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In vitro and in vivo Immunotherapy effect of Rosmarinus officinalis and Hygrophila spinosa cultivated from Nilgiri hills origin, India: A Comparative Study of Pharmacognostical, Toxicological, Phytochemical and Preclinical Analysis

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In vitro and in vivo Immunotherapy effect of *Rosmarinus officinalis and Hygrophila spinosa* cultivated from Nilgiri hills origin, India: A Comparative Study of Pharmacognostical, Toxicological, Phytochemical and Preclinical Analysis

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Abstract:

Introduction: Classic medical systems often use the medicinal plants *Rosmarinus officinalis* L. and *Hygrophila spinosa* for inflammatory and immune diseases. There is very limited information on their immunomodulatory potential, safety and pharmacognostical properties.

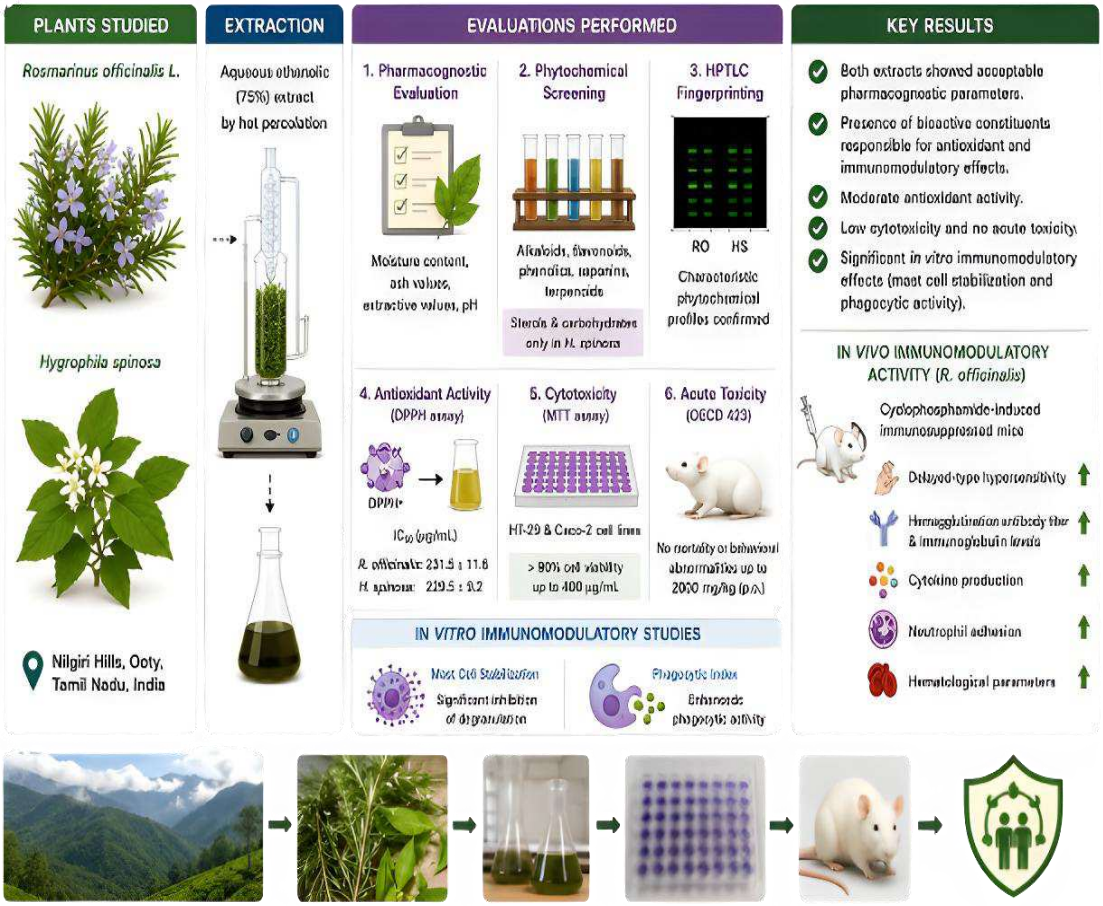
Objective: To compare the pharmacognostical, immunomodulatory , phytochemical, antioxidant, cytotoxic, toxicological and properties of *R. officinalis* and *H. spinosa* samples collected from Nilgiri Hills, Ooty, Tamil Nadu, India.

Methodology: Water Ethanolic (75%) extracts were prepared by hot percolation and physico-chemical testing was carried out and preliminary phytochemical and HPTLC fingerprinting was conducted. The strength of antioxidant activity was evaluated with DPPH assay. The MTT assay was conducted to determine the cytotoxicity of HT-29 and Caco-2 cell lines. Acute oral toxicity was determined according to OECD guideline 423. Immunomodulatory activity in cell culture was evaluated by mast cell stabilization and phagocytic index assay. Delayed type hypersensitivity, antibody titer, haematological parameters, immunoglobulin levels, cytokine production, and adhesion of neutrophils to nylon fibres were investigated to demonstrate the immunostimulatory effect of *R. officinalis* in mice immunosuppressed by cyclophosphamide.

Results: Both extracts had similar pharmacognostical features and were rich in phenolics, flavonoids, alkaloids, saponins and terpenoids. HPTLC showed good-quality phytochemical fingerprints. Moderate free radical inhibit conducted shows both extracts having inhibitory concentration values were 231.5 ± 11.6 micro gram per millilitre and 229.5 ± 9.2 micro gram per millilitre. Cell viability was above 90% in the highest dose and there were no acute toxicity levels up to 2000 mg/kg body weight. Both extracts inhibited histamine release and stimulated phagocytic activity more strongly. *R. officinalis* normalized immune and haematological measures in immunosuppressed mice.

Conclusion: It was concluded that *R. officinalis* and *H. spinosa* are promising antioxidants, safe, immune modulators and *R. officinalis* has better immunostimulatory potential and hence could be used as a natural immune modulator therapy. Genetic or multi-omics studies with bioinformatics will be further researched.

Key words: *Rosmarinus officinalis*; *Hygrophila spinosa*; Immunomodulatory activity; HPTLC; Antioxidant activity; Cyclophosphamide; Medicinal plants.



Introduction:

Medicinal plants have been recognized as remedial measures since the time immemorial and are still providing effective service to world's primary health care systems. As stated by World Health Organization (WHO), traditional and herbal medicines are consumed by lot many in several countries of the world as it is available, accessible, inexpensive and culturally acceptable [1]. The use of herbal medicine has also garnered huge interest from scientist, for its broad variety of pharmacological actions and considerably lower toxicity profile compared to its counterparts [2]. Due to the increasing global interest in plant-based therapies, there has been extensive research on plants as sources of immunomodulatory, antioxidant, and cytoprotective agents. The immune system is crucial for maintaining homeostasis within the body and safeguarding the host against invasions by microorganisms, toxins, and cellular dysfunction. Disruptions in immune function are implicated in a variety of diseases, including infectious conditions, inflammatory disorders, autoimmune diseases, and cancer [3]. While natural immunomodulatory drugs have shown great therapeutic potential in medicine, long-term administration can cause severe side effects such as liver toxicity, kidney toxicity, mutagenesis, and decreased immunity. This has stimulated efforts to find natural immunomodulators that improve immune function and not lead to toxicity [4]. Despite secondary metabolites being crucial

[5]. In many chronic diseases of metabolic disorders due to excessive production of reactive oxygen species (ROS). Antioxidants play a key role in removing free radicals and promoting normal physiological functions. We found that plant-based compounds have antioxidant and immunomodulatory properties and they are the subject of numerous studies [6,7]. Thus, plant-based medicinal plants with antioxidant and immunomodulatory properties are of interest for new phytopharmaceuticals. *Rosmarinus officinalis L*. (family: Lamiaceae) (rosemary) is an aromatic evergreen shrub, mainly used for cooking and for medicinal purposes Rosmarinic acid, carnosic acid, flavonoids, diterpenes, and phenolics are among the active ingredients of rosemary that are known to have a variety of roles in drug delivery [8]. R. officinalis is antioxidant, antimicrobial, anti-inflammatory, antiviral, hepatoprotective, neuroprotective, and immunomodulatory [9]. Additionally, experimental findings suggest that extracts from rosemary may influence cytokine secretion and immune cell function, indicating potential applications in treating immune-related disorders [10]. As a traditional medicinal plant *Hygrophila spinosa* T. Anders (family: Acanthaceae) is largely grown in India including other Asian countries for inflammation, asthma, urinary diseases, hepatic dysfunction, rheumatism and infectious disease. In many studies, the alkaloids, flavonoids, sterols, triterpenoids and phenolic compounds have been found in the plant and its pharmacological activity is determined [11]. Antioxidant, hepatoprotective, anti-inflammatory, antimicrobial, antidiabetic and immunomodulatory activity is reported in the plant [12]. However, there is a lack of studies of its immunomodulatory and toxicological activity. Despite the wide-ranging ethnomedicinal significance of *Rosmarinus officinalis* and *Hygrophila spinosa*, the immunomodulatory, antioxidant and toxicological activity of these two plants is still under study in the literature. In this work, we have analyzed the immunomodulatory, antioxidant and toxicological activity of aqueous ethanolic (75% ethanol) leaf extracts of *Hygrophila spinosa* and *Rosmarinus officinalis L* [13]. Both plant species were collected in Karnataka, India. Our investigators investigated the immunomodulatory and antioxidant activities of the two plants for the stable radical quenching assay and the intestinal epithelial carcinoma cells models. The immunomodulatory activity was evaluated by inhibition of histamine release from mast cells and phagocytic index by Candida albicans suspension assays. The results of this study may be relevant for the development of safe and effective plant-based immunomodulatory agents that can be applied in the medicinal field [14].

Materials and Methods:

Fresh leaves and young aerial parts of *Hygrophila spinosa* and *Rosmarinus officinalis L*. were collected in Nilgiri Hills region, Ooty, India. The plant samples were rinsed in running tap water, shade dried, pulverized and sieved through 100 mm mesh. The plants were verified at the National Institute of Science Communication and Information Resources (NISCAIR) in India (Voucher No.: NISCAIR/RHMD/3299/27). Dry powdered plant material was extracted in hot percolation with aqueous ethanol (75%) with solvents of different polarity. The extracts were concentrated in a rotating vacuum evaporator at low pressure after being filtered through Whatman No. 41 filter paper. Prior to additional examination, the extracted materials were weighed and kept in sealed containers at 8°C [15].

Pharmacognostical and Phytochemical studies:

The powdered leaf samples of *Hygrophila spinosa and Rosmarinus officinalis L*. have been physically examined to calculate the total ash, water-soluble ash, and moisture content. To check the total ash, about 1 gram of the dried powdered sample was placed in a silica crucible that had been previously ignited and then incinerated at 450°C to get rid of all the carbon in the powder [16]. Determine the overall ash value, the residual ash was weighed. After boiling the water-soluble ash in 20 millilitres of distilled water for five minutes, ash less paper was used to filter it. The non-physiological ash value was determined by deducting the insoluble ash value from the total ash after the insoluble ash was washed with boiling water, burned, and weighed (Table 1). Moisture content was determined by drying

powdered samples in an oven, and the powder was heated at temperatures between 100-110°C until a stable weight was found. An initial phytochemical study of the aqueous ethanolic leaf extract employed qualitative methods to identify the major phytoconstituents like nitrogenous heavy plant base, phenolic compounds, carbohydrates, foaming metabolites, and terpenoids. Phytochemical substances in the extracted plants have been identified and associated with the biological activity such as antioxidant and immunomodulatory benefits, as discussed in [17].

Chromatographical Studies:

HPTLC analysis was carried out on samples from *Hygrophila spinosa* and *Rosmarinus officinalis L*. to investigate the phytochemical character of the extracts and the quality of the extracts. The alcohol-free extracts were obtained by evaporating the solvent in a water bath and performing chloroform extraction (3 × 20 mL). After concentrating the chloroform fraction to 2 mL, it was added to silica gel 60 F254 HPTLC plates that had already been coated. *Hygrophila spinosa* was subjected to chromatographic analysis using chloroform (9.5:0.5, v/v) and *Rosmarinus officinalis* L. toluene (9:1, v/v). The plates were examined by UV light at 254 nm and 366 nm and iodine vapor to identify the phytoconstituents [18].

Cell culture:

The National Centre for Cell Science provided the human colon cell lines HT-29, which produces mucus, and Caco-2. Both were kept in Dulbecco's Modified Eagle Medium (DMEM) supplemented with ten% fetal bovine serum and 100 unit/millilitre of streptomycin, incubated at human body temperature (thirty-seven degrees Celsius) in a hydrated environment with five percent carbon dioxide [19]. The culture medium was routinely refreshed, allowing the cells to achieve approximately 90% confluency within 5 to 6 days. Prior to conducting the experimental analysis, the cells were transferred to DMEM containing 2% FBS to assess cell viability and stability [20].

Antioxidant activity evaluations:

The antioxidant activity of rosemary extract was assessed by DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay by Rani D. Different concentrations of the extract (10, 20, 40, 60, 80 and 100 µL) were prepared using dimethyl sulfoxide (DMSO). A 100 µL sample was mixed with 3.5 mL of 60 mM methanolic DPPH solution and incubated in the dark for 20 min at room temperature. The colour of DPPH changed from violet to yellow. The standard antioxidant agent was ascorbic acid. Absorbance was measured at 517 nm using a LAB INDIA 3200 UV-Visible spectrophotometer [22]. The percentage inhibition of free radicals was calculated using the following equation: The percentage inhibition of stable free radicals (DPPH) was evaluating using:
Percentage of Inhibition equal to $(A_{control} - A_{sample}) / A_{control} \times 100$
A sample represent the absorbance of control and test samples, respectively. Antioxidant activity was expressed as IC50, the concentration required to inhibit 50% of DPPH radicals.

Cytotoxicity evaluation by MTT Cell Proliferation

Human colonic cell lines, HT-29 (which secretes mucus) and Caco-2 (which does not secrete mucus), were cultivated to assess the cytotoxic effects of leaf extract from Rosmarinus officinalis. The cells were maintained in DMEM high-glucose medium enriched with fetal bovine serum and incubated at 37 °C within a humidified environment containing 5% CO_2. A total of 200 µL of cell suspension, consisting of 20,000 cells per well, was placed into 96-well plates and allowed to incubate for 12 hours [23]. The cells were then treated with different concentrations of extract (25, 50, 100, 200 and 400 µg/mL). After removing the media, the cells were kept in a CO2 incubator with 20 µL of MTT reagent (5 mg/mL) for an additional two hours. A microplate reader was used to measure the absorbance at 570 nm [24]. The

experiments were performed (n=3) and the percentage of cell viability was determined by standard protocols.

Toxicology with safety contour evaluation:

The Pinnacle Biomedical Research Institute, Bhopal's Institutional Animal Ethics Committee (IAEC) gave its approval for the rat experiments (Registration No. 1824/PO/Rc/S/15/CPCSEA). Eighteen rats weighing between 200 and 225 grams were divided into three groups (n=6).
Group I served as the control group,

Group II received an aqueous alcoholic extract, and

Group III was administered an ethyl acetate extract.

The acute toxicity assessment followed OECD Guideline 423, with doses gradually increases with 5, to 2000 mg/kg being utilized. Functional observational battery (FOB) measurements were taken after a single administration at intervals of 0, 10, 30, and 60 minutes [25,26].

Functional Observational Battery (FOB)

The negative effects of *Rosmarinus officinalis* extracts in the long-term, after single-dose treatment were assessed using the functional observer battery (FOB). Behaviour and neurological activity were measured at different time points and compared with the control group [27].

In-vitro Immunomodulatory Study

Inhibition of Histamine Release

Peritoneal cells were collected with Hank's Balanced Salt Solution (HBSS), centrifuged at 2000 rpm, and reconstituted in HBSS to get mast cells of about 10^5 cells/100 µL. Histamine release was measured with a fluorescence detector at excitation and emission wavelengths 350 and 450 nm respectively. The percentage inhibition of histamine release was calculated using the standard formula [28].

Phagocytic Index (PI)

Candida albicans were cultured in Sabouraud's broth and mixed with 1×10^8 cells/mL human serum in the ratio 4:1, using HBSS and human serum. Human polymorphonuclear leukocytes (PMNs) in blood samples were incubated in the test extracts and Candida albicans suspension at 37 °C. The phagocytosis was assessed microscopically in 100 granulocytes after the methanol fixing and Giemsa staining. The phagocytic index (PI) level and percentage of immunostimulant were calculated and compared to control groups [29]
In vivo* immunomodulatory effects of *Rosmarinus officinalis:

Healthy mice weighed 25 to 35 grams and of both sexes were housed in standard laboratory conditions where food and water were freely available to them.

Preparation of SRBC Antigen

Sheep blood was mixed with sterile Alsever's solution in a 1:1 ratio and centrifuged at 2000 rpm for 10 min at 4 °C. The pellet was washed in phosphate-buffered saline (PBS) and the erythrocyte suspension was then prepared with a concentration of 0.5×10^9 cells/mL using a Neubauer chamber to be used for antigen challenges and immunization on day zero [30].

Hemagglutination Antibody (HA) Titer

The body weights of the mice were measured before treatment and on day 1, 4, and 11 during the experiment. After 11 days of treatment, blood samples were drawn from the retro-orbital plexus and serum was isolated. Sera were serially diluted and then incubated with a suspension of SRBC in 96-well plates at 37 °C for one hour [31]. The highest dilution with visible hemagglutination was considered to be the antibody titer.

Delayed-Type Hypersensitivity (DTH) Response

The volume of the right hind paw was measured before and 24 hours after the SRBC challenge using a plethysmometer. The delayed-type hypersensitivity (DTH) response was evaluated by considering the change in the volume before and after the antigen challenge [32].

Neutrophil Adhesion Test

The blood samples obtained on day 11 were measured for total and differential leukocyte count before and after NIF incubation. The percent of neutrophil adhesion was calculated as follows: Neutrophil adhesion % = [(UTBS - TBS) / UTBS] x 100, where UTBS is the untreated blood sample and TBS is the treated blood sample [33].

Haematological and Cytokine Analysis

Moreover, levels of IgG, IgM, IL-2, TGF-β and IFN-γ were measured in ELISA kits in accordance with the manufacturer's guidelines [34].

Statistical analysis

Total experiments were performed in triplicate and mean ± standard deviation (SD) was taken in each group. The frequency of occurrences (FOBs) for each group was calculated based on the median and determined according to the Kruskal-Wallis one-way interpretation of dispersion by ranks. A probability data $p < 0.05$ (p-value less than 0.05) .

Results and Discussion:

Physiochemical evaluation:

Table 1. Physicochemical Parameters of *Rosmarinus officinalis* and *Hygrophila spinosa* (Mean ± SD, n = 3)

Parameter	*Rosmarinus officinalis* (Percentage of weight/weight	*Hygrophila spinosa* (Percentage of weight/weight
Foreign matter	1.85 ± 0.08	1.92 ± 0.07
Moisture content	3.70 ± 0.12	11.37 ± 0.25
Total ash (% w/w)	4.50 ± 0.15	8.12 ± 0.18
Physiological ash	0.50 ± 0.03	1.37 ± 0.05
Ethanol-soluble extractive	15.00 ± 0.42	3.81 ± 0.11
Aqueous-soluble extractive	17.00± 0.35	9.80 ± 0.23
pH	6.78± 0.06	6.20 ± 0.04

Phytochemical Evaluations: Both plants had rich phytochemical diversity with a lot of biologically active secondary metabolites. *Hygrophila spinosa* had a more diverse phytochemical composition due to the addition of carbohydrates and sterols while *Rosmarinus officinalis* was composed of phenolics, flavonoids, alkaloids, saponins, aromatic compounds and terpenes (Table 2). These phytoconstituents could be responsible for the medicinal and pharmacological properties of both plants.

Table 2: Comparative Phytochemical Screening of *Rosmarinus officinalis* and *Hygrophila spinosa* Extracts

S. No.	Screening Assay	*Rosmarinus officinalis*	*Hygrophila spinosa*
1	Carbohydrates	Absent	Present
2	Foaming metabolites	Present	Present
3	Nitrogenous base metabolites	Present	Present
4	Phenolic Compounds	Present	Present
5	Flavonoids	Present	Present
6	Aromaticity Test	Present	Present
7	Terpenes	Present	Present
8	Sterols	Absent	Present

Chromatographical studies :

The dark bands in UV 254 nm at Rf 0.16, 0.28, 0.48, 0.54, 0.60, 0.68 and 0.80 are identified. The six fluorescent bands are detected at Rf 0.42, 0.48, 0.60, 0.68 and 0.80 in iodine vapour . Seven brown bands with Rf 0.16, 0.28, 0.48, 0.54, 0.60 and 0.80 are collected in HPTLC for Rf 0.16, 0.28, 0.48, 0.54, 0.60 and 0.80 (Figure 1 and 2).

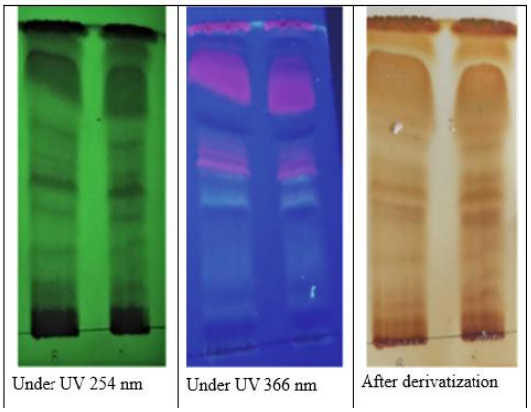


Figure 1: Rosmarinus officinalis extract's HPTLC fingerprint profile at UV 254 nm, UV 366 nm, and upon derivatization. The many phytochemical bands indicate that the extract contains a large number of bioactive components.

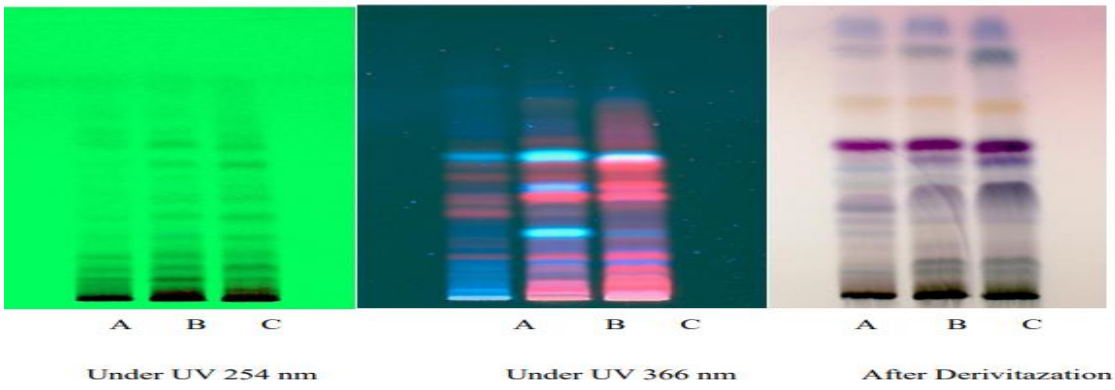


Figure 2. *Hygrophila Spinosa* samples A, B, and C's HPTLC fingerprint chromatograms under UV 254 nm, UV 366 nm, and after derivatization revealed distinctive bands that corresponded to several phytochemical ingredients. HPTLC analysis revealed five bands with Rf values 0.25, 0.33, 0.41, 0.53 and 0.61 at UV 254 nm. For UV 366 nm six fluorescent bands were found at Rf 0.18 (pink), 0.28 (blue), 0.40 (pink), 0.45 (pink), 0.52 (pink) and 0.56 (blue). Following derivatization with anisaldehyde-sulphuric acid reagent, five coloured bands with Rf values of 0.24 (brown), 0.45 (blue), 0.52 (purple), 0.66 (light yellow) and 0.83 (blue) were seen.

Free radical's prevention evaluation:

Redox-modulating activity of the hydroalcoholic separation from *Rosmarinus officinalis* and *Hygrophila spinosa* were evaluated using the DPPH method. Notably, H. spinosa exhibited a lower IC_{50} value (229.5 ± 9.2 µg/mL) compared to R. officinalis (231.5 ± 11.6 µg/mL), indicating its effectiveness in scavenging free radicals. However, both extracts demonstrated significantly reduced antioxidant activity when contrasted with Trolox, which had an Inhibitory $concentration_{50}$ of 5.36 ± 0.1 µg/mL. From redox modulating properties suggested presence of phenolic compounds including flavonoids (Table 3).

Table 3 : IC_{50} Values of *Rosmarinus officinalis* and *Hygrophila spinosa* Extracts in the DPPH Assay

Sample	DPPH IC_{50} (µg/mL)
Rosmarinus officinalis	231.5 ± 11.6
Hygrophila spinosa	229.5 ± 9.2
Trolox	5.36 ± 0.1

Comparative cytotoxicity evaluation of extracts by MTT Assay.

The MTT test was used to assess the cytotoxic effects of aqueous alcoholic extracts of Rosmarinus officinalis and Hygrophila spinosa on human colonic cell lines HT-29 and Caco-2 at concentrations ranging from 25 to 400 µg/mL for a full day. At all doses, the cell viability was robust (>90%), and neither extract exhibited cytotoxicity. The cell viability in the highest concentration (400 µg/mL) for R. officinalis and H. spinosa was 93.68 ± 0.0014% and 93.48 ± 0.004%, respectively, for the HT-29 cells and 90.28% for Caco-2 cells at the same concentration. The positive control, Camptothecin (15 µM), reduced cell viability to about 50% confirming the assay sensitivity with these two cells (Table 4).

Table 4: Comparative Cytotoxicity of extracts against HT-29 and Caco-2 Cell Lines (MTT Assay)

Concentration (µg/mL)	*Rosmarinus officinalis* HT-29 (%)	*Rosmarinus officinalis* Caco-2 (%)	*Hygrophila spinosa* HT-29 (%)	*Hygrophila spinosa* Caco-2 (%)
Cell Control	100 ± 0.0035	100 ± 0.035	100 ± 0.005	100 ± 0.05
Standard Control (Camptothecin)	50.23 ± 0.0028	50.23 ± 0.0026	50.23 ± 0.008	50.23 ± 0.006
25	99.84 ± 0.0014	97.23 ± 0.021	98.84 ± 0.004	96.32 ± 0.010
50	98.61 ± 0.0021	95.91 ± 0.0026	97.61 ± 0.001	95.71 ± 0.006
100	96.97 ± 0.0028	93.52 ± 0.0021	96.77 ± 0.008	94.82 ± 0.001
200	95.59 ± 0.0028	92.29 ± 0.0029	94.69 ± 0.008	92.19 ± 0.009
400	93.68 ± 0.0014	90.28 ± 0.0028	93.48 ± 0.004	90.28 ± 0.008

Comparative Acute Oral Toxicity evaluation of extracts:

The acute oral toxicity of aqueous alcoholic extracts from *Rosmarinus officinalis* and *Hygrophila spinosa* was evaluated following oral administration at doses of 5, 50, 300, and 2000 mg/kg, utilizing FOB parameters. The subjects were observed for behavioral, neurological, autonomic, and sensorimotor responses at intervals of 0, 10, 30, and 60 minutes post-treatment. In comparison to the control group, neither extract resulted in mortality or exhibited any toxicological signs during the evaluation period. Responses related to spontaneous activity, posture, respiration, gait, arousal levels, somatosensory reactions, muscle tone assessment, visual and olfactory responsiveness as well as palpebral reflexes and pupil reactions including aerial righting reflexes and tail pinch responses were consistent with those of the control group. There were no observable behavioral or neurotoxic alterations even at the highest dose of 2000 mg/kg, indicating a favorable safety profile for both extracts (Table 5). These findings imply that the aqueous alcoholic extracts of *R. officinalis* and H. spinosa are non-toxic under acute exposure conditions and possess a substantial safety margin for potential pharmacological investigations.

Table 5. Comparative acute toxicity assessment of extracts *by* using functional observational battery (FOB) parameters

Parameter	*Rosmarinus officinalis*	*Hygrophila spinosa*	Observation
Mortality	Nil	Nil	No mortality up to 2000 mg/kg
Spontaneous activity	Normal	Normal	No change
Posture	Normal	Normal	No change
Respiration	Normal	Normal	No change
Gait	Normal	Normal	No change
Arousal	Normal	Normal	No change
Excitation	Normal	Normal	No change
Muscle tone	Present/Normal	Present/Normal	No change
Visual/Palpebral reflex	Present	Present	Normal response
Olfactory response	Present	—	Normal response
Aerial righting reflex	—	Present	Normal response
Pupil reaction	—	Present	Normal response
Tail pinch response	Present	Present	Normal response

Urination	Normal	Normal	No change
Salivation	Absent	Absent	No toxicity observed
Lacrimation	Absent	Absent	No toxicity observed
Tremors	Absent	Absent	No toxicity observed
Convulsions	Absent	Absent	No toxicity observed
Fasciculations	Absent	Absent	No toxicity observed
Clonus	Absent	Absent	No toxicity observed
Straub tail	Absent	Absent	No toxicity observed
Writhing	Absent	Absent	No toxicity observed
Retropulsion	Absent	Absent	No toxicity observed
Diarrhoea	Absent	Absent	No toxicity observed
Piloerection	Absent	Absent	No toxicity observed
Ptosis	Absent	—	No toxicity observed
Exophthalmia	Absent	Absent	No toxicity observed
Safety conclusion	Non-toxic	Non-toxic	LD_{50} > 2000 mg/kg

Screening study for Immunomodulatory activity

Comparative In-vitro Immunomodulatory Activity of *Rosmarinus officinalis* and *Hygrophila spinosa* Extracts:

The immunomodulatory effects of aqueous alcoholic extracts from *Rosmarinus officinalis* and *Hygrophila spinosa* were assessed through their ability to inhibit histamine release from mast cells and enhance phagocytic activity against *Candida albicans*. Both extracts exhibited a concentration-dependent immunomodulatory effect. In the mast cell stabilization test, *R. officinalis* demonstrated an inhibition of mast cell degranulation by 78.94% and 79.48% at concentrations of 250 µg/mL and 400 µg/mL, respectively, while *H. spinosa* showed inhibition rates of 71.40% and 73.33% at the same concentrations. In terms of phagocytic activity, both extracts significantly increased the phagocytic index to values of 12.45 ± 0.4 at 250 µg/mL and 17.34 ± 0.2 at 400 µg/mL. The two extracts also suppressed the *Candida albicans* cells by 24.50% and 73.40%, respectively (Table 6). The overall results suggest that both extracts have significant immunomodulatory activity but R. officinalis has slightly more mast cell stabilizing capacity.

Table 6. Comparative effect of extracts on mast cell degranulation

Treatment	Concentration (µg/mL)	Number of Mast Cells (*R. officinalis*)	% Inhibition (*R. officinalis*)	Number of Mast Cells (*H. spinosa*)	Percentage of Inhibition (*H. spinosa*)
Gallic Acid (Standard)	—	8 ± 0.2	—	8 ± 0.2	—
Plant Extract	250	38 ± 4	78.94	28 ± 4	71.40
Plant Extract	400	39 ± 2	79.48	30 ± 2	73.33

Table 7. Comparative Effect of Plant Extracts on Phagocytic Index Against *Candida albicans*

Treatment	Concentration (µg/mL)	Phagocytic Index (PI)	% Inhibition
Gallic Acid (Standard)	—	10 ± 0.2	—
Plant Extract (*R. officinalis*)	250	12.45 ± 0.4	24.50
Plant Extract (*R. officinalis*)	400	17.34 ± 0.2	73.40
Plant Extract (*H. spinosa*)	250	12.45 ± 0.4	24.50

Plant Extract (*H. spinosa*)	400	17.34 ± 0.2	73.40

Hence, the immunomodulatory response of *Rosmarinus officinalis and Hygrophila spinosa* in an alcoholic extract has been demonstrated in vitro. These extracts were effective in stabilizing mast cells and reducing histamine release as well as improving the phagocytic activity of polymorphonuclear leukocytes against *Candida albicans*. The immunomodulatory response increased with concentration and reached 400 µg/mL. *R. officinalis* had a slightly higher mast cell stabilization rate of 79.48% compared to 73.33% for H. spinosa (Table 7). These results confirm and further promote the use of both plants as agents for immune protection.

***In-vivo* Immunomodulatory studies:**

Effect of *plant materials extracts* on delayed-type hypersensitivity response brought on by cyclophosphamide

All values are shown as follows. *** $p<0.001$ indicates very significant differences with NC compared to normal control. Significant differences with the pathological control group are indicated by ## $p<0.01$ and ### $p<0.001$. Specifically, the antibody titer is much larger at 500 mg/kg body weight as compared to the normal control group, but it drastically drops after taking cyclophosphamide. This is confirmed across all experiments. Cyclophosphamide reduces the delayed-type hypersensitivity (DTH) response and the paw thickness decreases from 0.513 ± 0.032 in normal control group to 0.267 ± 0.015 ($p<0.001$), and cell-mediated immunity is also impaired. In addition, *Rosmarinus officinalis* showed a dose-dependent restoration of the DTH response, as it was increased by two hundred fifty milligrams per kg to 0.313 ± 0.025 ($p<0.01$) and 500 mg/kg to 0.360 ± 0.020 ($p<0.001$). In addition, the standard drug showed an effect of 0.440 ± 0.046 ($p<0.001$) that is close to normal physiological responses (Table 8). These results indicate that *R. officinalis* effectively counteracts the immunosuppressive effects of cyclophosphamide on T cell-mediated immune responses.

Table 8: The effect of *Rosmarinus officinalis* against Cyclophosphamide induced delayed-type hypersensitivity response:

Groups	**Delayed-type hypersensitivity response**
Normal control	0.513±0.032
Disease group	0.267±0.015***
Standard group	0.440±0.046###
Test drug 1 (250mg/kg)	0.313±0.025##
Test drug 2 (500mg/kg)	0.360±0.020###

Effect of Rosmarinus officinalis extract on the Cyclophosphamide-induced hemagglutination titer assay

All values are shown as follows ***$p<0.001$ is for highly significant ### $p<0.001$ are for very significant and highly significant difference from disease group. Compared to the control group, the antibody titer was significantly higher after 500 mg/kg body weight, but rapidly decreased after cyclophosphamide. This is statistically significant in all trials. In normal mice, the hemagglutination antibody titer is 6.81 ± 0.102, while in the cyclophosphamide treatment group it was 3.167 ± 0.050

(p<0.001), implying a substantial suppression of humoral immunity. The extract of *R. officinalis* greatly increased antibody production; at the 250 mg/kg dose, the titer was 4.52 ± 0.105 (p<0.01) and at 500 mg/kg it rose to 4.90 ± 0.071 (p<0.001). In the standard treatment group, the titer was 5.26 ± 0.029 (p<0.001) (Figure 3). When rosemary extract was taken, B-cell activity was increased and antibody production increased in immunocompromised subjects.

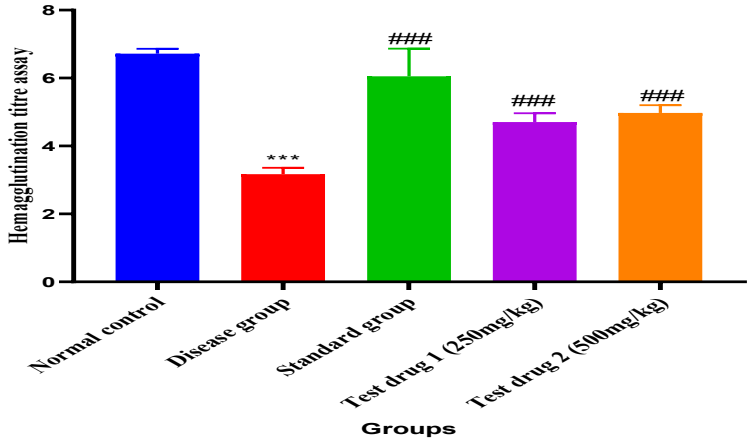


Figure 3: The effect of *Rosmarinus officinalis* against Cyclophosphamide induced hemagglutination titer assay

Effects of Rosemary ethanolic extract on Cyclophosphamide-induced IgG levels

The IgG levels in blood were lower after taking Cyclophosphamide from 16.02 ± 0.030 to 10.80 ± 0.780 (p<0.001) as a result of reduced humoral immune response. After treatment with *R. officinalis* extract, the IgG level was restored in a dose-dependent manner. In the first dosage of 250 mg/kg, IgG levels increased to 12.39 ± 0.357 (p<0.01) and in the second dose of 500 mg/kg, IgG increased to 13.37 ± 0.322 (p<0.001). In the standard group, IgG levels were 15.14 ± 0.316, and this is indicative of the increased production of immunoglobulin and strengthens the humoral immune system (Figure 4).

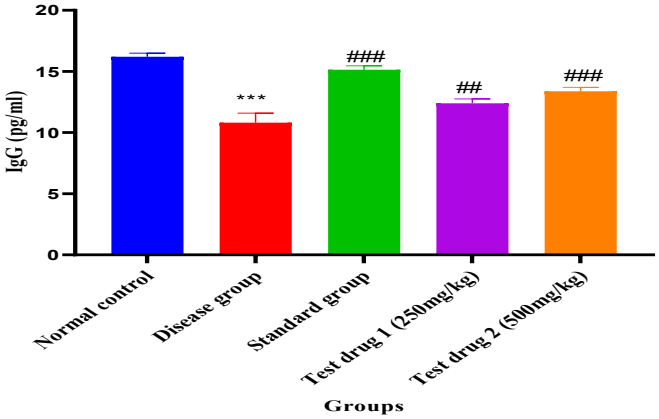


Figure 4: The effect of *Rosmarinus officinalis* against Cyclophosphamide induced IgG level

Effect of Rosmarinus officinalis extracts on Cyclophosphamide-induced IgM level:

There was a significant decrease in IgM levels after cyclophosphamide (10.72±0.161 to 5.22±0.293 ($p<0.001$)), and on the other hand, R. officinalis was higher in IgM at 250 milligrams per kilogram ($p<0.01$) and 7.78±0.201 for 500 mg/kg ($p<0.001$) (Figure 5). These results suggest rosemary extract promotes primary antibody responses and B-cell-mediated immunity.

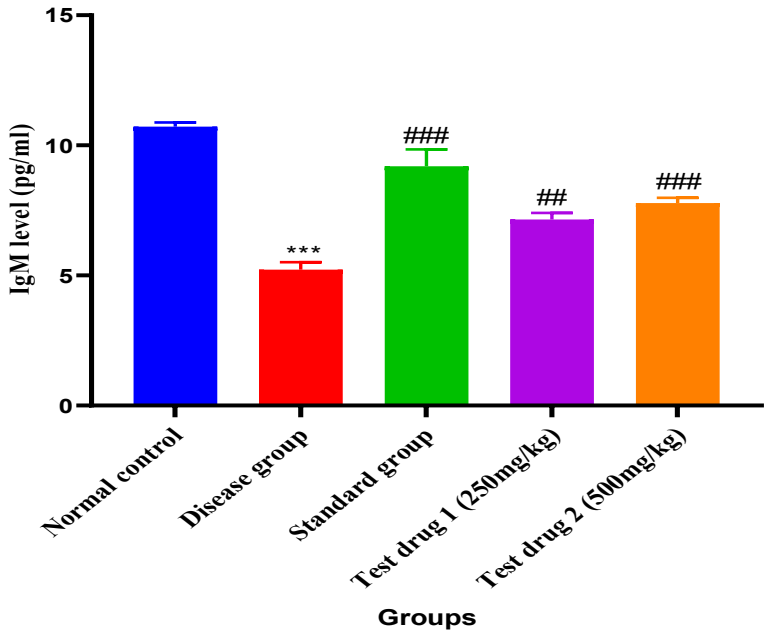


Figure 5: **The effect of ethanolic extracts of *Rosmarinus officinalis* against Cyclophosphamide induced IgM level**

Effect of Rosmarinus officinalis ethanolic extract on Cyclophosphamide-induced IFN-ɣ level:

In reality, cyclophosphamide decreased the production of IFN-γ from 51.65 ± 3.012 pg/ml to 34.98 ± 1.38 pg/ml ($p<0.001$), demonstrating a suppression of the Th1-mediated immune response. Conversely, R. officinalis elevated IFN-γ levels to 41.97 ± 1.82 pg/ml across levels of dosages two hundred fifty milligram per kilograms with 44.14 ± 1.50 pg/ml across dosages on five hundred milligram per kilo grams ($p<0.001$) (Figure 6). Meanwhile, standard treatment brought IFN-γ back up to 48.18 ± 1.14 pg/ml.

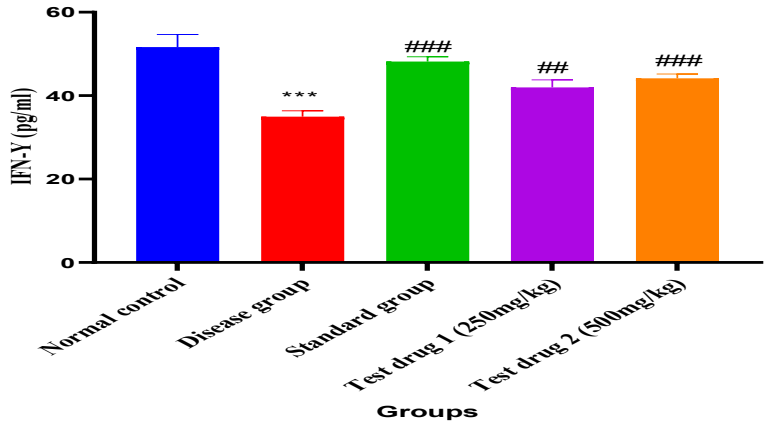


Figure 6 : The effect of *Rosmarinus officinalis* against Cyclophosphamide induced IFN-ɣ level

Effect of ethanolic extract against Cyclophosphamide induced IL-2 level:

The level of cyclophosphamide decreased from 75.39 ± 4.872 pg/ml to 52.71 ± 0.902 pg/ml ($p<0.001$) in patients receiving treatment with *R. officinalis*. The production of IL-2 was recorded at 59.38 ± 1.273 pg/ml for the dosage of 250 mg/kg and at 61.52 ± 1.541 pg/ml for the dosage of 500 mg/kg. In comparison, the standard drug elevated IL-2 levels to 67.71 ± 2.587 pg/ml ($p<0.001$). These findings indicate that rosemary extract stimulates a T-cell-mediated immune response by enhancing IL-2 secretion (Figure 7).

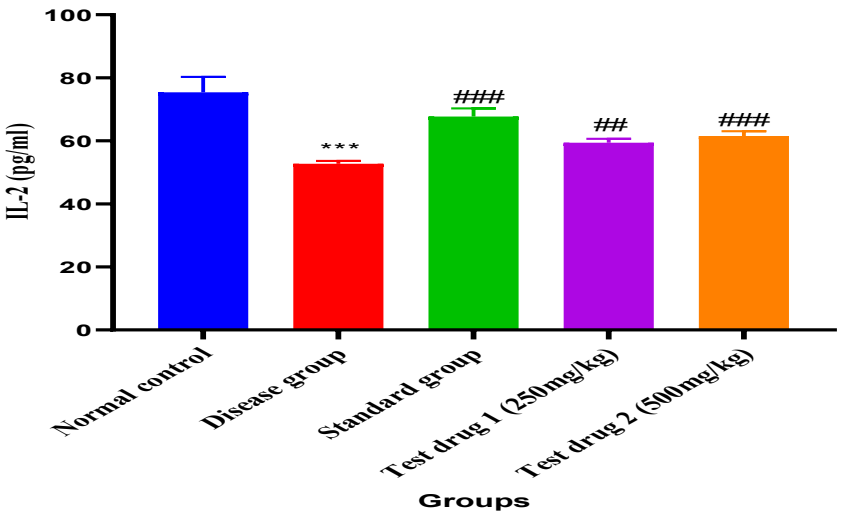


Figure 7: The effect of extracts of *Rosmarinus officinalis* against Cyclophosphamide induced IL-2 level

Effect of extracts against Cyclophosphamide induced IL-4 level:

The administration of cyclophosphamide resulted in an increase of IL-4 levels from 44.41 ± 1.126 pg/ml to 93.54 ± 0.809 pg/ml ($p<0.001$), suggesting a disruption in cytokine regulation. Treatment with R. officinalis led to a reduction in IL-4 levels by 72.87 ± 1.953 pg/ml across a levels of dosages two hundred milligrams per kilograms including with 65.41 ± 1.447 pg/ml across levels of dosages five hundreds milligrams per kilograms ($p<0.001$). IL-4 levels in the pathological control group were 53.96 ± 1.133 pg/ml ($p<0.001$). These results suggest that rosemary extract reduces excessive Th2-mediated responses and helps to stabilize cytokine homeostasis (Figure 8).

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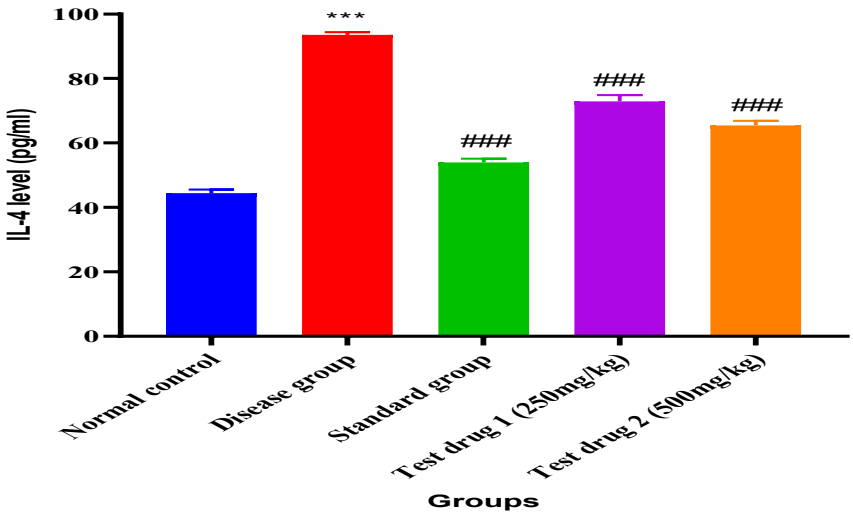


Figure 8: Effect extracts of *Rosmarinus officinalis* against Cyclophosphamide induced IL-4 level

Effect of extracts against Cyclophosphamide induced IL-10 level:

The IL-10 levels were significantly improved by cyclophosphamide-induced immunosuppression, which reached 93.54 ± 0.809 pg per mililitre in the normal pathological control group (similar to 44.41 ± 1.126 pg/ml across the range of dosages of two hundred fifty mg/kg ($p<0.001$). However, IL-10 levels were significantly lower after R. officinalis treatment, with 72.87 ± 1.953 pg/ml for 250 mg/kg and 65.41 ± 1.447 pg/ml for 500 mg/kg ($p<0.001$). IL-10 levels in the standard group were 53.96 ± 1.133 pg/ml ($p<0.001$). This reduction in IL-10 is indicative that the immune system's response can be restored after treatment and cytokine signalling in the immune system can be normalised (Figure 9).

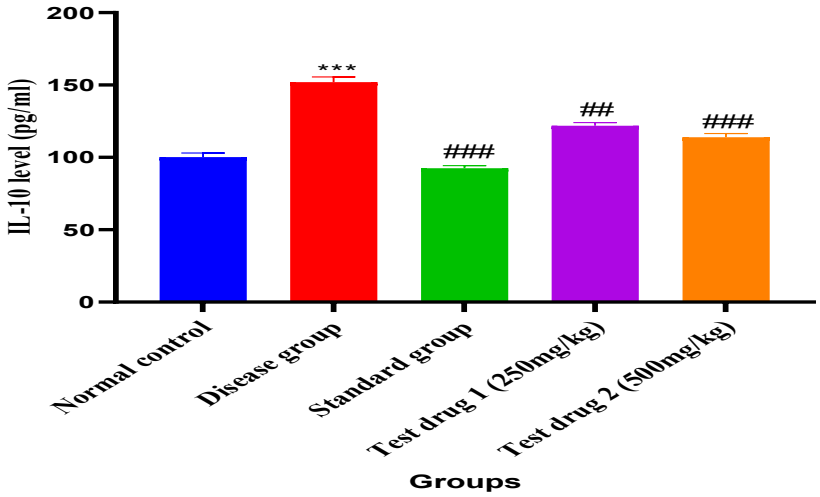


Figure 9: Effect of extracts against Cyclophosphamide induced IL-10 level

Effect of ethanolic extracts of *Rosmarinus officinalis* against Cyclophosphamide induced neutrophil adhesion test, WBC, RBC and Hb

The findings indicate that cyclophosphamide is linked to a reduction in neutrophil adhesion (14.71 ± 0.762%), white blood cell (WBC) count (2.45 ± 1.09 ×10⁹/L), red blood cell (RBC) count (6.02 ± 0.65 ×10⁹/L), and hemoglobin concentration (9.65 ± 0.12 g/dl) in normal control patients, while treatment with Rosmarinus officinalis significantly enhanced all hematological parameters. Specifically, at a dosage of 250 mg/kg, the metrics for neutrophil adhesion, WBC, RBC, and hemoglobin showed improvement from 17.94 ± 0.751%, 2.99 ± 1.17 ×10⁹/L, 5.98 ± 0.45 ×10⁹/L, and 10.9 ± 0.49 g/dl ($p<0.01$) to 21.79 ± 0.831%, 3.55 ± 0.09 ×10⁹/L, 7.06 ± 1.85 ×10⁹/L, and 11.0 ± 1.62 g/dl ($p<0.001$). These results imply that rosemary therapy aids in the recovery of hematopoietic activity and immune cell function (Table 9).

Table 09: Effect of ethanolic extracts against Cyclophosphamide induced neutrophil adhesion test, WBC, RBC and Hb

Groups	**Percentage of neutrophil adhesion**	**WBC (10^9/L)**	**RBC (10^9/L)**	**Hb (g/dl)**
Normal control	31.23±1.338	4.98±2.06	8.20±0.16	12.2±0.36
Disease group	14.71±0.762***	2.45±1.09***	6.02±0.65***	9.65±0.12***
Standard group	27.76±1.108###	3.72±0.06###	7.92±0.32###	11.9±0.37###
Test drug one (250mg/kg)	17.94±0.751##	2.99±1.17##	5.98±0.45##	10.9±0.49##
Test drug two (500mg/kg)	21.79±0.831###	3.55±0.09###	7.06±1.85###	11.0±1.62###

***Rosmarinus officinalis* against Cyclophosphamide treated TGFβ**

Cyclophosphamide administration resulted in an elevation of TGF-β levels, increasing from 82.69 ± 2.300 pg/ml in normal control subjects to 153.1 ± 2.522 pg/ml ($p<0.001$), indicating a robust immune response. Conversely, R. officinalis treatment reduced TGF-β levels to 65.88 ± 3.853 pg/ml at a dosage of 250 mg/kg and to 73.01 ± 1.857 pg/ml at 500 mg/kg, while the standard group exhibited nearly normative TGF-β levels at 80.85 ± 1.733 pg/ml ($p<0.001$). These findings imply that rosemary extract plays a significant role in mitigating cyclophosphamide-induced immunosuppression through the modulation of TGF-β-related pathways, thereby aiding in the restoration of immune equilibrium (Table 10).

Table 10: *Rosmarinus officinalis effects* against Cyclophosphamide induced TGFβ by ethanolic extracts.

Groups	**TGFβ (pg/ml)**

Normal control	82.69±2.300
Disease group	153.1±2.522***
Standard group	80.85±1.733###
Test drug 1 (250mg/kg)	65.88±3.853##
Test drug 2 (500mg/kg)	73.01±1.857###

***Rosmarinus officinalis* extracts impact on body weight**

Table 11: Impact on *Rosmarinus officinalis* alcoholic extracts on body weight

Group	Body Weight (Day 1) (g)	Body Weight (Day 4) (g)	Body Weight (Day 11) (g)
Normal Control	22.69 ± 0.40	23.74 ± 1.03	24.01 ± 0.72
Disease Control	20.19 ± 1.02	18.20 ± 0.36	16.74 ± 0.16***
Standard Treatment	22.15 ± 1.62	22.65 ± 0.98	22.98 ± 0.04###
Test Drug 1 (250 mg/kg)	20.88 ± 1.83	20.96 ± 0.12	21.65 ± 0.36##
Test Drug 2 (500 mg/kg)	20.01 ± 1.75	20.98 ± 0.01	21.00 ± 0.17###

Values are expressed as Mean ± SD (n = 6).
P < 0.001 compared with the Normal Control group.
##P < 0.01 and ###P < 0.001 compared with the Disease Control group

Discussion:

In this work, we have investigated the pharmacognostic, phytochemical, antioxidant, cytotoxic, toxicological and immunomodulatory properties of *Rosmarinus officinalis* and *Hygrophila spinosa*. Both plants were analyzed in terms of physicochemical parameters where they were found to have acceptable quality control parameters such as minimal foreign matter, moisture content, and standard ash and extractive value. These are very important to understand the purity, identity, and quality of crude drugs and are in accordance with the pharmacognostic standards for medicinal plants. The higher extractive value of *R. officinalis* in alcohol and water indicates that its polar bioactive compounds are more concentrated than those in *H. spinosa*. Despite secondary metabolites in alkaloids, flavonol, phenolic and foaming glycosides in both extracts. Sterols and carbohydrates were only present in *H. spinosa*, and these findings confirm previous studies that both species are rich in biologically active secondary metabolites related to antioxidant, anti-inflammatory and immune function. Phenolics and flavonoids are particularly important because they can scavenge reactive oxygen and alter immune response. UV absorption bands and derivatization patterns confirmed the chemical complexity of the extracts were determined through chromatographic-HPTLC fingerprints. The unique chromatographic fingerprints identified in this study could help in authentication and quality assessment of herbal products containing *R. officinalis* and *H. spinosa*. Chromatographic information and phenolics, flavonoids and terpenoids in these plants are similar. We assessed the free radical reducing function for both extracts via stable-DPPH assay with both extracts and found that both extracts have free radical scavenging potential and that *H. spinosa* showed an inhibitory concentration of 229.5 ± 9.2 microgram per millilitre and *R. officinalis* showed a lower inhibitory concentration of 231.5 ± 11.6 microgram per

millilitre with slightly higher activity. While the antioxidant activity was lower than we found in the standard Trolox, it could be because of phenolics and flavonoids in the extract. According to earlier research, polyphenols derived from rosemary, such as carnosic acid and rosmarinic acid, exhibit antioxidant properties by neutralizing free radicals and donating hydrogen atoms. The MTT assay showed that both extracts still have greater than 90% cell viability in HT-29 and Caco-2 cells at the highest concentration (400 μg/mL). The IC_{50} values above 16 mg/mL are also consistent with their safety for intestinal epithelial cells. Similar observations have been made for rosemary and *H. spinosa* extracts, which have low toxicity at the appropriate dose level. There was no death or treatment side effects up to 2000 mg/kg. The functional battery parameters were normal throughout the study period, which suggests a good level of safety. According to the OECD toxicity classification, substances that do not cause mortality at 2000 mg/kg are practically non-toxic. The results support the traditional medicinal use of both plants and also indicate that both plants are safe for future pharmacological studies [35]. In vitro studies of mast cell stabilization and phagocytosis against *Candida albicans* showed strong immunomodulatory activity in *R. officinalis*, which was slightly higher in mast cell degranulation (79.48%) than *H. spinosa* (73.33%), indicating superior anti-allergic activity. The higher phagocytic index suggests the immune system is stimulated as well. Mast cell stabilization is the major mechanism of allergic and inflammatory disorders as it inhibits histamine release. Extracted phenolic chemicals, flavonoids and terpenoids may be the reason for the immunomodulatory effect [36]. The immunomodulatory activity of *R. officinalis* in vivo with cyclophosphamide-induced immunosuppression confirmed the immune-enhancing effect of *R. officinalis* and significantly improved delayed-type hypersensitivity to antibody production, IgG and IgM levels, cytokine production (IFN-γ, IL-2, IL-4 and IL-10) and haematological parameters. Cyclophosphamide is known to suppress humoral and cellular immunity by cytotoxic action on rapidly proliferating lymphocytes and *R. officinalis* extract significantly reversed these changes. This indicates that Th1- and Th2-mediated immune responses were enhanced and hence the immune response was enhanced. Neutrophil adhesion, WBC count, RBC count and haemoglobin concentration show recovery of immune and hematopoietic function in the treated groups. Moreover, TGF-β levels are reduced after treatment, meaning that inflammatory and immunosuppressive pathways are regulated as well. The restoration of body weight in treated animals supports the protection of the extract from cyclophosphamide-induced systemic toxicity [37]. The higher dose (500 mg/kg) was found to provide better response than the lower dose, indicating an immunostimulatory effect at the dose level. *Rosmarinus officinalis* and *Hygrophila spinosa* as a whole show that the two have significant pharmacological prospects. Both plants had antioxidant, safety and immunomodulatory effects but *R. officinalis* showed the strongest mast cell stabilization and in vivo immunostimulatory effects [38]. The synergistic activity of terpenes, flavonoids, phenolic compounds and other secondary metabolites is probably responsible for the observed activities. These findings are useful to identify and characterize the active ingredients responsible for these biological activities, as well as to justify the traditional use of these plants as immunomodulatory medicines.

Conclusion: The pharmacological characteristics of the aqueous ethanolic extracts of *Rosmarinus officinalis* and *Hygrophila spinosa* are confirmed to be favorable, with no cytotoxic effects on HT-29 and Caco-2 cell lines and no acute toxicity at doses up to 2000 mg/kg. They also have moderate antioxidant properties. Both extracts showed significant in vitro immunomodulatory effects by stabilizing mast cells and enhancing phagocytic activity, and R. officinalis protected the immune system from cyclophosphamide-induced immunosuppression of the body (hence the delayed-type hypersensitivity response, hemagglutination antibody titers, immunoglobulin levels, hematological parameters, and cytokine balance were restored). The beneficial effects of both are probably due to the interplay of phenolics, flavonoids, terpenoids, etc. The results demonstrate that medicinal plants are well-suited for immunomodulation. *R. officinalis* is a promising candidate for plant-based immunotherapy. Further studies of bioactive compound isolation, molecular mechanisms, pharmacokinetics, and multi-omics are needed to confirm its therapeutic potential.

Authors contributions-

Dolly Rani: Conceptualization, methodology, investigation, data curation, formal analysis, writing—original draft preparation.

Puja Saha: Pharmacognostical studies, phytochemical analysis, validation, data interpretation, writing—review and editing.

Paramita Ganguly: Toxicological and preclinical investigations, statistical analysis, data curation, manuscript review.

Sandip Chatterjee (Corresponding Author): Study design, supervision, project administration, resource acquisition, interpretation of results, manuscript preparation, review and editing, correspondence with the journal.

Pawan Kumar Goswami: Supervision, validation, critical review of the manuscript, scientific guidance, and final approval of the submitted version.

All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work.

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