water ad libitum. All procedures were conducted in accordance with the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines and were approved by the Institutional Animal Ethics Committee (IAEC).

GROUPING AND TREATMENT DESIGN

The rats were randomly allocated into five groups, with each group comprising five animals (n = 5):

All groups received their respective treatments once daily for a duration of 14 consecutive days. On the 7th day of the study, all animals were immunized intraperitoneally with 0.1 mL of a 10% suspension of sheep red blood cells (SRBC) to stimulate an immune response.

RESULT AND DISCUSSION

Percentage Yield of extract:

Percentage Yield

Group	Treatment	Dose	Route
I	Control (Normal saline)	10 mL/kg	Oral
II	Standard (Levamisole)	50 mg/kg	Oral
III	Saponin extract – Low dose	100 mg/kg	Oral
IV	Saponin extract – Medium dose	200 mg/kg	Oral
V	Saponin extract – High dose	400 mg/kg	Oral

Formula:

% Yield = (Weight of dried extract / Weight of plant material) × 100

Calculation:

(42.5 g / 100 g) × 100 = 42.5%

Conclusion:

The process achieved a 42.5% yield, reflecting a high extraction efficiency and successful recovery of bioactive components from the plant material.

The 42.5% yield of the ethanolic extract (Table 3) indicates efficient extraction using 70% methanol via Soxhlet

Table 7.1.1: Yield Percentage of *Tribulus terrestris* Crude Extract

Plant Material	Solvent Used	Weight of Plant Material (g)	Weight of Dry Extract (g)	Percentage Yield (%)
Tribulus terrestris (Fruits)	70% ethanol	100	42.5	42.5

Phytochemical Screening: The table shows the phytochemical test results for plant species: Tribulus terrestris fruits. The presence (+) or absence (-) of phytochemicals

The phytochemical screening (Table 7.2.1) confirmed saponins, flavonoids, glycosides, and triterpenoids

Thin Layer Chromatography (TLC)

Table 7.2.1 Phytochemical Screening of *Tribulus terrestris* Ethanolic Extract

Phytochemical Constituent	Test Performed	Result
Saponins	Froth Test	Positive
Flavonoids	Shinoda Test	Positive
Alkaloids	Dragendorff's Test	Negative
Glycosides	Keller-Kiliani Test	Positive

Rf Calculation Formula:

Rf = (Distance traveled by the compound) / (Distance traveled by the solvent front)

RESULTS:

- Spot 1: 4.5 cm / 10 cm = 0.45
- Spot 2: 5.8 cm / 10 cm = 0.58
- Standard (Protodioscin): 4.6 cm / 10 cm = 0.46

CONCLUSION:

The Rf value of Spot 1 (0.45) is nearly identical to that of the standard protodioscin (0.46), indicating the probable presence of protodioscin in the extract and validating the efficiency of the extraction process.

Table 7.3.1: TLC Rf Values of *Tribulus terrestris* Ethanolic Extract

Sample	Distance Traveled by Compound (cm)	Distance Traveled by Solvent (cm)	Rf Value
Extract Spot 1	4.5	10	0.45
Extract Spot 2	5.8	10	0.58
Standard Protodioscin	4.6	10	0.46

Figure 7.3.1 Thin layer of chromatography (TLC)

Figure 7.3.2

The positive froth test and TLC Rf values (0.45 and 0.58, Table 7.3.1) closely matching protodioscin (0.46) validate the presence and purity of saponins. The second Rf value (0.58) suggests additional saponins (e.g., tribulosin), warranting further characterization.

ACUTE TOXICITY

Acute oral toxicity of the saponin-rich extract was assessed using Wistar albino rats (150–200 g), with six animals per group, following the guidelines outlined in OECD 423. No deaths or significant behavioral abnormalities were observed in any group during the study. Histopathological analysis of the liver, kidney, and spleen revealed no detectable tissue damage or structural changes. These results suggest that the extract is safe at the tested doses, with an estimated LD_{50} exceeding 2000 mg/kg, indicating a low level of acute toxicity and a wide safety margin.

Table 7.4.1: Acute Toxicity Results of *Tribulus terrestris* Saponin Extract

Dose (mg/kg)	Mortality (n=5)	Behavioral Changes	Body Weight Change (%)	Histopathology (Liver, Kidney, Spleen)
300	0/5	None	+2.1 ± 0.3	Normal
1000	0/5	None	+2.3 ± 0.4	Normal
2000	0/5	None	+2.0 ± 0.3	Normal

*Body weight change (%) = [(Final weight - Initial weight) / Initial weight] × 100

Acute toxicity results (Table 7.4.1) showed no adverse effects up to 2000 mg/kg, supporting an LD50 >2000 mg/kg. This confirms the safety of the saponin extract for pharmacological studies.

IMMUNOSTIMULATORY ACTIVITY

The immunomodulatory efficacy of the saponin-enriched extract from Tribulus terrestris was evaluated in Wistar albino rats (n = 6 per group). All treatments were administered once daily for 14 consecutive days.To induce an immune response, all animals were immunized intraperitoneally on Day 7 with 0.1 mL of a 10% suspension of sheep red blood cells (SRBCs). The following immunological markers were assessed:

HUMORAL IMMUNE RESPONSE

Antibody titers were measured using the hemagglutination assay.The results, presented in Table 7.5.1, demonstrated a clear dose-dependent enhancement in all measured immune parameters, indicating the significant immunostimulatory activity of the saponin-rich extract.

CONCLUSION

In summary, the findings validate the traditional use of Tribulus terrestris as an effective immunostimulant. The saponin-rich fraction significantly activated both innate and adaptive immune mechanisms without inducing toxicity, making it a strong candidate for further development as a natural immunomodulatory agent.These findings

suggest that saponins from Tribulus terrestris hold promising potential as a safe, natural alternative or adjunct to synthetic immunotherapeutics. Further investigations, including clinical trials and mechanistic studies, are recommended to fully explore their therapeutic applications and support the development of standardized herbal immunomodulators for clinical use.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

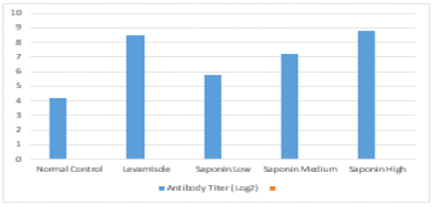

Figure 7.5.1 Immunostimulatory Effects of Tribulus terrestris Saponin Extract Antibody Titer

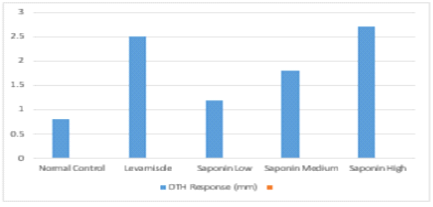

Figure 7.5.2 Immunostimulatory Effects of Tribulus terrestris Saponin Extract DTH Response

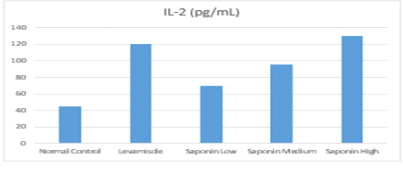

Figure 7.5.3 Immunostimulatory Effects of Tribulus terrestris Saponin Extract *IL-2 (pg/mL)*

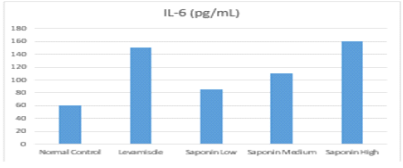

Figure 7.5.3 Immunostimulatory Effects of Tribulus terrestris Saponin Extract

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