Immunosuppressive activity of crude saponins from the leaves of *Calotropis gigantea*, *Calamus roteng* and *Artocarpus integrifolia*

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Abstract

Plants can be the ultimate source of various traditional medicines that play an important role in human health care. The objective of our present study is to investigate the immunosuppressive activity of the variable doses of crude saponin (0.625 – 2.5 mg) extracted from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* on lymphocytes, monocytes and granulocytes count and also observed nitric oxide estimation and haemolytic activity using human whole blood. The results showed that the crude saponin extracted from three medicinal plants showed inhibition of monocytes and granulocytes count as compared to control. In addition, these saponins also showed the inhibition of nitric oxide and increase in haemolytic activity at higher doses (2.5 mg) as compared to control. The data suggests that saponins extracted from these medicinal plants i.e. *Calotropis gigantea*, *C. roteng* and *A. integrifolia* showed immunosuppressive activity.

Key words: *Calotropis gigantea*, *Calamus roteng* and *Artocarpus integrifolia*

INTRODUCTION

Saponins are one of the groups of secondary metabolites and it is generally composed of triterpenoid or steroid aglycone moiety with complex substituent of oligosaccharide [1, 2]. These saponins are heterogeneous or diverse group of glycosides which is produced mainly by medicinal plants or natural products and also by lower marine animals and some bacteria [1, 2, 3, 4]. The combination of hydrophilic and lipophilic properties give saponins their surfactant properties which in turn give rises to their ability to form stable aqueous foams as well as forming complexes with membrane steroids and lipid compounds [3, 4, 5, 6]. Due to these properties, there is an increasing demand for natural plant products along with their physicochemical properties and numerous immuno as well as micro-biological activities has led to the emergence of saponins as commercially significant compounds with expanding applications in pharmaceutical industries [5, 7, 8, 9]. Saponins are also one of the important raw materials for the production of steroidal hormones and drugs and also used as immunopharmacological adjuvants in the formulation of vaccine antigen due to their immune enhancing properties [9, 10, 11, 12, 13, 14]. Information on the immunobiological activities of saponins from variety of sources provides lead for the development and design of new drugs.

Calotropis gigantean (commonly called as Rui or giant milkweed or crown flower), which belongs to the family *Asclepediace* [15, 16, 17]. This medicinal plant showed lot of potential to cure number of diseases and disorders. For the last ten years, number of scholars or researchers isolated the secondary metabolites from different parts of the plant and showed number of immunopharmacological activities e.g. anti-microbial [18], anti-bacterial [19], wound healing activity [20] and also reported to cure fits and convulsions [21].

C. rotang (commonly called as Vet), medicinal plant which belongs to the family *Arecaceae* [22]. This medicinal plant showed maximum number of medicinal uses e.g. fruits consumed directly or it is generally used in the preparation of pickles; leaves especially sap and alkaloid is generally used for eye problem and cramps; stem containing saponin and flavonoid in the root are generally used in convulsions and cramps [22]; methanolic extracts of seed showed CNS depressant, analgesic and anti-inflammatory effects [23].

Artocarpus integrifolia (commonly called as Phanas), belongs to the family *Moraceae* [24] which is an important source or requirement of edible fruit and is widely used in traditional medicines. In Ayurveda, *A. integrifolia* is reported to possess antibacterial, anti-peptic ulcer, anti-diabetic, anti-oxidant and immunomodulatory properties [24]. This previous ideas of three medicinal plants has inspired us to find the immunosuppressive effects of crude saponin from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* using human whole blood .

2. MATERIALS AND METHODS

2.1. Collection of plant material

Fresh plant leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* were collected from the garden of Vidya Pratishthan's School of Biotechnology (VSBT), Baramati (Pune), Maharashtra.

2.2. Preparation of saponins

The dried powdered leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* (40 g) were prepared in phosphate buffered saline (80 ml). After the aqueous preparation, the aqueous extract was extracted two to three times with diethyl ether (40 ml). After extraction, the diethyl ether, layer in the form of supernatant was discarded and retained the aqueous layer extracted or settled at the bottom further with 120 ml n-butanol (three to four times). The n-butanol extracts were bulked together and washed three to four times using 10 ml of five percent NaCl. The washed extract was concentrated at < 75 °C in an oven and air dried at room temperature to yield 500 mg of crude saponin residue. The residue was screened for saponin using the foaming test [25].

2.3. Human blood samples and determined the nitric oxide production and estimation of lymphocytes, monocytes and granulocytes count using human EDTA whole blood

During these studies, human whole EDTA blood samples were collected for Mangal Pathology Laboratory, Baramati, District Pune, Maharashtra, India. Briefly, human whole blood were lysed with lysis buffer (ammonium chloride, potassium bicarbonate and ethylene diamine tetraacetic acid) and washed two times with fresh RPMI medium containing 10 % FBS. Whole blood cells (100 µl, 10^6 cells/ml) were cultured in 96 well plates with serial dilutions of saponins (0.625 – 2.5 mg) extracted from *Calotropis gigantea*, *C. rotang* and *A. integrifolia* at 37°C for 24 h. The plates were centrifuged at 2500 rpm for 10 minutes and then the supernatant (100 µl) was collected for the estimation of nitric oxide and also estimates the lymphocytes, monocytes and granulocytes profile count in those cells which are settled at the bottom of 96 well plate.

In NO estimation, cell culture supernatant were collected from human lysed whole blood was mixed with same volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated the flat bottom 96 well 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 nitrite quantity was determined from a sodium nitrite standard curve. All experiments were performed in triplicates [26].

In addition, cells were cultured in 96 well plates in the presence of variable concentration of saponins (0.625 – 2.5 mg) extracted from *Calotropis gigantea*, *C. rotang* and *A. integrifolia* at 37°C for 24 h. The samples were centrifuged (300 – 400 × *g*) and the supernatant was aspirated or discarded and washed two to three times with phosphate buffered saline. After centrifugation, cells in the form of pellet dissolved in PBS and observed the cells through flow cytometer (FACS Calibur, BD Biosciences) [27, 28].

2.4. Preparation of erythrocytes suspension and determined its hemolytic activity

EDTA human whole blood (3 - 5 ml) samples were collected from Mangal Pathology laboratory, Baramati, District Pune. The EDTA blood was centrifuged at 1500 rpm for five minutes at 4 °C in a refrigerated centrifuge. After centrifugation, the supernatant (plasma) was discarded and the pellet containing red blood cells were washed continuously two to three times with phosphate buffer saline solution by centrifugation at 1800 rpm for 10 minutes. The human red blood cells were resuspended in phosphate buffered saline. For this experiment, 1% human red-blood cell suspension dissolved in pH 7.4 phosphate buffer saline was used throughout the experiment. Saponins extracted from *Calotropis gigantea*, *C. rotang* and *A. integrifolia* with variable concentrations transferred into test tubes containing a fixed volume of human red-blood cell suspension. The saponins were tested or screened at different concentrations. Negative controls (blanks) contained 1 % distilled water in red-blood cell suspension. The result for each test concentration of crude saponin was interpreted qualitatively *in vitro* hemolytic action either being present or absent. Overall, the result was a semi-quantitative evaluation of hemolytic activity for each extract in accordance with international guidelines for the evaluation of this activity in medicinal plant materials [29].

2.5. Statistical analysis

All values are mentioned as Mean ± S.E. Data is represented by One way ANOVA test (Boniferroni multiple comparison test).

3. RESULTS

3.1. Estimation of NO production from human whole blood

The effect of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* was observed in human whole blood as shown in **Fig .1**. Cells were cultured for 24 – 48 h in the presence of variable doses of saponin and the supernatants were collected for the estimation of nitric oxide (NO) content and measured in the supernatants as a function of

macrophage activation. There was a significant decreased in NO production elicited by the saponins at higher doses (2.5 mg). The inhibitory concentration of saponin at 2.5 mg as compared to control.

3.2. Estimation of lymphocytes, monocytes and granulocytes count using flow cytometry

The effect of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* on human EDTA whole blood on lymphocytes, monocytes and granulocytes count as shown in **Fig 2**. The results showed that there is dose dependent decrease in monocytes and granulocytes count at higher doses (2.5 mg) as compared to control. The inhibitory effect of saponin in human EDTA whole blood on monocytes and granulocytes count at higher doses (i.e. 2.5 mg) as compared to control.

3.3. Hemolytic activity

The effect of crude saponins extracted from *Calotropis gigantea*, *C. rotang* and *A. integrifolia* was observed in hemolytic activity of human red blood cells as shown in **Fig .3**. The data showed that there was a significant increased in hemolytic activity of saponins at higher doses (2.5 mg) as compared to control.

4. Discussion

The present study showed the immunopharmacological effect of crude saponin extracted from three medicinal plants i.e. *Calotropis gigantea*, *C. rotang* and *A. integrifolia* on various parameters of the immune system especially nitric oxide (NO) production, estimation of lymphocytes, monocytes and granulocytes count and also observed its haemolytic activity against human red blood cells. The preliminary screening of these crude saponins using human whole blood for determined its immunomodulatory effects whether the saponin showed immunostimulatory or immunosuppressive effect. The main advantage of the preliminary screening for the determination of secondary metabolites of various medicinal plants is due to its high sensitivity, general feasibility, low cost value and possibility of large scale performance. It has been suggested that production of NO from macrophages, dendritic cells and human lysed whole blood may depend on the cell types and their species origin [30, 31], different cells of our immune system having obviously different requirements for signal transduction pathways [32]. In nitric oxide (NO) production, there is phenomenon that there is an extensive knowledge showing that the decrease in the NO production from different types of immune cells is determined by a number of blood counts i.e. lymphocytes, monocytes and granulocytes count. There is direct correlation between the NO production and estimation of blood counts; direct NO-stimulatory or inhibitory function is dependent on monocytes as well as granulocytes count that triggers or suppress NO production on its own [30, 31, 32]. In this study, the results showed that there is dose dependent decrease in NO production as compared to control. In contrast, saponin showed dose dependent increase in haemolytic activity as compared to control.

In addition, saponins isolated from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* have been shown to inhibit or reduction of monocytes and granulocytes profile count in a dose dependent manner in human whole blood which is determined through flow cytometric analysis. Several studies related to immunohaematology have shown that the reduction of the count of monocytes and granulocytes profile contributes to the incidence of intracellular or extracellular infections in our immune system [33]. It should be noted that the inhibitory effects observed in this study could be considered as the immunosuppressive effect of these medicinal plants because in each case the viability of cultured cells are decreased in case of monocytes as well as granulocytes profile count were observed. The results showed that there is significant decrease in level of macrophages at higher doses as compared to control. Finally, the saponins from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia* showed immunosuppressive activity.

CONCLUSION

In the present study, we found that *Calotropis gigantea*, *C. rotang* and *A. integrifolia* significantly inhibited the NO production and monocytes as well as granulocytes profile. Further investigations will focus on the *in vivo* assessment of the immunobiological activity of these crude saponins and the chemical identification of the major active components responsible for the immunosuppressive activity in the efficacious extracts

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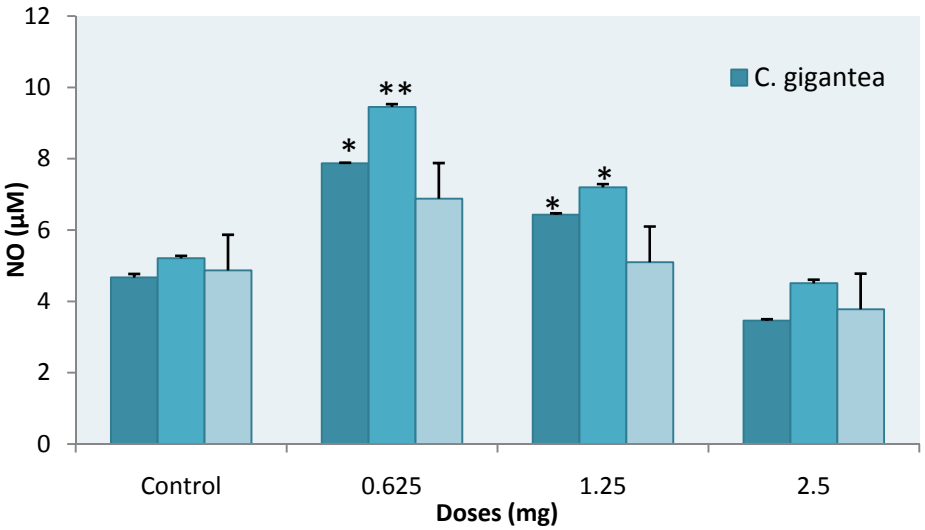

Fig.1. Production of nitric oxide (NO) from human whole blood. The supernatant nitrite concentration was determined by Griess reagent after the 24 h culture of cells in presence of variable doses of crude saponins (0.625 – 2.5 mg) from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia*. Values are expressed as Means ± S.E. The difference between the control and treated groups is determined by One way ANOVA test (Bonferroni multiple comparison test). *$P < 0.05$; **$P < 0.01$.

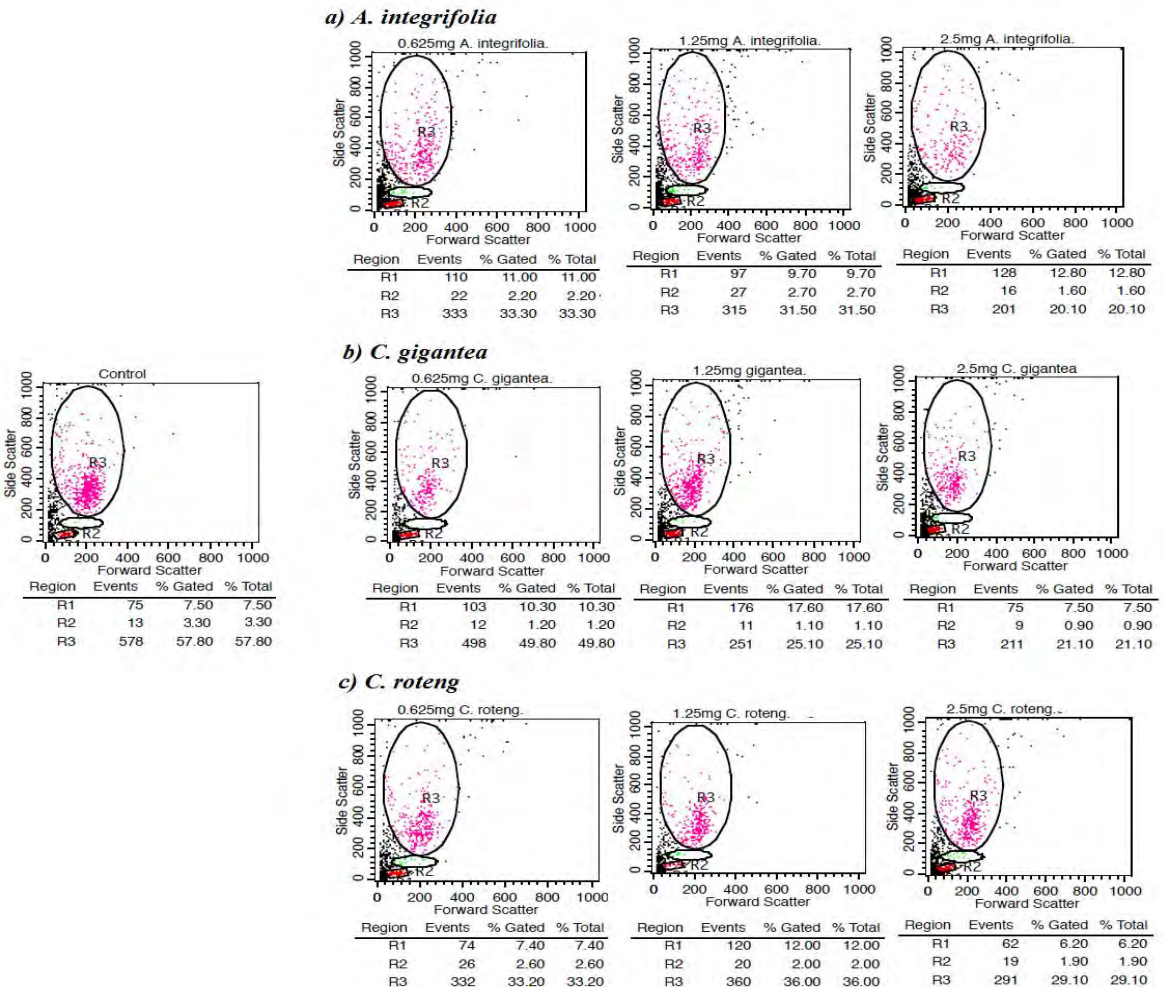

Fig.2. Flow cytometric analysis of crude saponins on lymphocytes, monocytes and granulocytes count. Human whole blood samples were incubated with serial dilutions of crude saponins and incubated the samples at 37°C, 5% carbon dioxide incubator for 2 h. After 2h, lysed the blood samples and wash the samples two times with phosphate buffered saline and then observed the cells in flow cytometer (FACS Calibur). Data acquisition of 10000 events and fraction or separation of cell populations representing different phenotypes analyzed using cell quest software

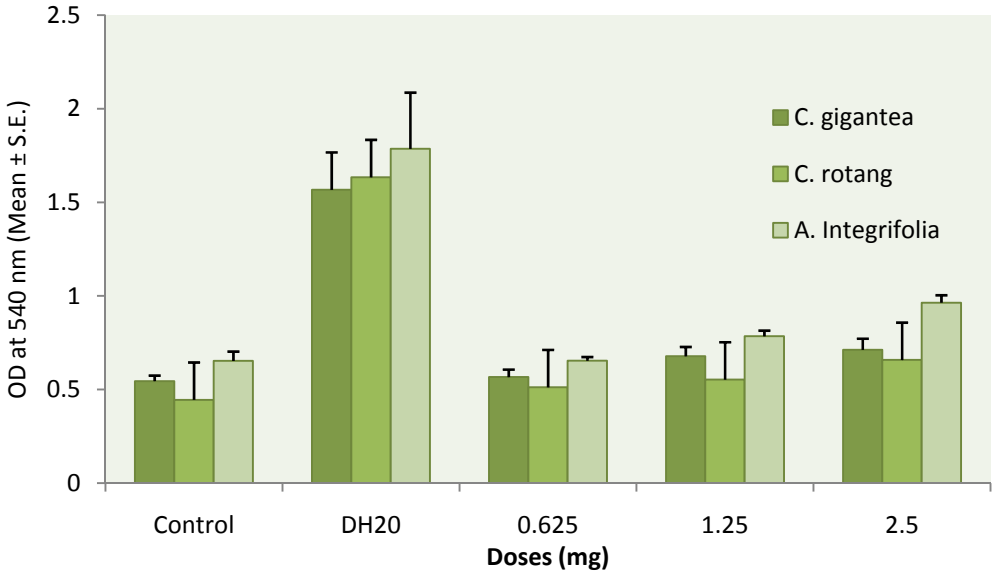

Fig.3. Haemolytic activity of crude saponins from the leaves of *Calotropis gigantea*, *C. rotang* and *A. integrifolia*. on human erythrocytes. Data are represented as Mean ± S.E. of human whole blood samples. Distilled water and phosphate buffered saline used as positive and negative control.