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Evaluation of Immunomodulatory Activity of *Clerodendrum inerme* on Human PBMC

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Abstract: Medicinal and ornamental plants have been used for many centuries in various ayurvedic treatments and chinese medicine. Many of which have proven to possess many bioactive compounds which may treat many deadly ailments. One such medicinal and ornamental plant is the *Clerodendrum inerme*. The Anti-Inflammatory Activity of *Clerodendrum inerme* is well proven by many previously established works of literature. This provides a pathway to evaluate the immunomodulatory activity of the plant since anti-inflammatory and immunomodulatory activity go hand in hand. This study aims to evaluate the immunomodulatory activity of the methanolic activity of *Clerodendrum inerme.* The leaves were harvested, dried and extracted using methanol. The phytochemical analysis for the extract was conducted and a column chromatography was performed to separate the extract into various fractions. Cell proliferation and NO inhibition assay was performed for each fraction and a GC-MS analysis for the best fraction was performed to identify the key compounds responsible for the immunomodulatory activity. The crude extract showed the presence of many key phytochemical groups. The column chromatography yielded eight different fractions and the sixth fraction was found to possess the best immunomodulatory activity as it showed the most significant reduction in cellular proliferation as well as nitric oxide production. The GC-MS analysis showed the presence of fourteen compounds in the key fraction.

Keywords: Anti-inflammation, Cell Proliferation Assay, Clerodendrum Inerme, Column Chromatography, GC-MS, Immunomodulatory, NO Inhibition Assay

1. Introduction

Inflammation is part of the non-specific immune response that occurs in reaction to any type of bodily injury [1]. It can result from various conditions such as physical injury, autoimmune disorders, infection and much more. It is characterized by heat, swelling and redness. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing inflammation as opposed to opioids, which affect the central nervous system to block pain signalling to the brain [2]. Inflammation could be considered as a base for various immunological reactions. The structural and functional changes of the immune system caused by drugs and plants are responsible for the immunosuppression or immunostimulation, which, in turn, may cause adaptation of the host defence mechanism against cancer and infection. These changes may also stimulate the abnormal immune response causing autoimmunity and allergy [3]. The need for immunosuppression is a major factor to consider for a variety of condition some of which include organ transplantation and autoimmune disease such as lupus erythematosus, rheumatoid arthritis etc… Supressing the immune system is a major task since the immune system is a complex network of cells signalling. Many cases require surgery to cause immunosuppression and very few effective drugs are present currently in the market. And the ones present are ineffective, costly and cause

adverse side effects. Thus, a need for an effective immunosuppressive agent is the current need of the transplant immunology field. *Clerodendrum inerme* also known as *Volkameria inermis*, is a species of flowering plant in the genus *Volkameria* of the family *Lamiaceae,* found in India and other tropical countries. This plant is commonly used as an ornamental plant for creating hedges and much more landscaping plants. *Clerodendrum inerme* also has found to possess many bioactivities such as hepatoprotective activity [4], antifungal activity [5], antidiabetic activity, antimicrobial, antiparasitic and many other pharmacologically important activities [6]. This study aims to identify the anti-inflammatory activity of the methanolic extract of the plant and the chemical compounds responsible.

2. Materials and Methods

2.1. Extraction

Healthy leaves of *Clerodendrum inerme* were harvested during January in and around Chennai, Tamil Nadu. A sample was taken to the PRAC (Plant Anatomy and Research Centre) Chennai, Tamil Nadu. And authenticated by Dr. Jayaram. (reg id: PARC\2020\4215). The leaves were separated and dried for a week and then ground to a coarse powder. 100g of course powder was placed in a large conical flash to which 1000ml of methanol was added. The extraction proceeded for 72hrs. after 72hrs the contents were filtered using a Whatman no 2 filter paper and the solvent was allowed to evaporate to obtain a concentrated paste under reduced pressure via rotary vacuum evaporator and the yield was calculated.

2.2. Phytochemical Analysis

the evaluation of the main phytochemicals present in the extract were performed according to [7]. All the phytochemical analysis was qualitative and was used to find the presence of saponins, steroids, terpenoids, alkaloids, flavonoids, glycoside, cardiac glycoside, tannins, reducing sugars, coumarins and quinones.

2.3. Column Chromatography

The column chromatography was performed according to [8] with slight modifications. Silica gel was placed in a clean glass beaker, suspended in two volumes of hexane, and allowed to settle. Chromatographic columns were made from 30 cm x 4 cm I.D. glass column by plugging their constricted ends with glass wool. Silica gel was suspended in hexane, and it was transferred to the columns with a glass pipette. Columns were packed to a bed height of 21 cm above the glass wool plug. They were rinsed twice with 20 ml of hexane (100%) before the sample application. After sample loading the solvent system consisted of 20ml of each, hexane (100:0, v/v), hexane: ethyl acetate (50:50, v/v), ethyl acetate (100:0, v/v), ethyl acetate: methanol (50:50, v/v) and methanol (100:0, v/v) in the respective order. All solvent mixtures used in this work were prepared by additive combination of the named solvents in the stated proportions by volume (e.g. hexane-ethyl acetate 20: 1, v/v was prepared by combination of 20 volume units of hexane with 1 volume unit of ethyl acetate).

2.4. Lymphocyte Proliferation Assay

The lymphocyte proliferation assay was performed according to [9]. Blood samples from human volunteers were collected from Hare Diagnostic Centre and Fertility Lab. The human peripheral blood mononuclear cells (PBMC, 105 cells/ml) was isolated using Ficoll–Hypaque gradient centrifugation and plated in 96 well flat bottom tissue culture plates and incubated in the presence of hepatitis vaccine (1 µg) along with a varying concentration (20-100µg/ml) at 37°C for 24 h. After incubation exchange the old media with an equal volume of fresh medium. Add MTT solution (5 mg/ml, 10 µl) were added to each well and then incubated for 4 h. Again, the 96 well flat-bottom plates were spun or centrifuged at 1500 rpm for 4 minutes and the supernatant was discarded. Add 100 µl of DMSO solution to the formazan crystals and the absorbance was evaluated in an ELISA reader at 570 nm. All experiments were performed in triplicate.

2.5. Nitric Oxide Inhibition Assay

The nitric oxide inhibition assay was performed according to [10, 11]. Human peripheral blood mononuclear cells were incubated for 24 h with variable doses of flavonoids in the presence of the hepatitis vaccine antigen as mentioned above. After incubation, 96 well flat-bottom plates were centrifuged at 1500 rpm for 4 minutes and then the supernatant (50 µl) was collected was mixed with 50 µl of Griess reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in presence of 2.5% phosphoric acid) and incubated in 96 well flat-bottom plates at room temperature for 10 minutes, and the absorbance at 540 nm was measured in a microplate reader. The fresh culture medium (RPMI containing 10% fetal bovine serum) was used as a blank. The quantity of nitrite was determined from a sodium nitrite standard curve.

2.6. Gas Chromatography-Mass Spectrophotometer (GC-MS) Analysis

GC-MS analysis was carried out according to [12] for the methanolic extract of *Clerodendrum inerme* to identify the extracted phytocompounds. The Gas Chromatogram (Clarus 680) was used in the analysis employed a fused silica column, packed with Elite-5MS (5% biphenyl 95% dimethyl polysiloxane, 30m × 0.25 mm ID × 250µm df) and the components were separated using Helium as carrier gas at a constant flow of 1 mL/min. The injector temperature was set at 260°C during the chromatographic run. The 1µL of extract sample injected into the instrument the oven temperature was as follows: 60°C (2min); followed by 300°C at the rate of 10°C min−1; and 300°C, where it was held for 6 min. The

mass detector conditions were the transfer line temperature of 240°C, ion source temperature of 240°C and ionization mode electron impact at 70 eV, scan time 0.2 sec and scan 0.1 sec. The fragments were obtained from 40 to 600 Da. The spectra of the obtained components were compared with the database stored in the GC-MS NIST (2008) library.

3. Results

3.1. Phytochemical Screening

The phytochemical screening of the methanolic extract of Clerodendrum inerme detected the presence of carbohydrates, saponins, flavonoids, quinone, terpenoids, coumarins and steroids.

3.2. Column Chromatography

The column chromatography for the crude methanolic extract of *Clerodendrum inerme*, with stationary phase as silica gel and mobile phase as a solvent system mixture with varying polarities yielded eight different fractions, each fraction was differentiated by measuring the difference in optical density. Each fraction was reduced into a concentrated paste at reduced pressure.

3.3. Lymphocyte Proliferation Assay

The lymphocyte proliferation assay performed for all the eight fractions obtained from column chromatography of crude methanolic extract of *Clerodendrum inerme*, showed that fraction no: 6 has the highest inhibition of lymphocyte proliferation. The results are displayed in Figure 1, a decrease in OD at 570 nm. The results showed that there a significant decrease in proliferation when compared to activation induced by hepatitis B antigen

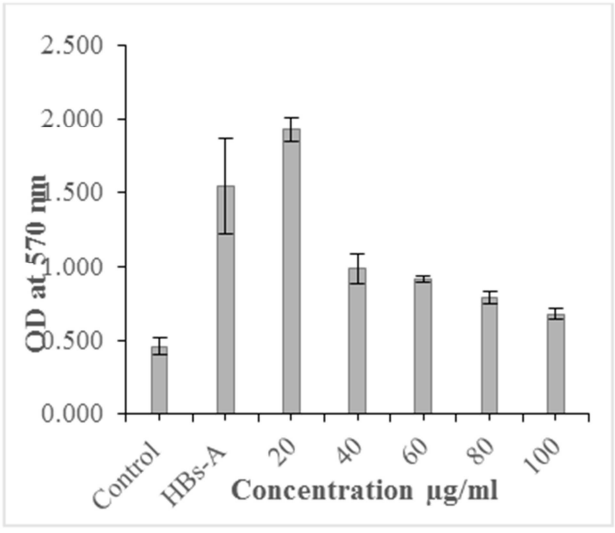

Figure 1. *The lymphocyte proliferation assay for HBs-A induced proliferation of PBMC shows a stark decrease in proliferation of lymphocytes with increasing concentration of Fraction 6. Values is presented as Mean ± S.D.*

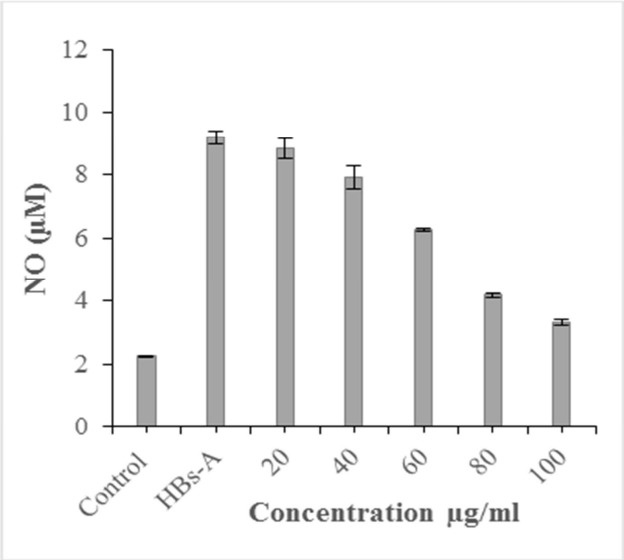

Figure 2. *The Nitic Oxide inhibition assay for HBs-A induced proliferation of PBMC shows a stark decrease in NO production by lymphocytes with increasing concentration of Fraction 6. Values were compared with the standard curve of sodium nitite and presented as Mean ± S.D.*

3.4. Nitric Oxide Inhibition Assay

The effect of variable doses of each fraction obtained from column chromatography of crude methanolic extract of *Clerodendrum inerme* on nitric oxide production was observed in the cell culture supernatant of human peripheral blood mononuclear cells. The results clearly show that fraction no: 6 showed the most inhibition of NO production as shown in Figure 2. The results showed that there was a significant dose-related decrease in NO production as compared to activation induced by the hepatitis B antigen.

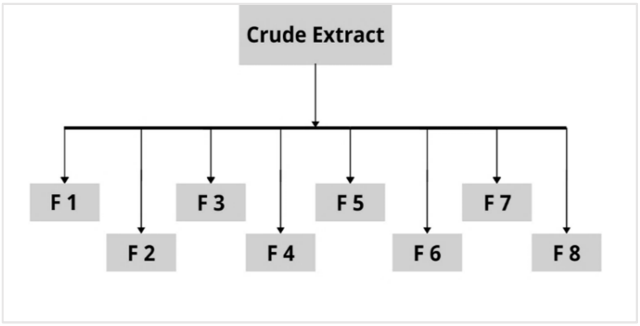

Figure 3. *The column chromatography yield eight different fractions, the fractions were separated based on the difference in OD and each fraction was reduced into a concentrated paste under reduced pressure.*

3.5. Gas Chromatography-Mass Spectrophotometer (GC-MS) analysis

The GC-MS analysis of fraction number 6 yielded 14 different compounds present in the sample, the gas chromatogram for the fraction is shown in Figure 4. From the gas chromatogram, it is evident that compound 2-Phenyl-4,7-chromanediol with RT= 15.25 is the key compound present in this fraction and thus establishing the immunomodulatory

activity of *Clerodendrum inerme*

4. Discussion

Inflammation means tissue injury induces the complex cascade of nonspecific events and is generally induced by physical stress, infectious agents (bacterial or viral), toxins, and other factors. The immune system is composed of a large variety of cells and mediators that interact in a complex and dynamic network to ensure protection against foreign pathogens, which may be encountered during one's life-time, while simultaneously maintaining self-tolerance [13]. Inflammation is one of the key functions of the immune system. There are many cases wherein the immune system needs to be suppressed to treat an array of condition and this is accomplished through immunosuppressive drugs. immunosuppressive drugs, also known as immunosuppressive agents, immunosuppressants and antirejection medications are drugs that inhibit or prevent the activity of the immune system [14]. Progress in immunosuppressive drugs during the past decade has been phenomenal. However, newer drugs with higher therapeutic ratios are needed, the mechanisms of action of the outstanding drugs need to be resolved at the cellular and intracellular level, and better methods are needed to restrict the action of drugs to the immunocompetent precursor cells that are responsive only to the test antigen [15]. most research into phytopharmacology is now moving onto ornamental plants as there have been promising results in selected plants before. One such ornamental plant is the *Clerodendrum inerme.* This plant has a rich literature of various bioactivities [4-6]. And from the results above we can say with some degree of assurance that this plant possesses various phytochemicals responsible for its immunomodulatory activity. Furthermore, research into the actual mechanism of actions and its effect on immune cells in the cellular and intracellular level are required to further understand the capabilities of this compound and its future use as a lead compound for a new immunosuppressive drug.

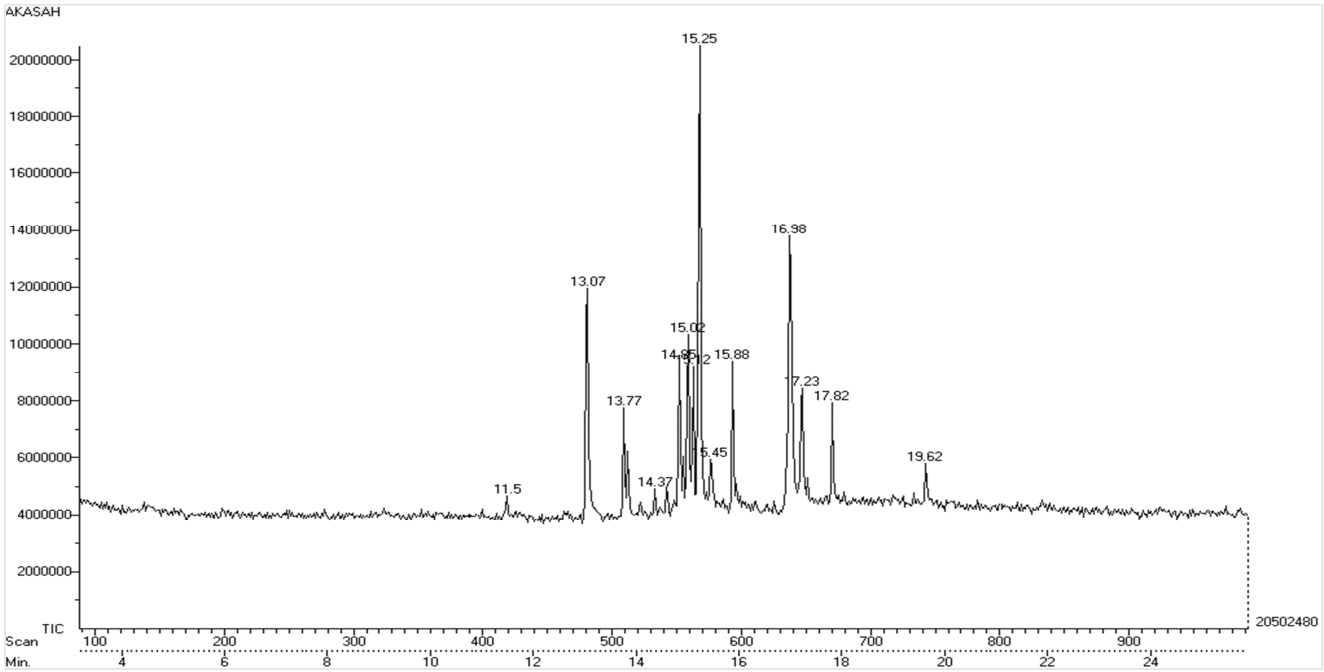

Figure 4. *Gas Chromatogram for Fraction 6. This gas chromatogram showed the presence of 14 different compounds*

Table 1. *The GC- Ms analysis of Fraction 6 showed the presence of fourteen different compounds present.*

S.No	RT	Name		Molecular Formula	MW g/mol
		Common	IUPAC		
1	11.5	Alpha.-Santoline alcohol	2,5-Dimethyl-3-vinyl-4-hexen-1-ol	$C_{10}H_{18}O$	154.3
2	13.07	Humulen-(v1)	4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-3-ene	$C_{15}H_{24}$	204.4
3	13.77	Tumerone	2-Methyl-6-(4-methyl-1,3-cyclohexadien-1-yl)-2-hepten-4-one	$C_{15}H_{22}O$	218.3
4	14.37	Hydroxyacetophenone	3-[4-Hydroxyisopent-2(Z)-enyl]-4-hydroxyacetophenone	$C_{13}H_{16}O_3$	220.3
5	14.85	Reynosin	naphtho[1,2-b]furan-2(3H)-one	$C_{15}H_{20}O_3$	248.3
6	15.02	7-Hydroxyflavone	4H-1-Benzopyran-4-one, 7-hydroxy-2-phenyl	$C_{15}H_{10}O_3$	238.2
7	15.12	Lauroylaceton	Pentadecane-2,4-dione	$C_{15}H_{28}O_2$	240.4
8	15.25	2-Phenyl-4,7-chromanediol	2-phenyl-3,4-dihydro-2H-chromene-4,7-diol	$C_{15}H_{14}O_3$	242.3
9	15.45	Quinazoline	8-methoxybenzo[f]quinazoline-1,3-diamine	$C_{13}H_{12}N_4O$	240.3
10	15.88	Pratol	7-Hydroxy-2-(4-methoxyphenyl)-4H-chromen-4-one	$C_{16}H_{12}O_4$	268.3
11	16.98	Methyl oleate	Methyl cis-9-octadecenoate	$C_{19}H_{36}O_2$	296.5
12	17.23	Octadecenoic acid	Octadecenoic acid	$C_{18}H_{34}O_2$	282.5

S.No	RT	Name		Molecular Formula	MW g/mol
		Common	IUPAC		
13	17.82	4-Androstenediol	4-Androstenediol	$C_{19}H_{30}O_2$	290.4
14	19.62	Methyl vouacapenate	methyl (11*b*-trimethyl-1,2,3,4*a*,5,6,6*a*,7,11,11*a*-decahydronaphtho[2,1-f, 1]benzofuran-4-carboxylate	$C_{21}H_{30}O_3$	330.5

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Conflict of Interest

No conflict of interest to be declared by any of the authors.

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Compliance with Ethical Standards

This article does not contain any studies with human participants or animals performed by any of the authors.

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