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Flow cytometric evaluation of *Calotropis gigantea* for determining its antimicrobial activities in infected human whole blood samples

Amit Gupta*, Sushama R Chaphalkar

Department of Immunology and Virology, Vidya Pratishthan's School of Biotechnology (VSBT, Research Centre affiliated to Savitribai Phule Pune University), Baramati, Maharashtra, India

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ABSTRACT

Introduction: Medicinal plants are considered to be safer, non-toxic and less harmful as compared to synthetic based drugs that are available. In this study, we focused on aqueous leaves extract of *Calotropis gigantea* for determining its antimicrobial activity in infected (dengue) human whole blood samples using flow cytometry.
Methods: Infected dengue human blood samples (n = 5; confirmed on the basis of NS1 antigen to dengue virus;) were collected from pathology lab and evaluated its blood counts (lymphocytes, monocytes and granulocytes count); forward scatter (FSC) and side scatter (SSC) including CD14 monocyte surface marker following the use of variable doses of aqueous leaves extract of *C. gigantea*.
Results: In this study, the results showed that aqueous leaves extract of *C. gigantea* caused enhancement in case of granulocytes FSC (shape and size) and SSC (granularity) counts but this aqueous extract inhibited CD14 monocyte surface marker population at higher doses. In contrast, dengue infected human blood samples used as control showed sudden decline in granulocytes count but there was enhancement in CD14 monocyte surface marker as compared to control group.
Conclusion: Overall, *C. gigantea* in the form of aqueous leaves extract showed anti-dengue activity in infected human whole blood samples.

Implication for health policy/practice/research/medical education:
Aqueous leaves extract of *Calotropis gigantea* showed anti-dengue activities in human whole blood samples.
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Introduction

According to Ayurveda, medicinal plant products are continuously used in the form of traditional medicine especially in Asian countries including India. These medicinal plant products are generally very effective for various ailments and their activities could be due to phytochemicals that are present in the forms of primary and secondary metabolites (1,2). These metabolites extracted from medicinal plants play important roles especially in the fields of immunology and virology for the treatment and prevention of animal and human diseases (3,4). Natural compounds in the forms of extract or fraction or pure molecules isolated from medicinal plants represent a major source of molecules with traditional medicinal properties (1,2,5). Therefore, researchers should have sufficient knowledge regarding primary and secondary metabolites extracted or isolated from medicinal plants because of their widespread uses and properties (1,2).
In India, every year thousands of people are died because of dengue disease. It is generally observed or occurred in children. This disease is generally transmitted through mosquitos and reported in the form of types 1 to 4 in number (6,7). When a person is suffering from dengue disease, he/she will encounter major symptoms such as fever, erythema, low platelet count etc. (9,10). If any person is infected once again with a different dengue virus serotype (after getting recovery from another serotype) he/she may develop more severe than before known as dengue haemorrhagic fever (DHF) (9-11). There is no specific treatment or medication for this disease. In the present study, we focused on medicinal plants especially *Calotropis gigantea* to show its effectiveness against dengue positive human blood samples and to determine its antimicrobial activity.

***Corresponding author:** Dr. Amit Gupta;
Emails: amitvsbt@gmail.com; amitgupta@vsbt.res.in

Calotropis gigantea (commonly known as Rui; family *Asclepiadacea*) has shown special attraction because of major phytochemical i.e. calotropin. It has shown various properties including immunopharmacological activities, as well as anti-inflammatory, antifungal, free radical scavenging activities, etc. (12-15). The present investigation is to observe the anti-microbial activity of aqueous leaves extract of *C. gigantea* on dengue positive human whole blood samples.

Materials and Methods

Plant material

Calotropis gigantea medicinal plant was collected in the month of January 2016 from Nakshatra garden of Vidya Pratishthan, Baramati. This medicinal plant was taxonomically identified by Late Dr Sharadini Dahanukar (founder of Nakshatra udyan, Vidya Pratishthan).

Extraction of plant material

Medicinal plant leaves sample (2 g) of *C. gigantea* was macerated in mortar and pestle using liquid nitrogen in order to prepare fine powder. This powder was macerated in phosphate buffered saline (PBS, pH 7.2) for 5-10 minutes at room temperature. The samples were centrifuged at high speed (10 000 rpm, 10 minutes at 4°C) and the supernatant was collected or store at 4°C for immunological studies.

Phytochemical investigation of secondary metabolites

During qualitative studies, *C. gigantea* revealed the presence of secondary metabolites i.e. terpenoids (acetic anhydride test); flavonoids (Lead acetate test); saponins (Foam test) and phenolics (ferric chloride test) in aqueous leaves extract. High performance thin layer chromatography (HPTLC) was performed for this aqueous extract using ethyl acetate: n-Butanol (6:4 ratio) as mobile phase and the results are shown in Figure 1.

Flow cytometric analysis

Infected positive human blood samples of dengue (n = 5) were collected from Mangal Pathology lab, Baramati, District Pune, Maharashtra. These samples were collected on the basis of platelets count (decline in number) including NS1 antigen to dengue virus; which was confirmed through pathology lab (data not shown).

For estimation of human blood counts in case of dengue human whole blood samples for analysing lymphocytes, monocytes and granulocytes count including forward and side scatter using aqueous leaves extract of *C. gigantea* were used. For analysing these data assets of 10 000 events/counts of cell populations representing different phenotypes cell quest software was used. In this study, dengue whole blood samples of human were incubated with various concentrations of aqueous leaves extracts. The samples were incubated (30 minutes), lysed (FACS lysing solution), centrifuged (2500 rpm, 10 minutes) and washed (using PBS). Finally, these cells were analysed through flow cytometry and also analysed its forward and side scatter (14-16).

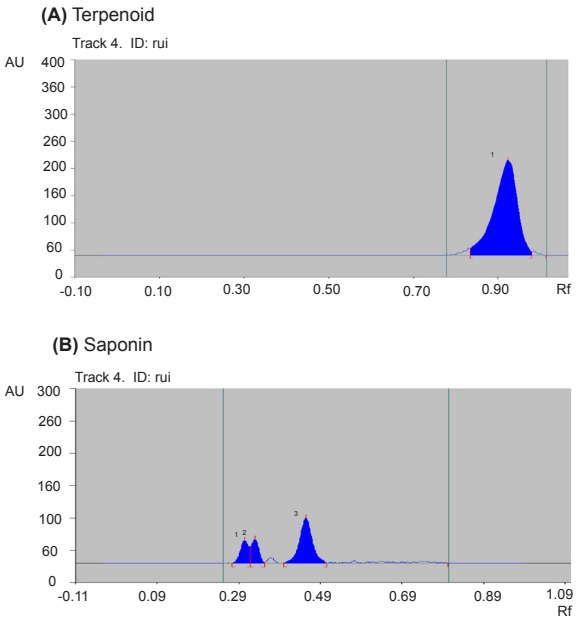

Figure 1. HPTLC analysis of *Calotropis gigantea*.

Similarly, lysed whole blood samples of positive dengue (10^5 cells/well) were cultured for 24 hours in presence of aqueous leaves extract of *C. gigantea*. These cells were stained with CD14 FITC and incubated for 30 minutes. After incubation, the cells were washed with PBS and then analysed through flow cytometer (14-16).

Data analysis

The difference between normal control, infected (dengue) sample and variable doses of aqueous leaves extract of *Calotropis gigantea* was determined through one-way analysis of variance (ANOVA) test (Bonferroni multiple comparison test).

Results

Qualitative estimation

The data revealed that *C. gigantea* possessed secondary metabolites such as flavonoids, terpenoids, alkaloids and saponins.

Blood counts and analysing scattering counts

The effects of various doses of aqueous leaves extract on human blood counts pertaining to analysing its lymphocytes, monocytes and granulocytes counts are shown in Figure 2. The data showed that there was enormous loss of granulocytes count in case of positive blood samples of dengue but after treating with various doses of aqueous extract especially 0.625 mg showed sudden enhancement in case of granulocytes count as compared to dengue control sample. Similarly, there was the same pattern observed in case of forward scatter (FSC) and side scatter (SSC) (Figure 3).

CD14 monocyte surface marker

Aqueous extract of the plant caused decline in CD14 surface marker at higher doses as compared to control

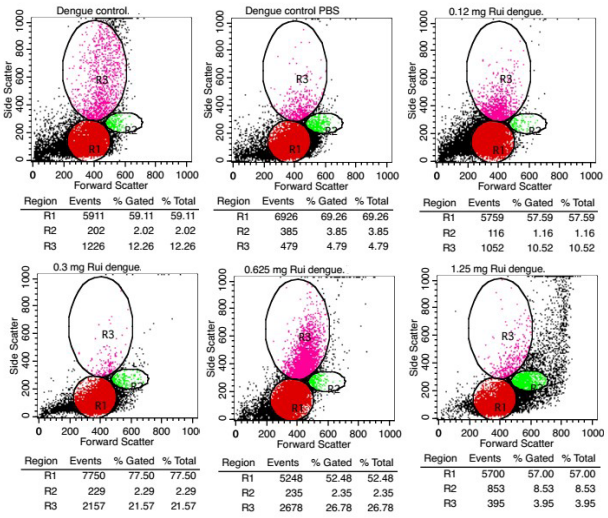

Figure 2. Flow cytometric analysis of aqueous leaves extract of *Calotropis gigantea* on lymphocytes, monocytes and granulocytes count in infected (dengue) human whole blood.
Infected anticoagulant positive dengue human whole blood samples were treated with aqueous extract and then lysed the cells with red cell lysis buffer and washed the cells with phosphate buffered saline and analyzed the samples through flow cytometry.

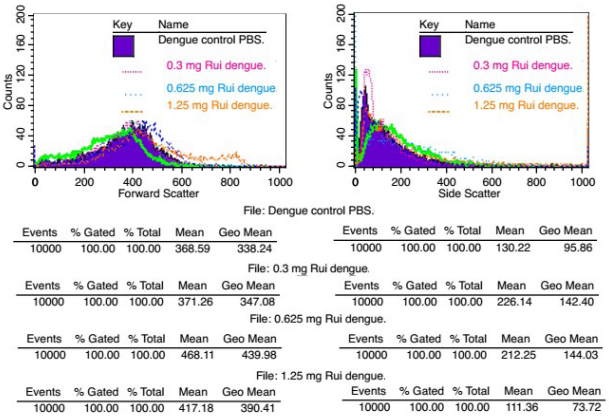

Figure 3. Effect of various doses of aqueous leaves extract of *Calotropis gigantea* on shape and size (FSC) including granularity (SSC) in infected dengue human whole blood samples.
All these samples were analyzed through flow cytometry.

group. On the other hand, dengue control sample showed enhancement in CD14 monocyte surface marker as compared to control group (Figure 4).

Discussion

In the present study, positive dengue human blood samples were analysed at different time intervals for flow cytometric studies. Generally, this disease is caused by arthropode-borne flavivirus named dengue virus (DENV), transmitted by *Aedes aegypti* mosquito. Till date, only four antigenically but distinct virus serotypes (DENV-1, 2, 3 and 4) have been reported belonged to the genus *Flavivirus* in the *Flaviviridae* family. Recently, researchers focused on international public health awareness e.g. dengue which is more prevalent in remote areas, cases of dengue are distributed mostly in urban and

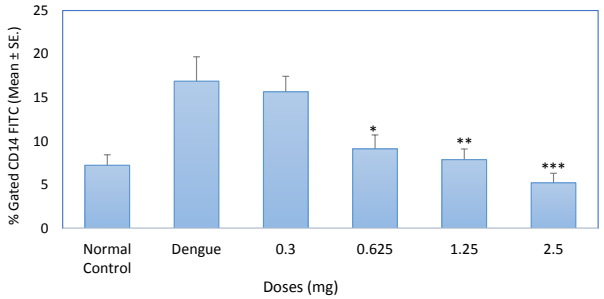

Figure 4. Effect of various doses of aqueous leaves extract of *Calotropis gigantea* on CD14 FITC monocyte surface marker in infected dengue human whole blood samples.
All these samples were analyzed through flow cytometer. The difference between dengue control and various concentration of aqueous leaves extract is determined through one way ANOVA test. $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

sub-urban areas. In this disease, the serotype 2 (DENV-2) is directly bound to human platelets causing drastic decline in platelet count (thrombocytopenia) (7-9). In this regard, we focused on *C. gigantea* for determining its antimicrobial properties in infected dengue human whole blood samples. Flow-cytometer is generally used for measuring immunophenotyping i.e. cell surface markers (CD3/CD4/CD8 etc.), cell cycle analysis (G0, G1, S and M phase), cell viability (live versus dead), total protein, enzyme activity, gene expression, etc (17). The identification of these cells in the form of lymphocytes, monocytes including granulocytes count, present in a fluid, are measured through light scatter properties in the form of FSC and SSC. As per the literature, there is strong interrelationship between FSC (related to shape and size) and SSC (granularity) pertaining to flow-cytometric operation (16,17). This technique is commonly used and applied in preclinical and clinical research studies. As per the literature, live cells show enhancement in FSC and decrease in SSC whereas dead cells have higher SSC and lower FSC (16,17). As per the data in this study, aqueous leaves extract showed enhancement in FSC and SSC at higher dose (0.625 mg) as compared to control group. Similarly, the results showed that aqueous extract increased the level of granulocytes count after treating with variable doses of aqueous leaves extract as compared to control group. Overall, the antimicrobial activity of aqueous extract was affected in dose dependant fashion and was more effective in higher doses.

Further confirmation for antimicrobial activity of *C. gigantea* against positive dengue blood samples of human was CD14 monocyte surface marker which was determined by flow-cytometry. In the literature, CD14 (55 KDa glycoprotein) surface marker was observed on the surface of monocytes/macrophages including several monoclonal antibodies to the same epitope on human monocytes assigned to a provisional CD14 cluster, which was categorized or designated as leucocyte differentiation antigen (18). In this study CD14 monocyte marker production markedly declined at higher doses of aqueous

leaves extract of *C. gigantea* and this marker might depend on cell types and their species origin as well as different cells having different requirements for signal transduction pathways.

Conclusion

This is a preliminary study to report its antimicrobial property of aqueous leaves extract of *C. gigantea*. It showed some scientific proof for the use of this extract for the treatment of various infectious diseases.

Conflict of interests

Authors have declared that no competing interests exist.

Ethical considerations

These studies were conducted under IBSC guidelines and approved by Savitribai Phule Pune University, Pune.

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