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ORIGINAL ARTICLE

Stimulation of Murine Immune Response by *Clerodendrum infortunatum*

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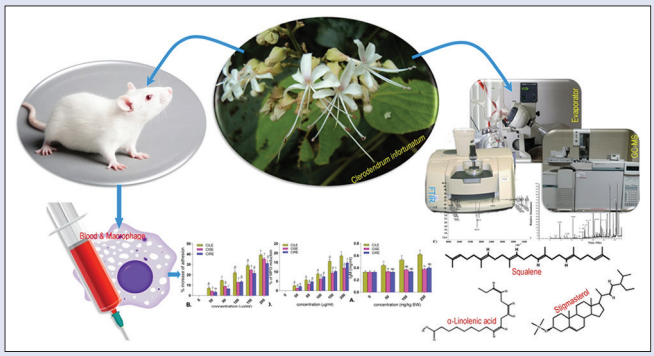
ABSTRACT

Background: *Clerodendrum infortunatum* is a medicinal plant found especially in the Sub-Himalayan regions of West Bengal. Various tribal communities of this region have been using these plants as a source of natural medicine. **Objective:** Medicinal values of *C. infortunatum* are well established, but the immunomodulatory properties have never been studied. Therefore, we wanted to investigate various immunomodulatory activities of this plant. **Materials and Methods:** Several parameters such as plaque-forming cell assay, hemagglutination titer, phagocytic activity of macrophages, and inhibition of lipopolysaccharide (LPS) were performed. Moreover, effects of *C. infortunatum* on the weight of various organs and total serum protein, albumin, and globulin levels were also determined. The phytochemical fingerprints of *C. infortunatum* extracts were obtained from Fourier transform infrared and gas chromatography-mass spectrometry analysis. **Results:** The macrophage stimulation and plaque-forming cell numbers (using sheep red blood cell as antigen) were significantly proliferated and found optimum at 100 and 250 mg/kg doses of leaf and root extracts simultaneously. The extent of stimulation in mural humoral immunity was in the order of leaf > root > stem. The anti-inflammatory activity of *C. infortunatum* was confirmed by the inhibition of LPS-induced nitric oxide synthesis by macrophages. **Conclusion:** It can be concluded that 70% hydromethanolic extract of *C. infortunatum* has profound immunomodulation potentials in the murine model, stimulating both humoral and innate form of the immune system.

Key words: *Clerodendrum infortunatum,* Fourier transform infrared, gas chromatography-mass spectrometry, immunomodulation, plant extract

SUMMARY

- *Clerodendrum infortunatum* is used as traditional medicine to cure common ailments such as bronchitis, asthma, fever, blood infection, and inflammation
- The macrophage stimulation and plaque-forming cell numbers (using sheep red blood cell as antigen) were significantly proliferated and found optimum at 100 and 250 mg/kg doses of leaf and root extracts simultaneously
- Our study revealed that the hemolytic activity of *C. infortunatum* is very negligible in case of all the extracts compared to standard Triton X and therefore may be considered safe from the hemolytic perspective
- It can be concluded that 70% hydromethanolic extract of *C. infortunatum* has profound immunomodulation potentials in the murine model, stimulating both humoral and innate form of the immune system.



Abbreviations used: PFC: Plaque-forming cell assay; HA titer: Hemagglutination titer; LPS: Lipopolysaccharide; FTIR: Fourier transform infrared; GC-MS: Gas chromatography-mass spectrometry; SDS: Sodium dodecyl sulfate; sRBC: Sheep red blood cell; PBS: Phosphate buffer saline; PC: Phagocytic capacity; PI: Phagocytic index; NaCl: Sodium chloride; $CaCl_2$: Calcium chloride; NBT: Nitro blue tetrazolium; DMSO: Dimethyl sulfoxide; KOH: Potassium hydroxide; H_2SO_4: Sulfuric acid; HOCl: Hypochlorous acid; H_2O_2: Hydrogen peroxide; EDTA: Ethylenediaminetetraacetic acid.

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INTRODUCTION

Currently, there is a worldwide increase in diseases, especially the infectious disease. This requires a proportionate increase in body's defense mechanisms to control the wrath through a process called immunomodulation. In developing nations, malnutrition and infectious diseases have always worked hand in hand. This remains a challenge as they compromise the body's immune responses, especially in the affected individuals.[1] Effective immune responses typically involve antigen presentation, activation of T- and B-lymphocytes, and secretion of immune effectors molecules such as antibodies and cytokines. Various tribal communities confined in different parts of India use medicinal plants for the treatment of several diseases. Even in ancient civilizations of Mesopotamia, Egypt, Greece, India, and China, the plant-derived medicines were extensively consumed for the treatment of various inflammation and tumor.[2,3] Many immunomodulatory agents derived from plant products are used to either suppress or stimulate the immune responses of an organism against the antigens. In the rural areas of the

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Sub-Himalayan region, there are lots of tribal populations such as Munda, Oraon, Santhal, Rava, Gurkha, and Lepcha, who are suffering from malnutrition and various infectious diseases. These tribal populations do not fulfill their required amount of dietary supplement to have a proper defensive immune system. The allopathic medicines are obviously the first choice when it comes to counter any sort of infection. These allopathic drugs stimulate or suppress the immune functions rapidly but have many side effects. Moreover, these drugs are very expensive for the poor people. In distant places and rural areas, these drugs are not easily available, and in the most cases, they are associated with advance drug reactions. As a result, the majority of tribal populations turn to use alternative herbal medicine that is widely accepted, is easily available, and assumed to have fewer side effects.[4] In the present investigation, *C. infortunatum* Linn. is selected to ascertain its ethnopharmacological importance with scientific evidence. *C. infortunatum* Linn. is a shrub found abundantly in the Sub-Himalayan regions of West Bengal and Sikkim provinces of India. This plant is used as traditional medicine to cure common ailments such as bronchitis, asthma, fever, blood infection, inflammation,[5] burning sensation, tuberculosis, and hepatoprotective.[6] It is also used as an antiepileptic in Indian folk medicine.[7,8] Conventionally, the Kuki and Rongmai Naga tribes found in Northeast India use this plant extract orally to cure fever, bowel troubles, and scorpion sting. In Sub-Himalayan region of North Bengal, various tribal communities use fresh root bark as a remedy for diarrhea. Likewise, Kachari, Hmar, and Riang tribal communities of Barak Valley and North Cachar Hills use this plant to combat stomach pain and diabetes.[9] This plant is also used as a bandage in swelling[10] and vermifugation and in the treatment of malaria.[11] There are some bioactive compounds that were isolated from *C. infortunatum* such as β-sitosterol, clerosterol, clerodolone, clerodone, and luperol, which have tremendous medicinal properties.[12,13] Since no extensive immunomodulatory study in animal model has been carried out yet with *C. infortunatum* crude extract, we have attempted to evaluate the stimulation of murine immune responses by *C. infortunatum*. Besides these, we have also done the phytochemical fingerprinting of *C. infortunatum* using gas chromatography-mass spectrometry (GC-MS) and Fourier transform infrared (FTIR) analysis to identify the bioactive compounds which stimulate or enhance the murine immune system.

MATERIALS AND METHODS

Plant material

C. infortunatum was collected between April and May 2016 from the medicinal plant garden of Department of Botany, University of North Bengal. The plant was identified by a plant taxonomist of Department of Botany, University of North Bengal. The specimen (Accession no. 9617) was deposited at the Herbarium of the same department.

Chemicals

All solvents and materials were obtained from HiMedia Laboratories Pvt. Ltd (Mumbai, India), unless otherwise indicated. Lipopolysaccharide (LPS), Freund's incomplete adjuvant, and nystatin were procured from Sigma Aldrich, MO, USA. Sodium dodecyl sulfate (SDS) was procured from Merck Specialties Pvt. Ltd, Mumbai, India, and phosphoric acid was obtained from Thomas Baker Chemicals Pvt. Ltd, Mumbai, India.

Preparation of plant extract

The fresh and disease-free parts of the plant such as leaf, stem, and root were washed twice with double distilled water. After proper washing, leaf, stem, and root were separated and shade-dried at room temperature for 21 days. Dried parts were pulverized into fine powder using a mechanical grinder. The powdered samples (10 g) were extracted in a Soxhlet apparatus using 70% methanol (the ratio of plant material to solvent was 1:10 m/v) for 6–7 h. The extracts were then concentrated under reduced pressure and controlled temperature (40°C–50°C) using rotary evaporator (Buchi Rotavapor R-3, Switzerland). After that, the extracts were further lyophilized using Eyela Freeze Dryer (FDU-506, USA) to obtain dry powder and stored at −20°C until required. The yield of the extract was calculated following the formula:

$$y = \frac{W_2 - W_1}{W_0} \times 100$$

where W_2: Weight of lyophilized extract and container, W_1: Weight of container, and W_0: Weight of the initial dried plant sample.

Animals and care

Both male and female Swiss albino mice of 6–8 weeks of age and 25 ± 2 g body weight (BW) were used for all the experiments. Mice were kept in polypropylene cages, with paddy husk as bedding material. The experimental mice were maintained in the animal house of the Department of Zoology, University of North Bengal, with sufficient food and water *ad libitum* under a constant 12-h dark/light cycle at an environmental temperature of 25°C. Complement was collected from guinea pigs (250 g) to perform plaque-forming cell (PFC) assay. Immunization was done using sheep red blood cell (sRBC) which was preserved at the Departmental Animal House. Animal Ethical Committee (Registration No. 840/ac/04/CPCSEA) was approved for performing all the *in vivo* experiments.

Immunomodulatory activity

Both *in vivo* and *in vitro* experiments were performed to investigate the immunomodulatory activities of *C. infortunatum*.

In vivo experiments

Acute toxicity test

Acute toxicity study of *C. infortunatum* was done according to the Organization for Economic Cooperation and Development guidelines (test 423: acute oral toxicity–acute toxic class method; 2002).[14] Mice were divided into six groups ($n = 6$) for leaf, stem, and root extracts and were made to fast overnight before the experiment. Hydromethanolic extract of *C. infortunatum* was administered orally at 1000 and 1500 mg/kg of BW dose and carefully observed for the development of any clinical or toxicological symptoms up to 48 h.

Dosage

Sixty Swiss albino mice were divided into ten groups ($n = 6$ per group) for *in vivo* immunomodulatory study. All the extracts were received orally for 20 days to perform the *in vivo* experiments. Group I (normal) received normal saline. Groups II, III, and IV (experimental) received *C. infortunatum* leaf extract (CILE) 50, 100, and 250 mg/kg BW, respectively. Groups V, VI, and VII (experimental) received *C. infortunatum* stem extract (CISE) 50, 100, and 250 mg/kg BW, respectively, and Groups VIII, IX, and X (experimental) received *C. infortunatum* root extract (CIRE) 50, 100, and 250 mg/kg BW, respectively.

To measure the body and organ weight and count the splenocyte and leukocyte at day 21, 24 h after the last dose, all the animals were sacrificed under a proper anesthetic condition, and relative organ weight (organ weight/100 g of BW) of the liver and spleen and the percentage BW gain/loss were measured. Single cell suspensions of the spleen (prepared in RPMI containing 5% fetal bovine serum [FBS]) and total leukocyte were prepared (using Leishman stain) to count the cellularity by hemocytometer.[15]

Plaque-forming cell assay

The PFC assay, the key experiment to determine the effect of immunomodulation, was performed according to the standard method with slight modifications.[16] Mice were immunized with sRBC at day 17, and on the 4th day, all the mice were sacrificed under proper anesthesia. A single cell suspension of the spleen was prepared in phosphate buffer saline (PBS) and cells were adjusted to a concentration of 10^6 cells/ml. 0.1 ml cell suspension was mixed with 0.05 ml guinea pig complement and 0.05 ml of 25% sRBC. Glass slide and bi-gummed tape (Scotch Brand, St. Paul, MN) were used to prepare the Cunningham chambers.[16,17] Then, the prepared samples were placed in the chambers and sealed with paraffin and petroleum jelly (1:1). After incubation for 4 h at 37°C, the plaques were counted under a phase-contrast microscope.

Estimation of total immunoglobulin M

The blood sample was collected from the immunized mice. The collected blood sample was kept at room temperature for 30 min for the separation of serum. After separation, straw-colored serum was isolated using centrifugation at 1000 rpm for 5 min. The isolated serum was diluted 10,000-fold for the estimation of immunoglobulin M (IgM) level using commercially available kit (MyBiosource, USA) according to the manufacturer's instruction.

Hemagglutination titer assay

Hemagglutination (HA) titer assay was performed according to the standard method with slight alterations.[17] Serum was collected from the blood on the 4th day of immunization with sRBC. After that, serum was kept in a water bath at 56°C for 45 min for the inactivation of complement activity. Khan tubes were used for the determination of HA titer. Serum (0.1 ml) and PBS (0.9 ml) were added to the first Khan tube. In the remaining Khan tubes, 0.5 ml of PBS was added. Then, 0.5 ml of mixture was added to the second Khan tube from the first one and 0.5 ml of the mixture was simultaneously added to the third tube from the second one. In the same manner, eight such dilutions were prepared as double-fold dilution; 0.5 ml of the solution was thrown away from the last tube, thus yielding a serial dilution of 1/10, 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280. 0.1 ml of 10% sRBC was added to each tube. Then, all the Khan tubes were incubated at 37°C for 12 h in a humidified atmosphere, and after incubation, visible HA was observed and the degree of agglutination was also noted.

Counting of peritoneal macrophages

The peritoneal macrophages were enumerated according to the standard protocol.[18] 0.5 ml of Freund's incomplete adjuvant was injected in the peritoneum, 1 day before the experiment at day 20, to count the peritoneal macrophages. The peritoneal exudates were collected by washing the peritoneum with PBS. The peritoneal exudate cells were collected after a spin for 5 min at 1000 rpm in a centrifuge followed by washing two times in PBS; the remaining pellet was resuspended in PBS. The total mixture was incubated for 45 min at 37°C in a Petri dish. After incubation, the Petri dish was washed with 2% ethylenediaminetetraacetic acid in chilled PBS and centrifuged for 5 min at 1000 rpm. After centrifugation, the pellet was mixed in PBS. Neutral red, a vital dye, was added to the cell suspension, and the macrophages were counted using hemocytometer under the phase-contrast microscope.

Assessment of phagocytic activity of macrophages

Stimulation of phagocytic activity of macrophages was performed according to a standard method with little modifications.[19] The procedure of macrophage collection was described previously. After the collection of macrophages from the *C. infortunatum*-treated mice (50 mg/kg BW, 100 mg/kg BW, and 250 mg/kg BW, respectively for 20 days), the cell suspension was prepared in a way that the density became 5×10^6 cells/ml. Then, 0.1 ml cell suspension, 0.1 ml of 20% FBS, and heat-treated yeast cell suspension (100×10^6 cells/ml) were mixed and incubated at 37°C for 1 h with occasional shaking. After incubation, the 50 µl mixture was smeared on a glass slide and stained with Wright–Giemsa. The stained slide was observed and 500 cells were counted under a light microscope. The phagocytic activity was expressed as phagocytic capacity (PC). Phagocytic index (PI) was calculated using the following formula:

PI = AB

where A = percentage of yeast-ingesting phagocytes and B = number of yeast-ingested per phagocytes; PC = mean percentage of cells that engulfed ≥4 yeast cells.

Estimation of total serum protein, albumin, and globulin

Serum was collected on the 21st day from the tail vein of a mouse for the estimation of protein, albumin, and globulin. Commercially available kits (Crest Biosystems, India) were used to determine the total serum protein, albumin, and globulin.

In vitro experiments

Hemolytic assay

Blood samples were collected from Swiss albino mice (25 ± 2 g) followed by three times washing of blood cells with 20 mM Tris-HCl containing 144 mM NaCl (pH 7.4). Two percent erythrocyte suspension was prepared for hemolytic assay.[20] The experiment was done in a 96-well plate, and 100 µl of 0.85% NaCl solution containing 10 mM $CaCl_2$ was added in each well. The first well containing the solvent alone served as control, and from the second well onward, 100 µl of plant extract was added with various concentrations (0–200 µg/ml). Triton X (0–200 µg/ml) was added to the last well which served as positive control. Thereafter, 2% erythrocyte suspension was added in each well and incubated at room temperature for 30 min. After incubation, the suspension was centrifuged and the supernatant was collected. Then, the supernatant was used to measure the absorbance of liberated hemoglobin at 540 nm. The hemolytic activity was calculated using the following formula:

$$\text{Percentage of hemolytic activity} = \frac{H_0 \quad H_1}{} \times 100$$

where H_0 is the absorbance of the blank and H_1 is the absorbance of sample and standard (Triton X).

In vitro cell adhesion assay

Cell adhesion assay was performed according to the standard method with slight modifications.[21] 0.5 ml Freund's incomplete adjuvant was injected intraperitoneally 1 day before the experiment. For the collection of macrophages, a midline incision was made in the abdomen of mice with proper anesthesia, and the peritoneum was carefully washed with RPMI-1640 on the 4th day. Then, the peritoneal exudate cells were collected and centrifuged at 1000 rpm at 4°C for 10 min. The supernatant was discarded after centrifugation and the remaining pellet was resuspended in RPMI-1640. The number of cell was adjusted to 5×10^6 cells/ml and seeded in 96-well plate with different concentrations of 0–200 µg/ml of *C. infortunatum* extract. Then, the total experimental setup was incubated for 60 min, and after incubation, the cells were gently washed with RPMI 1640. After washing, 100 µl crystal violet (0.5% dissolved in 25% of methanol) and 10% ethanol were added to each well and incubated for 4 h at 37°C under humidified condition. At the end of the incubation, 100 µl of 1% SDS was added and the absorbance was measured at 570 nm. Adherence property was measured using the following formula:

Percentage increase of adherence = $\frac{A_0 - A_1}{A_0} \times 100$

where A_0 = absorbance of the control (cells without treated plant extract) and A_1 = absorbance of the test sample.

Respiratory burst activity

The assay was performed according to the standard protocol with some modifications.[22] Murine peritoneal macrophages were collected in RPMI-1640 as previously described and seeded into 96-well plate. The plate was precoated with 0.2% poly-L-lysine along with various concentrations (0–200 μg/ml) of *C. infortunatum* extracts. Zymosan (0.1%) was added and the plate was incubated for 30 min at 37°C under humidified condition. After incubation, cells were washed thrice with RPMI-1640 followed by staining with 100 μl NBT (0.3%) at room temperature (RT). The NBT solution was discarded after 30 min, and the reaction was stopped by addition of 100 μl absolute methanol. The formazan which was generated was dissolved in 120 μl of 2M KOH and 140 μl of dimethyl sulfoxide and the absorbance was immediately taken at 630 nm.

Myeloperoxidase release assay

The assay was performed according to the standard protocol with slight modifications.[23] Murine peritoneal macrophages (2×10^6 cells/ml) were seeded into 96-well culture plate. To this, 100 ng/ml LPS and varying concentrations (0–200 μg/ml) of the plant extracts were added. The cells were then incubated at 37°C under a humidified condition for 60 min, and after incubation, the solutions from each well were centrifuged at 13,000 rpm for 10 min. After centrifugation, the supernatants were removed and 0.01% SDS (dissolved in RPMI-1640) was added to lyse the cells. The solution was centrifuged at 13,000 rpm for 10 min and the supernatant (100 μl) collected from each group was mixed with 100 μl substrate buffer (ortho-phenylenediamine). After 20 min, the reaction was stopped using 100 μl of 2N H_2SO_4 and the absorbance was read at 492 nm.

Inhibition of lipopolysaccharide-induced nitric oxide production

Inhibition of nitric oxide (NO) production was done according to the standard methods with slight modification.[24] Griess reagent method was applied to estimate the NO level. Macrophage was collected intraperitoneally by the method as described previously. 200 μl of cell suspension (2×10^6 cells/ml) was added in each well of 96-well plate with the addition of 50 U/ml penicillin, 50 U/ml streptomycin, 50 U/ml nystatin, 10% FBS, 100 μl of plant extract of different concentrations (0–200 μg/ml), and LPS suspension (20 μg/ml). After that, the total reaction mixture was incubated for 24 h under 5% CO_2 in humidified atmosphere of 90% air at 37°C temperature. After incubation, centrifugation was done at 5000 rpm for 5 min. 50 μl of the supernatant was mixed with 200 μl of Griess reagent in each well of 96-well plate and incubated for 20 min at room temperature. Purple azo dye was formed after incubation, and the dye was measured at 540 nm. The NO level was measured using the following formula:

Percentage inhibition of NO level = $\frac{A_0 - A_1}{A_0} \times 100$

where A_0 = absorbance of the control (cells without treated plant extract) and A_1 = absorbance of the test sample.

Fourier transform infrared analysis

The characteristic of functional groups in leaf, stem, and root of *C. infortunatum* was identified by using FTIR spectrophotometer. 10 mg of plant extracts of leaf or stem or root was taken in CaF_2 vessel and placed in a sample cup of a diffuse reflectance accessory. Shimadzu 8300 FTIR spectrophotometer was used to identify the functional group available in the extracts. The leaf, stem, and root extracts of *C. infortunatum* were scanned from 400 to 4000 cm^{-1} for 16 times to increase the signal-to-noise ratio.

Gas chromatography-mass spectrometry analysis

C. infortunatum extract was mixed with 5 ml n-hexane. Then, the mixture was incubated for 2 days at 4°C with occasional shaking and centrifuged at 15,000 rpm for 20 min at 25°C. The resultant supernatants were collected and filtrated using Whatman filter paper no. 1. Then, the supernatant (200 μl) was mixed with 20 μl of N, O-bis (trimethylsilyl) trifluoroacetamide + trimethylchlorosilane (99:1 v/v) mixture and incubated 60 min at laboratory temperature with occasional shaking.

Thereafter, *C. infortunatum* extract was analyzed using Thermo Scientific Trace 1300 gas chromatography instrument attached with Thermo Scientific ISQ QD single quadrupole mass spectrophotometer. The GC was equipped with TG-5MS column (30 m × 0.25 mm × 0.25 μm). The inlet temperature was maintained at 250°C and the initial temperature of the program was set at 60°C (solvent delay 5 min) with a hold of 4 min, followed by a ramp of 5°C to 290°C with a hold of 10 min (60-min program). Samples of leaf, stem, and root extract of *C. infortunatum* (1 μl) were injected in a splitless mode (split flow 50 ml/min) with a splitless time of 0.80 min, using a Thermo Scientific AI-1310 auto-sampler with a constant flow of helium gas (1 ml/min). MS transfer line temperature was set at 290°C with an ion source temperature of 230°C (electron ionization). The individual samples were analyzed at electron energy 70 eV (vacuum pressure: 2.21e-0.5 Torr) and the mass analyzer range was set to 50–650 amu for better performance. All the plant extracts (leaf, stem, and root) were analyzed thrice for confirmation of the result.

Statistical analysis

All data have been reported as mean ± standard deviation of six measurements. KyPlot version 2.0 beta 15 (32 bit) and Graph Pad Prism version 7.03 were used for statistical analysis. One-way analysis of variance with Dunnett's test was used to analyze the data.

RESULTS

Acute toxicity study

In the acute toxicity study, no sign of mortality of mice was observed following the concentration from 0 to 1500 mg of leaf, stem, and root extract of *C. infortunatum*/kg BW. Hence, 50 mg/kg BW, 100 mg/kg BW, and 250 mg/kg BW were selected for the *in vivo* immunomodulatory experiments.

Effect of plant extract on body weight, organs' weight, cellularity, and leukocyte count

Effect of various doses of *C. infortunatum* extracts in different organs on the basis of weight is shown in Table 1. No significant changes in terms of increase or decrease in the BW, liver weight, and spleen weight were observed in mice that were fed with leaf, stem, and root extract for 20 days when compared with their respective control groups.

Table 1: Effect of different doses of *Clerodendrum infortunatum* leaf extract, *Clerodendrum infortunatum* stem extract and *Clerodendrum infortunatum* root extract on liver, spleen, and body weights of mice after 20 days along with the phagocytic index of stimulation of phagocytosis assay

Dose (mg/kg)	Body weight	Liver weight	Spleen weight	PI of yeast cell phagocytosis
Control (0)	25.64±0.52	4.66±0.27	0.53±0.05	62.95±4.18
50	25.96±0.83 NS	5.06±0.07 NS	0.59±0.02 NS	72.06±3.57 NS
CILE				
100	25.62±1.00 NS	4.42±0.48 NS	0.46±0.03 NS	79.02±2.55*
250	25.77±0.26 NS	4.90±0.16 NS	0.48±0.03 NS	85.37±3.86*
50	25.97±0.56 NS	4.73±0.24 NS	0.54±0.04 NS	66.23±3.14 NS
CISE				
100	25.82±0.28*	4.99±0.30 NS	0.62±0.04 NS	70.61±2.46 NS
250	25.78±0.34 NS	5.59±0.26 NS	0.62±0.01 NS	74.17±2.25 NS
50	25.28±0.61 NS	5.51±0.22*	0.52±0.02 NS	69.08±2.27*
CIRE				
100	25.94±0.37 NS	5.17±0.52 NS	0.58±0.01 NS	73.98±1.75 NS
250	26.23±0.39 NS	5.08±0.29 NS	0.63±0.03 NS	81.81±2.17*

*$P \leq 0.05$. CILE: *Clerodendrum infortunatum* leaf extract, CISE: *Clerodendrum infortunatum* stem extract, CIRE: *Clerodendrum infortunatum* root extract; PI: Phagocytic index, NS: Nonsignificant

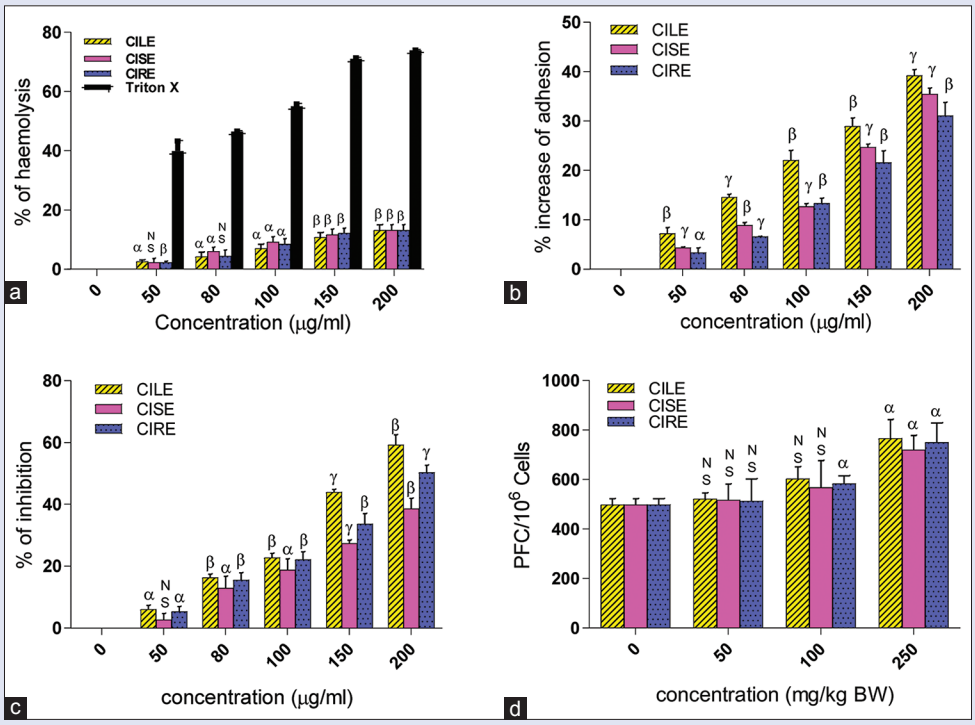

Figure 1: (a) Hemolytic activity, (b) *in vitro* cell adhesion assay, (c) inhibition of lipopolysaccharide-induced nitric oxide production, (d) plaque-forming cells assay. The value represents mean ± standard deviation of six sets of experiments. $^{\alpha}P \leq 0.05$, $^{\beta}P \leq 0.01$, $^{\gamma}P \leq 0.001$; when compared with control and standard (in case of hemolytic activity). NS: Nonsignificant; SD: Standard deviation

Assessment plaque-forming cell assay and hemagglutination titer

The potentiality of *C. infortunatum* extract to modulate the humoral immune response is presented in Figure 1d. The most poignant increase in PFC value was found at 250 mg/kg BW dose compared to the control in case of all the extracts. Results of the HA titer [Table 2] showed the highest visible agglutination at the 100 mg/kg dose, followed by 250 and 50 mg/kg dose in case of CILE, and 250 mg/kg followed by 100 and 50 mg/kg BW in case of CISE and CIRE when compared to the control.

Immunoglobulin M level

In vivo IgM level is displayed in Table 2 and Figure 2a. The IgM level at 0 mg/kg (control) was 0.33 ± 0.02 mg/ml. At 250 mg/kg, the level of IgM for CILE, CISE, and CIRE was 0.62 ± 0.02, 0.38 ± 0.01, and 0.40 ± 0.02 mg/ml, respectively. The dose-dependent correlation between PFC value and IgM level demonstrated high correlation between two interrelated parameters. The coefficient of determination (R^2) of PFC and IgM correlation for CILE, CISE, and CIRE was 0.8468, 0.8223, and 0.9769, respectively [Figure 2b-d].

Counting of peritoneal macrophage

Peritoneal macrophages elevated with the increase in the dose of leaf, stem, and root extract of *C. infortunatum,* supporting stimulation of murine immune response [Table 3]. The most significant increase of the macrophage population was found at the dose of 50, 100, and 250 mg/kg BW in case of CILE and 250 mg/kg BW in case of CISE and CIRE, respectively, when compared with control groups.

Table 2: The immunomodulatory effect of *Clerodendrum infortunatum* leaf extract, *Clerodendrum infortunatum* stem extract, *Clerodendrum infortunatum* root extract by plaque-forming cell assay, immunoglobulin M levels, and hemagglutination titer assay

Doses	Control		CILE			CISE			CIRE	
	0 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg	50 mg/kg	100 mg/kg	250 mg/kg
PFC	497.77±24.28	521.10±24.28	602.77±48.57	766.11±75.90	517.22±64.25	567.77±108.40	719.44±58.71	513.32±88.08	583.33±30.86	750.55±77.67
IgM	0.33±0.02	0.44±0.02	0.53±0.02	0.62±0.02	0.34±0.01	0.37±0.01	0.38±0.01	0.33±0.01	0.34±0.01	0.40±0.02
HA titre	1:40	1:40	1:160	1:80	1:40	1:80	1:160	1:40	1:80	1:160

PFC: Plaque forming cell; IgM: Immunoglobulin M; HA: Hemagglutination; CILE: *Clerodendrum infortunatum* leaf; CISE: *Clerodendrum infortunatum* stem extract; CIRE: *Clerodendrum infortunatum* root extract;

Stimulation of the degree of phagocytic activity of macrophages

The effect of CILE, CISE, and CIRE on the PC of murine peritoneal macrophage is illustrated in Figure 3a and b. The PC was measured by the mean percentage of macrophages engulfing >4 yeast cells. Among the three extracts, only CILE demonstrated significant increase ($P < 0.01$) in PC at 250 mg/kg dose as compared to 0 mg/kg dose (control group). In case of CILE, the PC at 0 mg/kg (control) was 12.66% ± 1.53% which was increased to 30.66% ± 2.52% at 250 mg/kg. In case of CIRE, the PC was 17.00% ± 2.00% at the highest dose. In case of CISE, the PC was 17.66% ± 2.09% at the highest dose. Figure 3b demonstrates the PI of CILE, CISE, and CIRE. The PI was calculated by multiplying the percentage of yeast-ingesting macrophages with the number of yeast-ingested per macrophages. Compared to 0 mg/kg (control group), the PI of CILE and CIRE was significant ($P < 0.05$) at 250 mg/kg dose. At the highest dose, the PI of C. *infortunatum* leaf extract (CILE) and C. *infortunatum* root extract (CIRE) was 162.66 ± 6.80 and 103.67 ± 11.06, respectively.

Determination of total protein, albumin, and globulin levels in serum

Total serum protein, albumin, and globulin levels poignantly increased with the increment in the doses of various extracts of *C. infortunatum* [Table 3]. The protein level was significantly increased at 100 mg/kg and 250 mg/kg BW in case of CILE and CIRE extracts and 250 mg/kg BW in case of CISE. The protein level is significantly elevated at 250 mg/kg BW in case of CISE.

Hemolytic activity

The hemolytic activity of *C. infortunatum* on murine erythrocytes was negligible when compared to the standard Triton X, as presented in Figure 1a. The percent of hemolysis increased in a dose-dependent manner in case of all the three plant part extracts.

In vitro cell adhesion assay

Adherence property of macrophages increased in a dose-dependent manner with the increase in *C. infortunatum* extract concentration [Figure 1b]. Impressive result was observed with every dose of all extracts.

Respiratory burst assay

An increase in absorbance signifies increased respiratory burst activity [Figure 3c]. Significance increases in absorbance were observed at highest dose (200 μg/ml) in case of CILE, CISE, and CIRE when compared with control group (0 μg/ml).

Myeloperoxidase release assay

Significant ($P < 0.001$) myeloperoxidase (MPO)-reducing capacity [Figure 3d] was observed in case of CILE, CISE, and CIRE. At 200 μg/ml, the amount of reduction of MPO for CILE, CISE, and CIRE was 18.59% ± 0.66%, 12.07% ± 0.75%, and 14.76% ± 0.71%, respectively.

Inhibition of lipopolysaccharide-induced nitric oxide production

The inhibition of NO production from LPS-stimulated macrophage by *C. infortunatum* extracts is represented in Figure 1c. The inhibition of NO is increased in a dose-dependent manner for all extracts. The plant extract showed a significant NO

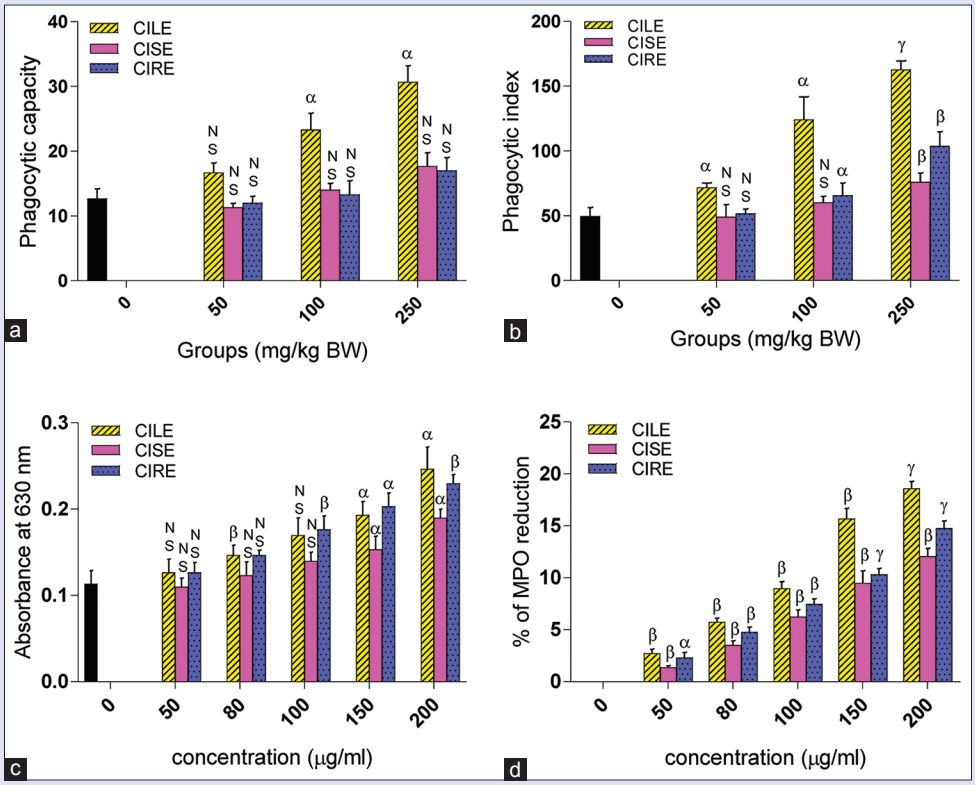

Figure 2: (a) Immunoglobulin M levels and linear correlation between (b) immunoglobulin M level and plaque-forming cell value of *Clerodendrum infortunatum* leaf extract, (c) immunoglobulin M level and plaque-forming cell value of *Clerodendrum infortunatum* stem extract (d) immunoglobulin M level and plaque-forming cell value of *Clerodendrum infortunatum* root extract. Where R^2: Coefficient of determination; NS: Nonsignificant; $^{\alpha}P \leq 0.05$, $^{\beta}P \leq 0.01$, $^{\gamma}P \leq 0.001$; when compared with control

Table 3: Effect of different doses of *Clerodendrum infortunatum* leaf extract, *Clerodendrum infortunatum* stem extract, *Clerodendrum infortunatum* stem extract and *Clerodendrum infortunatum* root extract on total serum protein, albumin, and globulin levels; splenocyte, leukocyte, and macrophage count after 20 days treatment

Doses mg/kg BW	Protein[a]	Albumin[a]	Globulin[a]	Spleenocyte[b]	Leukocyt[c]	Macrophage[b]
Control (0)	6.77±0.53	2.95±0.05	2.49±1.82	422.51±11.93	12395.36±135.87	16.00±0.50
50	8.06±0.39 NS	3.66±0.04**	4.39±0.39 NS	443.85±10.68 NS	12863.90±0.96*	16.97±0.40**
CILE						
100	8.51±0.20*	3.98±0.47 NS	4.53±0.40 NS	460.61±7.38*	13558.90±184.79**	18.64±0.24**
250	9.65±0.30**	4.20±1.34 NS	5.44±1.62 NS	476.6±5.35*	14591.42±116.53***	20.57±0.35**
50	6.97±0.27 NS	3.31±0.21 NS	3.66±0.44 NS	425.65±7.10 NS	12567.79±110.94 NS	16.08±0.76 NS
CISE						
100	7.22±0.20 NS	3.43±0.52 NS	3.77±0.60 NS	432.72±6.45 NS	13035.80±188.33*	16.813±0.16 NS
250	8.35±0.30*	3.96±0.72 NS	4.38±1.02 NS	453.91±4.65*	13700.85±164.64**	17.706±0.22**
50	6.77±0.37 NS	3.41±0.11 NS	3.36±0.26 NS	433.84±10.70 NS	12654.66±145.51 NS	16.79±0.47 NS
CIRE						
100	8.21±0.22*	3.73±0.44 NS	4.48±0.41 NS	450.61±7.42*	13604.36±238.53*	17.66±0.20 NS
250	9.31±0.09*	4.20±0.32*	5.11±0.41 NS	466.36±5.00*	14597.5±258.04*	18.99±0.30**

[a]Amount in g/dl, [b]Mean±SD×106 cells per ml, [c]Mean±SD cells/mm^3. *P≤0.05, **P≤0.01, ***P≤0.001; when compared with control. NS: Non significant, CILE: *Clerodendrum infortunatum* leaf extract, CISE: *Clerodendrum infortunatum* stem extract, CIRE: *Clerodendrum infortunatum* root extract; BW: Body weight; SD: Standard deviation

suppression capacity for each dose. At the highest concentration of 250 mg/kg BW, the percentage of inhibition was 59.25% ± 3.26% and 50.33% ± 2.27% by leaf and root, respectively, which is very convincing.

Fourier transform infrared analysis

The IR spectrum [Figure 4 and Table 4] of the leaf, stem, and root extract displayed different peaks corresponding to different functional groups present in *C. infortunatum*.

Gas chromatography-mass spectrometry analysis

Automated Mass Spectral Deconvolution and Identification System version 2.70 was used for MS data analyses. Mass fragmentation patterns using the database of National Institute Standard and Technology with an MS-Library Version 2011 were used for the identification of major and essential compounds. The identified several bioactive compounds from the leaf, stem, and root of *C. infortunatum* have diverse chemical nature; many of them possess distinct and definitive pharmacological activities [Table 5 and Figure 5].

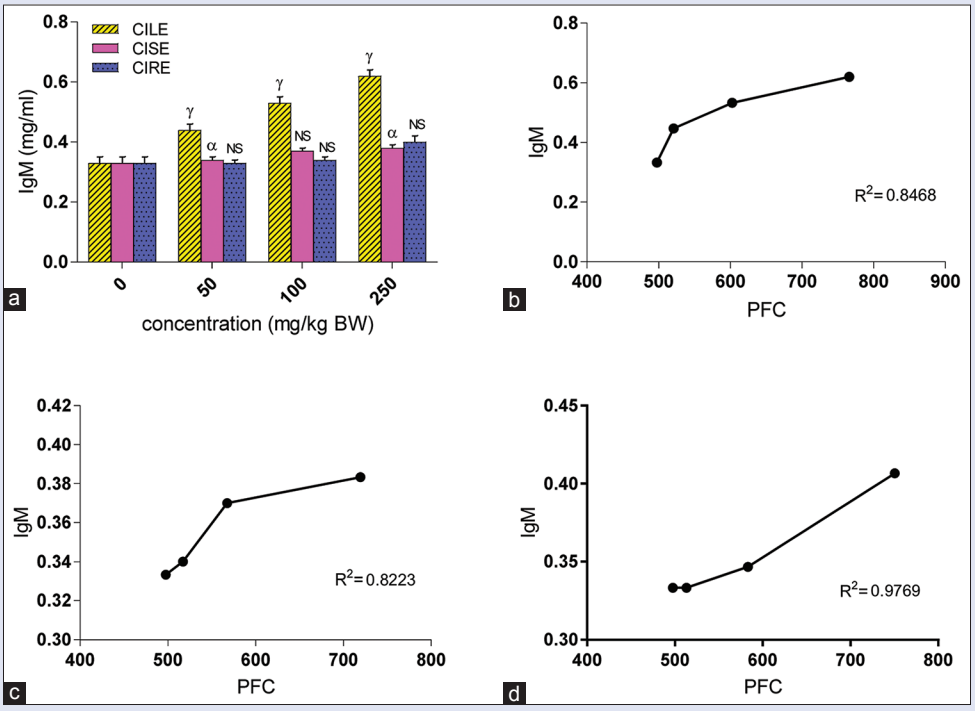

Figure 3: (a) Phagocytic capacity, (b) phagocytic index of murine peritoneal exudate macrophages, (c) respiratory burst activity of murine peritoneal exudate macrophages, (d) percentage of myeloperoxidase reduction in murine peritoneal macrophages. The value represents mean ± standard deviation of six sets of experiments. $^{\alpha}P \leq 0.05$, $^{\beta}P \leq 0.01$, $^{\gamma}P \leq 0.001$; when compared with control

DISCUSSION

Most of the multicellular living organisms possess immune system. The immune system works either through the humoral pathway or by the cell-mediated pathway to destroy the foreign materials and causative agents of many diseases. The 70% hydromethanolic extracts of *C. infortunatum* have been investigated to possess immense immunomodulatory activities. Hydromethanolic extracts of *C. infortunatum* were chosen as the sample material, because in ethnopharmacology and traditional system, medicines are prepared mostly in polar solvents and many ethnopharmacological medicines are actually tinctures prepared by hydroalcoholic solvent extraction processes. Immunomodulatory evaluation of CILE, CISE, and CIRE was performed using both *in vivo* and *in vitro* methods. Although the immunomodulatory potentialities of *C. infortunatum* have been claimed in the traditional medicinal systems, very few studies were performed to evaluate such claim. The BW and organ weight changes are one of the most crucial and sensitive indicators of an animal's health status, and it is widely accepted in the evaluation of test article-associated toxicities.[25] In the present study, no significant loss of BW and organ weight was observed supporting nontoxicity of *C. infortunatum*. Initial evaluation of the stimulation of murine humoral immune system was performed by PFC assay and corresponding IgM level estimation. The PFC assay is based on complement-mediated lysis of foreign antigen (sRBC) through immunoglobulin activity.[26] sRBC is a particulate T-cell-dependent natural antigen which activates T-cell to induce activation of enough B-cells in whole splenocyte population and evokes IgM-mediated immune response at higher multitude followed by the complement-mediated lysis of sRBC. IgM binds guinea pig complement more efficiently; thus, the PFC value is influenced by the generation of IgM level. sRBC-mediated antibody response is also routinely used in immunotoxicological studies.[27,28] In the present study, only CILE and CIRE demonstrated gradual dose-dependent stimulation of the murine humoral immune system as evaluated by PFC and IgM assay. The significant increase ($P < 0.001$) of the PFC and IgM value of CILE and CIRE at 250 mg/kg dose was higher than that of the control. The PFC value without any antigenic stimulation usually remains at basal level (5–30 PFC/10^6 cells).[26] HA titer assay is one of the key experiments to establish the immunomodulatory potential of *C. infortunatum*. Differentiation and proliferation of B-cells into antibody-secreting plasma cells were done by the interaction of naive B-cells with foreign antigens followed by the antibody binding to neutralize the antigen. Thus, a cross-linking latex was formed which was ingested by the phagocytic cells.[29] As the serum of the immunized mice contained antibody against sRBC, agglutination was visible. It was observed that leaf, stem, and root extracts of *C. infortunatum* exhibited optimum dose for the humoral antibody titer. Counting of peritoneal macrophages is another important parameter that could help in assessing the immunomodulatory activity of *C. infortunatum*. Macrophages are the important cells that control the cell-mediated and humoral immunity as antigen presenting cells engulf lead to digestion of cellular debris, foreign substances, microbes, and cancer cells in a process called phagocytosis.[29] Activation of macrophages may be responsible for immunostimulation. A significant increase in the number of peritoneal macrophages in case of *C. infortunatum*-treated mice, therefore, represents its immunomodulatory activity. The increase of PI of yeast cell in a dose-dependent manner in case of all the extracts demonstrated that *C. infortunatum* consumption had a significant effect on the phagocytic potential of the macrophages which stimulated the innate immunity as well.

Our present study showed the adherence property of the murine macrophages increased in a dose-dependent manner and is highest at 250 mg/kg BW in case of leaf extract, and this improvement might be primarily caused due to the increase in the expression of cell adhesion receptors such as integrin and mucin-like molecules. NO is released from macrophage as the primary effector molecule for inflammatory and cytotoxic activity. The release of NO can be used as a marker of macrophage

Table 4: Fourier transform infrared spectroscopy peak values of *Clerodendrum infortunatum*

Characteristic absorptions (cm^{-1})	Type of bonds	Functional group	Type of vibration	Intensity
3691.63	O-H	Alcohol	Stretch, free	Strong, sharp
3691.28				
3691.58				
3677.85				
3677.88				
3651.33				
3630.59				
3630.38				
3677.54				
3650.87				
3620.92				
3630.54				
3589.60				
3569.14				
3385.80	O-H	Alcohol	Stretch, H Bonded	Strong, broad
2925.28	C-H	Alkane	Stretch	Strong
2924.92				
2924.70				
2854.57				
2854.75				
2854.60				
2345.89	C=O	Aliphatic Ketone	Stretching	-
2345.93				
2345.75				
1735.77	C=O	Carbonyl	Stretch	Strong
1774.14				
1735.86				
1719.33				
1701.98				
1701.95				
1735.86				
1719.44				
1701.99				
1686.08	C=O	Amide	Stretch	Strong
1654.56				
1686.04				
1654.45				
1654.57				
1647.94				
1637.71	N-H	Amide	Bending	-
1637.80	N-H	Amide	Bending	-
1637.42				
1618.07				
1618.11				
1618.04				
1560.38				
1542.35				
1578.08				
1560.38				
1560.32				
1542.34	N-O	Nitro	Stretch	Strong, two bands
1542.31				
1508.35	C=C	Aromatic	Stretch	Medium-weak, multiple bands
1508.31				
1508.35				
1459.27				
1458.83				
1458.71				
1377.58	-C-H	Alkane	Bending	Variable
1376.89				
1376.50				
1053.21	C-O	Alcohol	Stretch	Strong
722.32	=C-H	Alkene	Bending	Strong

Table 5: Chemical fingerprints of *Clerodendrum infortunatum* revealed by gas chromatography-mass spectrometry analyses

Compound name	Molecular weight†	Formula	RT
CILE			
Lactic acid, bis-TMS	234	C9H22O3Si2	9.45
2-Methyl-4-pentenoic acid, trimethylsilyl ester	186	C9H18O2Si	9.79
Propane-1,2-diol, di-TMS	220	C9H24O2Si2	13.24
4-Hydroxybutyric acid, (2TMS)	248	C10H24O3Si2	14.35
Phosphoric acid, tris-TMS	314	C9H27O4PSi3	15.64
Succinic acid (2TMS)	262	C10H22O4Si2	16.47
(4-Allyl-2-methoxyphenoxy) trimethylsilane	236	C13H20O2Si	20.29
Malic acid (3TMS)	350	C13H30O5Si3	20.19
Tetradecane, 2,6,10-trimethyl-	240	C17H36	22.03
Dodecanoic acid, 1-methylethyl ester	242	C15H30O2	24.03
Octanedioic acid, bis (trimethylsilyl) ester	318	C14H30O4Si2	25.81
Azelaic acid, bis-TMS	332	C15H32O4Si2	27.91
Tetradecanoic acid, TMS	300	C17H36O2Si	28.87
n-Pentanoic acid, trimethylsilyl ester	314	C18H38O2Si	30.84
Palmitelaidic acid, trimethylsilyl ester	326	C19H38O2Si	32.23
Hexadecanoic acid, trimethylsilyl ester	328	C19H40O2Si	32.74
Heptadecanoic acid, TMS	342	C20H42O2Si	34.51
Linoleic acid, TMS	352	C21H40O2Si	35.70
α-Linolenic acid, TMS	350	C21H38O2Si	35.82
Stearic acid, trimethylsilyl ester	356	C21H44O2Si	36.26
Eicosanoic acid, trimethylsilyl ester	384	C23H48O2Si	39.50
Docosanoic acid, trimethylsilyl ester	412	C25H52O2Si	42.51
Squalene	420	C30H50	45.20
Tetracosanoic acid, trimethylsilyl ester	440	C27H56O2Si	45.32
Heptacosane	380	C27H56	48.73
(+)-α-Tocopherol, O-trimethylsilyl-	502	C32H58O2Si	49.58
Stigmasteroltrimethylsilyl ether	484	C32H56OSi	52.04
CISE			
Propanoic acid, 2-oxo-, trimethylsilyl ester	160	C6H12O3Si	5.25
2-Pyrrolidinone, 1-methyl-	99	C5H9NO	8.81
Propionic acid, 2-(trimethylsiloxy)-, trimethylsilyl ester	234	C9H22O3Si2	9.52
Hexanoic acid, trimethylsilyl ester	188	C9H20O2Si	9.70
Acetic acid, (trimethylsiloxy)-, trimethylsilyl ester	220	C8H20O3Si2	9.90
1-Trimethylsilyloxy-n-octene	200	C11H24OSi	10.04
Pentanoic acid, 2-oxo-, trimethylsilyl ester	188	C8H16O3Si	11.84
β-Hydroxybutyric acid (2TMS)	248	C10H24O3Si2	12.29
Tetradecane	198	C14H30	15.91
Succinic acid (2TMS)	262	C10H22O4Si2	16.48

Contd...

Table 5: Contd...

Compound name	Molecular weight†	Formula	RT
Glyceric acid, (3TMS)	322	C12H30O4Si3	17.15
4-Isopropylphenol, trimethylsilyl ether	208	C12H20OSi	17.24
Fumaric acid, bis-TMS ester	260	C10H20O4Si2	17.35
Nonanoic acid, trimethylsilyl ester	230	C12H26O2Si	17.59
Glutaric acid (2TMS)	276	C11H24O4Si2	18.85
(4-Allyl-2-methoxyphenoxy) trimethylsilane	236	C13H20O2Si	20.29
Malic acid (3TMS)	350	C13H30O5Si3	21.24
Adipic acid, bis (trimethylsilyl) ester	290	C12H26O4Si2	21.40
Hexadecane, 2,6,11,15-tetramethyl-	282	C20H42	22.04
Isopropyl laurate	242	C15H30O2	24.04
Dodecanoic acid, trimethylsilyl ester	272	C15H32O2Si	24.68
Suberic acid (2TMS)	318	C14H30O4Si2	25.81
L-(+)-Tartaric acid, bis (trimethylsilyl) ether, bis (trimethylsilyl) ester	438	C16H38O6Si4	27.31
Tetradecanoic acid, TMS	300	C17H36O2Si	28.89
Pentadecanoic acid, TMS ester	314	C18H38O2Si	30.84
Eicosane, 2-methyl-	296	C21H44	31.17
cis-9-Hexadecenoic acid, trimethylsilyl ester	326	C19H38O2Si	32.24
Palmitic acid, trimethylsilyl ester	328	C19H40O2Si	32.77
Heptadecanoic acid, TMS	342	C20H42O2Si	33.43
tert-Hexadecanethiol	258	C16H34S	33.64
Heptadecanoic acid, trimethylsilyl ester	342	C20H42O2Si	34.52
Linoleic acid trimethylsilyl ester	352	C21H40O2Si	35.43
Oleic acid, trimethylsilyl ester	354	C21H42O2Si	35.51
Stearic acid, trimethylsilyl ester	256	C21H44O2Si	36.31
Nonadecanoic acid, trimethylsilyl ester	370	C22H46O2Si	37.91
Eicosanoic acid, trimethylsilyl ester	384	C23H48O2Si	39.50
Behenic acid, trimethylsilyl ester	412	C25H52O2Si	42.52
Squalene	410	C30H50	45.21
Tetracosanoic acid, trimethylsilyl ester	440	C27H56O2Si	45.33
(+)-α-Tocopherol, O-trimethylsilyl-	502	C32H58O2Si	49.58
Cholesterol trimethylsilyl ether	458	C30H54OSi	49.75
Stigmasteroltrimethylsilyl ether	484	C32H56OSi	52.09
CIRE			
Propanoic acid, 2-oxo-, trimethylsilyl ester	160	C6H12O3Si	5.37
2-Pyrrolidinone, 1-methyl-	99	C5H9NO	8.85
Lactic acid, bis-TMS	234	C9H22O3Si2	9.55
Hexanoic acid, trimethylsilyl ester	188	C9H20O2Si	9.73
Acetic acid, [(trimethylsilyl) oxy]-, trimethylsilyl ester	220	C8H20O3Si2	9.94
2-Ethylhexanoic acid, trimethylsilyl ester	216	C11H24O2Si	12.31
Butane, 1,2,3-tris (trimethylsiloxy)-	322	C13H34O3Si3	13.27
Benzaldehyde, 2,5-dimethyl-	134	C9H10O	13.66
Caprolactam	113	C6H11NO	14.45
Phosphoric acid, tris-TMS	314	C9H27O4PSi3	15.66
2-Isopropyl-5-methyl-1-heptanol	172	C11H24O	16.10
Succinic acid (2TMS)	262	C10H22O4Si2	16.48
Fumaric acid, bis (trimethylsilyl) ester	260	C10H20O4Si2	17.35
Nonanoic acid, trimethylsilyl ester	230	C12H26O2Si	17.60
Tetradecane	198	C14H30	18.54
(4-Allyl-2-methoxyphenoxy) trimethylsilane	236	C13H20O2Si	20.29
1-Hexadecanol, 2-methyl-	256	C17H36O	20.40
Malic acid (3TMS)	350	C13H30O5Si3	21.20
Phenol, 2,4-bis (1,1-dimethylethyl)-	206	C14H22O	21.38
Hexadecane, 2,6,11,15-tetramethyl-	282	C20H42	22.04
1-Dodecanol, O-TMS	258	C15H34OSi	22.77
Hexadecane	226	C16H34	23.41
Dodecanoic acid, 1-methylethyl ester	242	C15H30O2	23.04
Dodecanoic acid, trimethylsilyl ester	272	C15H32O2Si	24.68
Dodecyl acrylate	240	C15H28O2	25.53
Nonadecane	268	C19H40	25.26
Suberic acid (2TMS)	318	C14H30O4Si2	25.81
L-(+)-Tartaric acid, bis (trimethylsilyl) ether, bis (trimethylsilyl) ester	438	C16H38O6Si4	27.31
Azelaic acid, bis-TMS	332	C15H32O4Si2	27.92
Tetradecanoic acid, trimethylsilyl ester	300	C17H36O2Si	28.89
Palmitelaidic acid, trimethylsilyl ester	326	C19H38O2Si	32.24
Hexadecanoic acid, trimethylsilyl ester	328	C19H40O2Si	32.79
Oleic acid, methyl ester	296	C19H36O2	33.69
Heptadecanoic acid, TMS	342	C20H42O2Si	34.53
Linoleic acid trimethylsilyl ester	352	C21H40O2Si	35.73
Oleic acid, trimethylsilyl ester	354	C21H42O2Si	35.83
Stearic acid, trimethylsilyl ester	356	C21H44O2Si	36.32
Docosanoic acid, trimethylsilyl ester	412	C25H52O2Si	42.53
Squalene	410	C30H50	45.21
Stigmasteroltrimethylsilyl ether	484	C32H56OSi	52.93
β-Sitosteroltrimethylsilyl ether	486	C32H58OSi	53.22

†$(M-H)^-$. RT=Retention time. CILE: Clerodendrum infortunatum leaf extract, CISE: Clerodendrum infortunatum stem extract, CIRE: Clerodendrum infortunatum root extract

activation.[30] NO reacts with free radical superoxide and forms peroxynitrite which stimulates the production of prostaglandins, a potent mediator of inflammatory conditions.[31] There are various disorders such as multiple sclerosis, arthritis, juvenile diabetes, and ulcerative colitis, which is associated with the release of NO[32] and the NO inhibitory effects of plant materials which directly correlated with their anti-inflammatory

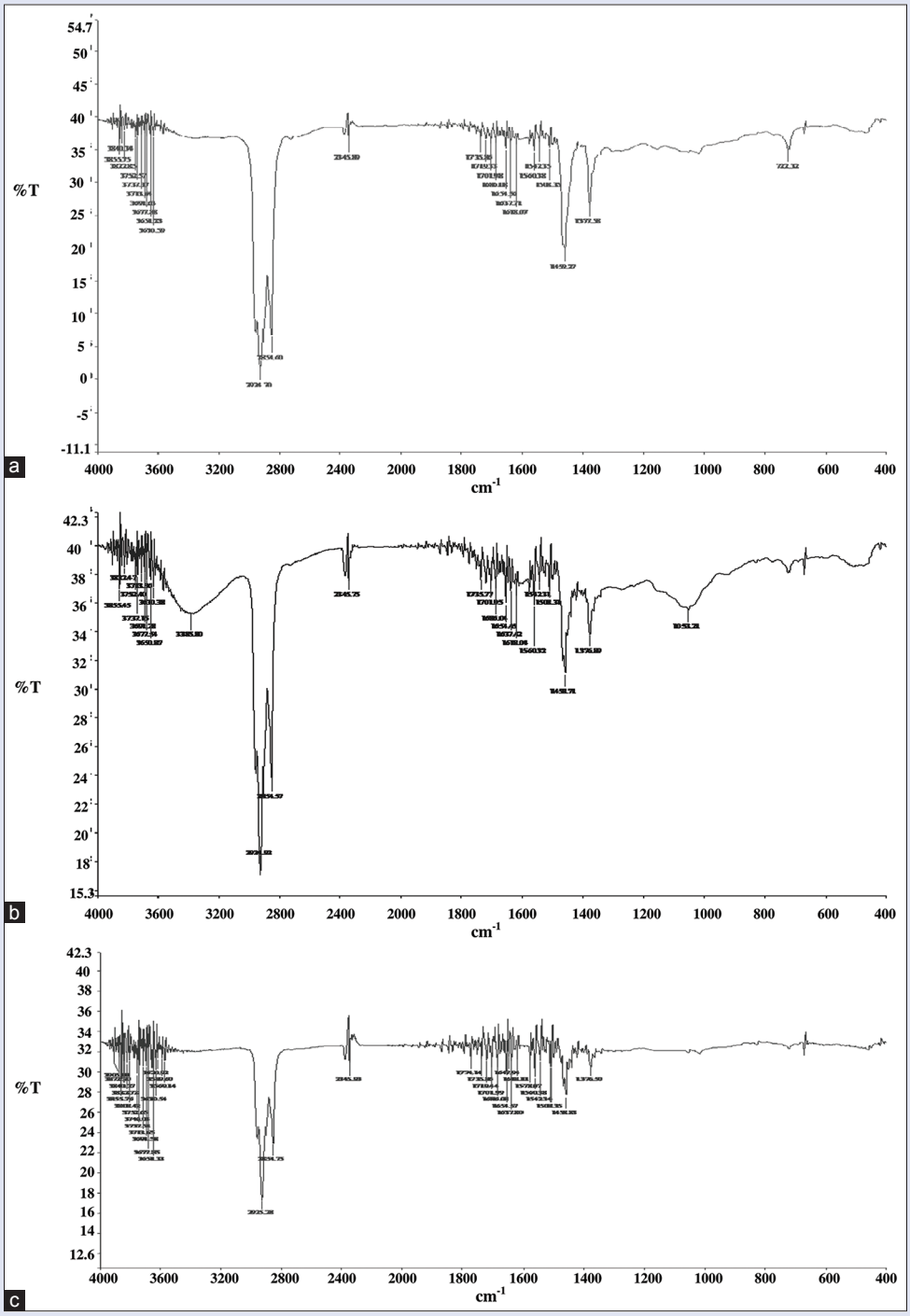

Figure 4: Fourier transform infrared of *Clerodendrum infortunatum*

potential.[33] Our present study demonstrates that leaf and root extracts of *C. infortunatum* have a higher potential of NO inhibition than stem extract when macrophages are stimulated by LPS. The inhibition of NO established the anti-inflammatory activity of *C. infortunatum*. In a certain pathological conditions such as hemoglobinopathies, oxidative stress, and glucose-6-phosphate dehydrogenase deficiency, hemoglobin is destructed and the heme liberates from it. Many components available in the food have the hemolytic activity which makes the erythrocyte lipid bilayer permeable irreversibly, and thus, hemoglobin is released into the medium.[34] Our study revealed that the hemolytic activity of *C. infortunatum* is very negligible in case of all the extract compared to standard Triton X and therefore may be considered safe from the hemolytic perspective.

The present study investigated the *in vitro* myeloperoxidase and respiratory burst activities of the isolated murine peritoneal macrophage. Recognition and internalization of invading bacteria are the primary functions of the macrophages, and inside the phagosome, activation of NADPH oxidase results in the generation of superoxide anion, which is deprotonated to form O_2 and H_2O_2, from which the highly reactive hypochlorous acid (HOCl) is generated through the myeloperoxidase

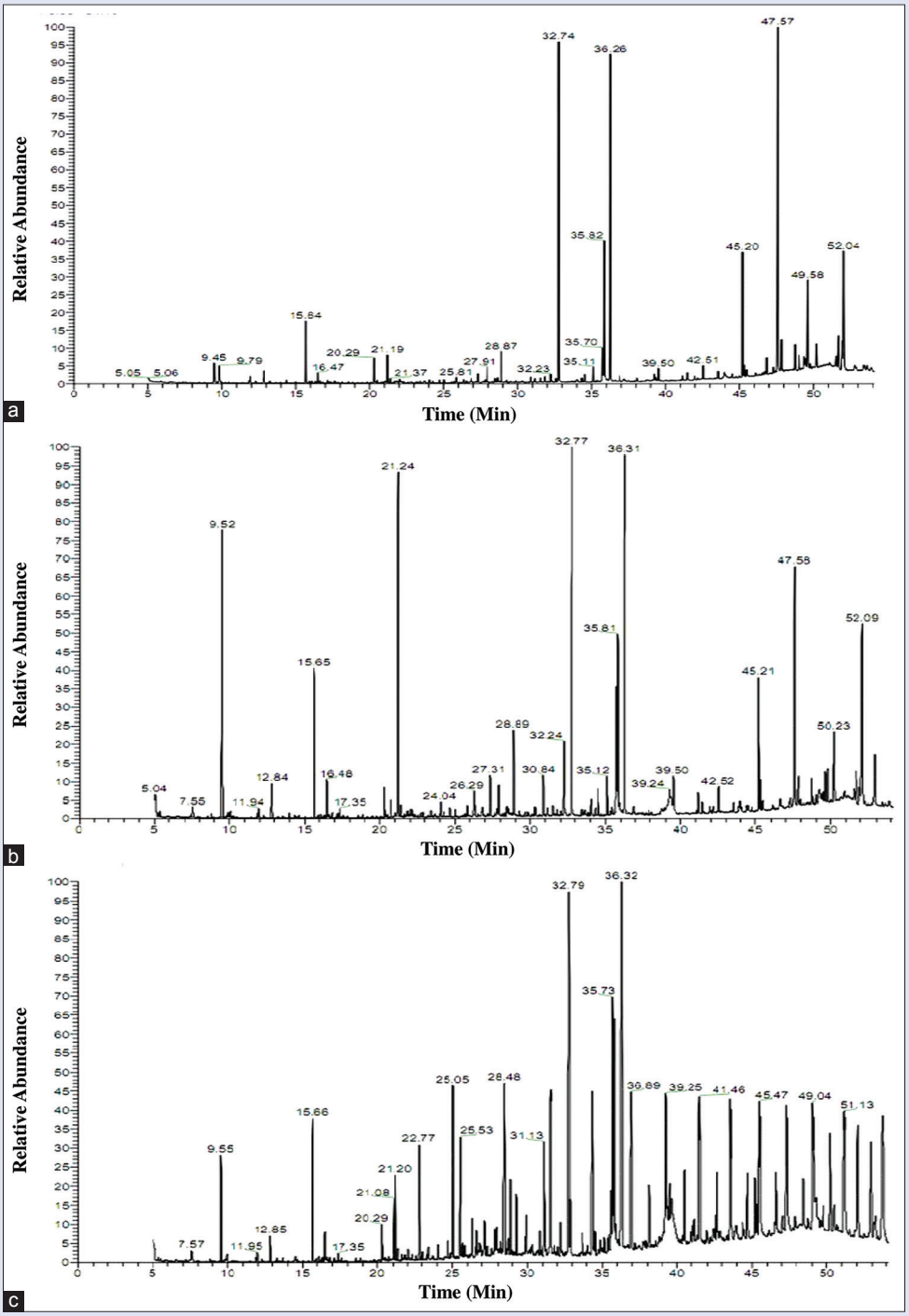

Figure 5: Gas chromatography-mass spectrometry identification of chemical fingerprints of *Clerodendrum infortunatum*

reaction.[35] Generation of a plethora of oxygenated radical such as superoxide radical, H_2O_2, hydroxyl radical, singlet oxygen, HOCl, chloramines, NO, snd peroxynitrite, to kill the internalized pathogen, is termed as respiratory burst activity. Total protein, globulin, and albumin level is very important for the immune system. A low protein level can lead to liver and kidney disorders, whereas the insufficient level of albumin and globulin may be the cause of autoimmune disorders. In our study, total protein, albumin, and globulin level was increased significantly by *C. infortunatum* in a dose-dependent manner, so it can be concluded that *C. infortunatum* may help in the protection of liver, kidney, and many autoimmune disorders. Functional group identified that using FTIR analysis had serious implications in the anti-inflammatory, antibacterial, and immunomodulatory activity of *C. infortunatum*. Various ketone analogs were demonstrated anti-inflammatory and immunosuppressive potential through the *in vivo* model.[36] The hydromethanolic extract of *C. infortunatum* has C=O, C-H, C=C, and C-O bond-stretching compounds exhibiting the zone of inhibition against the tested organism when compared with control. Hence, our investigation suggests that stem extract of *C. infortunatum* is a promising development of phytomedicine for antimicrobial properties.

There are numerous compounds possessing active nitro groups with diverse medicinal properties, which stimulate the immunomodulatory potential of *C. infortunatum*.[37] The immunomodulatory activity of *C. infortunatum* was also supported by chromatographic fingerprinting as the bioactivity of the identified phytochemicals. Azelaic acid present in the leaf of *C. infortunatum* can be used as an effective treatment for mild-to-moderate acne vulgaris.[38] Squalene present in the leaf, stem, and root of *C. infortunatum* is a polyunsaturated hydrocarbon and considered as an important substance in practical and clinical uses with a huge potential in nutraceutical and pharmaceutical industries. Squalene possesses anticancer, antioxidant, drug carrier, detoxifier, skin-hydrating, and emollient activities both in animal models and *in vitro* environments which support the immunomodulatory activity of *C. infortunatum*.[39] Acetic acid is known to kill a wide spectrum of fungi and bacteria present in *C. infortunatum* and directly correlated with our antimicrobial study. Stigmasterol present in *C. infortunatum* shows antioxidant and immunomodulatory activity.[40] Besides, literature review reveals that the phytocompounds present in *C. infortunatum* is the major cause of the immunomodulatory activity.

CONCLUSION

C. infortunatum displayed potent immunomodulatory activity which may prove beneficial and effective after further exploration. Stimulation of the immune system by herbal medicine is directly correlated to the improvement of depressed the immune system and downregulation of infectious diseases. Our investigations showed that *C. infortunatum* possess potent immunomodulatory activity by stimulating both innate and humoral arm of the murine immune system.

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Conflicts of interest

There are no conflicts of interest.

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