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Flow cytometry based assay of formulation from *Syzygium cumini* in human whole blood and gylcosylated red blood cells

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ABSTRACT

The aim of this study is to determine the anti-diabetic effect of formulation of Syzygium cumini in diabetic people of different age groups. This study determined whether or not Syzygium cumini could alter hyperglycemia in diabetic people. In this study, diabetic blood samples were collected and the whole blood and plasma samples were treated with formulation of Syzygium cumini (1.25 – 10 mg) to determine the lymphocytes, monocytes and granulocytes count and also estimate the blood counts using forward and side scatter through flow cytometry. In this study, we used huminsulin (50/50) as standard for our experiment. The results showed that huminsulin 50/50 showed increase in the number of lymphocytes but there is sudden decline in the level of monocytes while maintaining the level of granulocytes where as in formulation of Syzygium cumini showed decrease in the number of monocytes and increased in the level of granulocytes but the formulation retain the level of lymphocytes as compared to control. On the other hand, estimation of glycosylated RBC in plasma samples using flow cytometry. The results showed that the forward and side scatter changes in the level of blood counts in case of formulation of Syzygium cumini with respect to cell shape and granularity at a very low concentration which might be primarily linked to a continuing insulin deficiency or to secondary hyperglycemia occurring in the diabetic individual. Accordingly, effective control with formulation of Syzygium cumini is likely to be relevant in diabetic patients. In addition, formulation of Syzygium cumini showed no toxic effect in mice and there is enhancement of CD3, CD4 and CD8 count as compared to control.

Key words: *Syzygium cumini, Antidiabetic, Glycosylated, Huminsulin.*

INTRODUCTION

Diabetes mellitus is one of the group of metabolic diseases which is characterized through increase in blood glucose levels (hyperglycemia) resulting from defects in insulin secretion [1]. Insulin is a hormone which is produced from the beta cells of pancreas and is able to utilize the glucose from digested food items as an energy source. There are two common types of diabetes i.e. type 1 and type 2. In type 1, the body is not be able to produce enough insulin and daily insulin injections are required and this is usually diagnosed in childhood or early adolescence where as in type 2 diabetes is the result of failure to produce sufficient insulin [2, 3]. Elevated blood glucose levels are managed with reduced food intake, increased physical activity and eventually oral medications or insulin. The main aim is to treat both types of diabetes and to maintain the blood glucose levels near the control ones. Another aim is to promote the normal growth and development of healthy weight, normalize blood glucose levels and minimize hyperglycemia and hypoglycemia, achieve normal lipid levels, prevent and delay complications, promote optimal health and well-being [2-6]. Achieving these goals requires insulin that depends on the type of diabetes, medical nutrition therapy, frequent blood glucose monitoring and evaluating patterns and also provides the knowledge to people about diabetes. The major drawback in diabetes mellitus i.e. chronic hyperglycemia, which is associated with micro as well as macrovascular symptoms that can lead to visual impairment, blindness (loss of vision), nerve/neuron damage, heart disease and stroke [6, 7].

As per the literature, there are number of studies on oral antihyperglycemic agents derived from plants used in traditional system of medicine have been conducted and most of the plants were found to have good activity [8, 9]. According to World Health Organization (WHO) also recommended the evaluation of plants effectiveness whenever safe modern drugs are unavailable [8, 9]. This has led to an increased demand for research on natural products with antidiabetic activity as well as minimal to no side effects. Unfortunately, complete therapy for diabetes mellitus and its complications has not been established yet. On the basis of this criterion, our group focuses on medicinal plants especially *Syzygium cumini* for anti-diabetic activity [10].

Syzygium cumini belongs to the family Myrtaceae and these trees are commonly grown in Asian subcontinents including India. Different parts of the plant *Syzygium cumini* i.e. fruit, bark and leaves showed antioxidant [11], anti-inflammatory [12], anti-cancer [13], anti-bacterial, anti-HIV, antifungal, nitric oxide scavenging, free radical scavenging, anti-diarrheal, antifertility, gastroprotective and anti-ulcerogenic activities [10, 11]. Moreover, seeds of *Syzygium cumini* plant are generally used to treat variety of ailments, the most important and effective being diabetes mellitus [10, 11]. According to ayurveda, *Syzygium cumini* fruits bark and leaves are traditionally used and number of research papers and patents related to *Syzygium cumini* seeds which are mentioned about the anti-diabetic activity [10, 12]. In *Syzygium cumini*, seeds contained a glycoside called jamboline which is reported as anti-diabetic agent and also reported as anti-inflammatory agent [12]. On the basis of this study related to *Syzygium cumini* seeds, our group focused on the formulation of *Syzygium cumini* plant on human diabetic whole blood samples and also evaluated the *in vivo* effect of *Syzygium cumini* on normal mice.

MATERIALS AND METHODS

1. Preparation of extracts:

Fresh explants of leaf from theme based organic plantation of *Syzygium cumini* were collected. The explants of *Syzygium cumini* were soaked in tap water for ten minutes and then dried into a shady area. The maceration step of explants with liquid nitrogen to form the fine powder and then appropriately used for specific formulation. Experimental doses were prepared for final immunological studies. Appropriate homogenous formulation prepared in phosphate buffered saline (pH 7.2). The solids were separated by centrifugation at 5000 rpm for 15 minutes from the formulation.

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2. High performance thin layer chromatography (HPTLC) fingerprinting:

The formulation was purified from *Syzygium cumini* and detects various metabolites using HPTLC. The solvents, reagents and HPTLC plates (10 x 10 cm) were purchased from Qualigens and Merck. Generally, the solvent system used in mobile phase and detect its wavelength at 366 nm. The stock solution of formulation of *Syzygium cumini* was prepared for HPTLC studies and dissolved the 5 g of weighed compound in phosphate buffered saline or with different solvents in a final volume of 50 ml. Further dilutions were made to obtain working standards. The formulation of *Syzygium cumini* showed the presence of terpenoids, flavonoids, phenolics, saponin and carbohydrates in the phytochemical profile of *Syzygium cumini*. The retardation factor *(Rf)* values of terpenoids and flavonoids are 0.96 and 1.78.

3. Formulation of *Syzygium cumini:*

In formulation, main constituents are hydroxyibuprofen, estradiol acetate, 5-Ethyl-5-(1-methyl-3-carboxypropyl) barbituric acid, Camptothecin, Cyclosporin A, Dihydrostreptomycin 6-phosphate etc. All these values are within the range between -5 to + 5 ppm. The information related to this formulation is mentioned in the supplementary studies..

4. Diabetes cases:

The 54 diabetic cases included in this study were analyzed at the VSBT, Baramati, Maharashtra, India, between July to September 2014. Diabetic and non-diabetic blood samples collected from *Mangal Pathology Laboratory*, Maharashtra, India. The median age at diagnosis was 51 years (range, 34-73 years), with a male/female ratio of 2:1 as shown in **Table 1**. Response of blood counts and plasma cells was monitored by means of flow cytometric analysis. For standardization procedures, we used normal whole blood human non diabetic samples as negative control samples. Informed consent was obtained from all patients before performing these procedures.

5. Flow Cytometric analysis:

Flow cytometry analysis is for counting and examines the cells count suspended in a stream of fluid. For the hematological analysis, blood samples were collected one hour after the meals and the plasma collected for the estimation of biochemical parameters using forward and side scatter through flow cytometric analysis.

5.1. Human whole blood and plasma samples:

For the estimation of variable doses of formulation of *Syzygium cumini* (1.25 – 10 mg) in human whole blood and plasma samples using forward and side scatter gating applied for data acquisition of 10000 events of cell populations representing different phenotypes analyzed using cell quest software. Huminsulin 50/50 used as standard for this study.

a) **Whole blood: -** In this experiment, 100 µl of human whole blood was taken in each tube. Add serial dilutions of formulation on human whole blood and then incubated the samples in dark for 2 h at 37°C carbon dioxide incubator. Subsequently, 2 ml of 1× FACS lysis solution was added at room temperature with gentle mixing followed by incubation for 10 min. The samples were spinned (300 – 400 × *g*) and the supernatant was aspirated and washed two times with phosphate buffered saline. After centrifugation, pellet dissolved in PBS and observed the cells through flow cytometer [14, 15].

b) **Plasma samples**:- Another set of this experiment, collect the plasma samples from diabetic and non-diabetic people and then treated with serial dilutions of formulation (1.25 -10 mg) and then incubated the samples for 2h at 37°C carbon dioxide incubator. The plasma samples were lysed and washed the samples 2-3 times with phosphate buffered saline and then analyzed in flow cytometer.

6. Animal studies:

6.1. *In vivo flow cytometric studies:*

The *in vivo* effect of formulation was evaluated in Swiss mice. Briefly, the serial dilutions of formulation (0.625 – 2.5 mg) were mixed together in a final volume of 0.2 ml. These samples were incubated for 1 h at room temperature. After incubation, 0.2 ml of the formulation was injected subcutaneously from day 0 to day 7 in mice. The mice were then observed daily for the clinical signs viz., weight loss, flaccid paralysis followed by death. On 10th day, the mice spleen cells were collected and estimate the cell surface markers i.e. CD3, CD4/CD8 count in mice.

For flow cytometric analysis, spleen cells (100 µl, 10^6cells/ml) were taken in each tube [16]. FITC labeled CD8, CD3 and PE labeled CD4+ monoclonal antibody were added directly to 100 µl of cells. Tubes were incubated in dark for 30 min at room temperature. Subsequently, 2 ml of 1× FACS lysis solution was added at room temperature with gentle mixing followed by incubation for 10 min. The samples were spinned (300 – 400 × *g*) and the supernatant was aspirated and sample was given three washings of PBS (pH 7.4). The resulting stained cell pellet was resuspended in 500 µl of PBS and was run on a flow cytometer. The forward and side scatter gating applied for data acquisition on 10,000 events in FACS Calibur and fraction of FSC abd SSC cell populations representing different phenotypes analyzed using cell quest software.

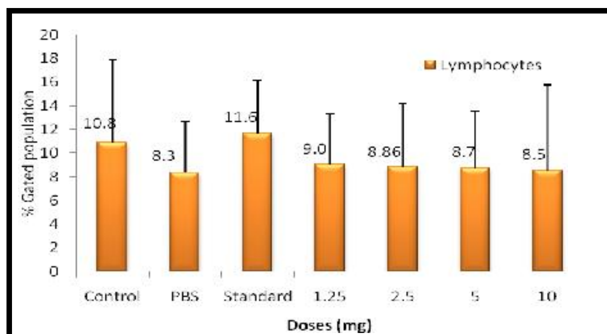
RESULTS

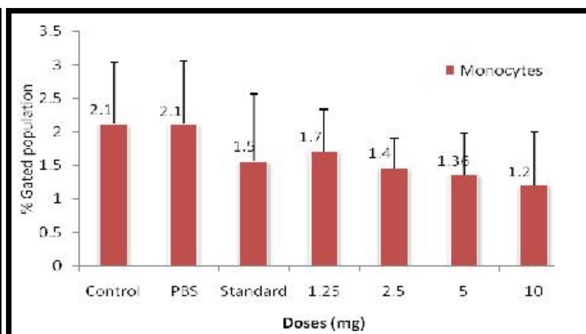
1. Effect of formulation of *Syzygium cumini* on human whole blood and plasma samples:

The effect of formulation on the number of lymphocytes, monocytes and granulocytes count as shown in **Fig. 1**. The results showed that the formulation showed dose dependent decrease in the number of lymphocytes and monocytes count as compared to control while huminsulin 50/50 showed increase in the number of lymphocytes and decrease in monocyte count. In contrast, the formulation increased the number of granulocytes count where as huminsulin 50/50 retains the number of granulocytes count as compared to control. In addition, the formulation increased the dose dependent increase in forward scatter (shape and size) and side scatter (granularity) as compared to huminsulin 50/50 and controls (**Fig. 2)**.

2. Effect of formulation of *Syzygium cumini* on CD3//CD4/CD8 count in mice:

The effect of formulation on CD3//CD4/CD8 count in mice as shown in **Fig. 3**. The results showed that the formulation showed dose dependent decrease in CD4/CD8 ratio as compared to control while huminsulin 50/50 showed moderate increase in the number of CD4/CD8 count. The effective concentration of the formulation is at 0.625 and 1.25 mg as compared to control. In contrast, the maximum effect of formulation in CD3 surface marker is at 1.25 mg as compared to control **(Fig. 4)**.





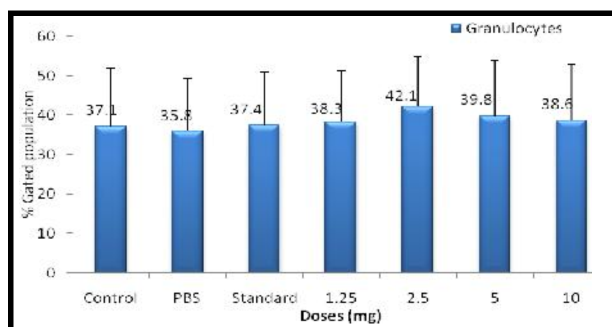


Fig.1. Flow cytometric analysis of formulation of *Syzygium cumini* on lymphocytes, monocytes and granulocytes count. EDTA human whole blood were treated with formulation and then lysed and wash the cells with phosphate buffered saline and analyzed through flow cytometer. Values are expressed in Mean ± S.E. of fifty four human whole blood samples. Huminsulin 50/50 used as standard for anti-diabetic studies.

Fig.2. Flow cytometric analysis of formulation of *Syzygium cumini* on human plasma cell count. Plasma cells were treated with variable concentration of formulation and then lysed and wash the cells with phosphate buffered saline and analyzed through flow cytometer. Values are expressed in Mean ± S.E. of fifty human whole blood samples. Huminsulin 50/50 used as standard for anti-diabetic studies.

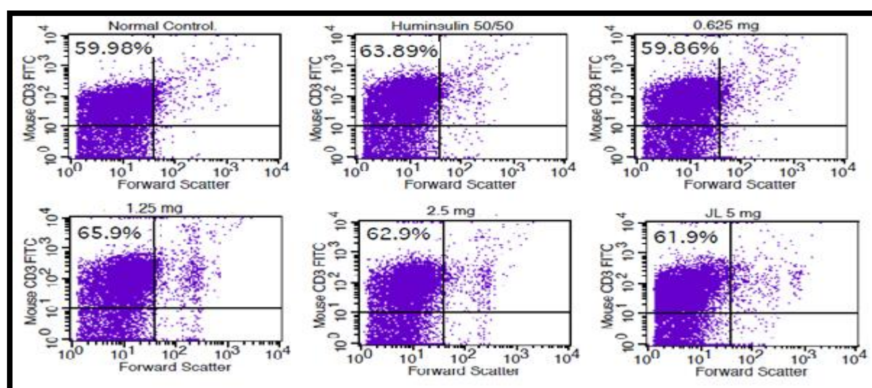


Fig.3. Flow cytometric analysis of formulation of *Syzygium cumini* on CD3, CD4 and CD8 count in mice. Swiss mice were immunized subcutaneously with variable concentration of *Syzygium cumini* from day 0 to day 7. Huminsulin 50/50 used as standard for anti-diabetic studies. On day 10, spleen cells were collected and stained with CD3 FITC surface marker. After 30 minutes incubation with monoclonal antibody, cells were lysed and washed the cells with phosphate buffered saline and analyzed through flow cytometer (FACS Calibur).

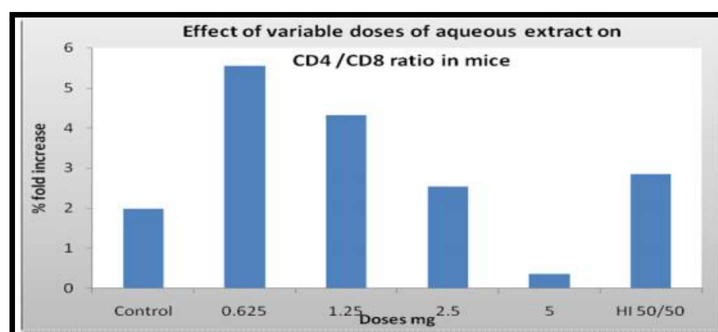


Fig.4. Flow cytometric analysis of formulation of *Syzygium cumini* on CD4/CD8 ratio in mice. Swiss mice were immunized subcutaneously with variable concentration of *Syzygium cumini* from day 0 to day 7. Huminsulin 50/50 used as standard for anti-diabetic studies. On day 10, spleen cells were collected and stained with CD4 PE and CD8 FITC surface marker. After 30 minutes incubation with monoclonal antibody, cells were lysed and washed the cells with phosphate buffered saline and analyzed through flow cytometer (FACS Calibur).

Table No. 1: Case studies of human diabetes blood samples

No. of Samples	Name of Patient	Sex	Age (years)				
Sample 1	Umesh Chavan	M	34	Sample 27	Anaspure Gajanan Yashwant	M	74
Sample 2	Rajesh M Kalyankar	M	40	Sample 28	Zagade Ambadas Dashrath	M	32
Sample 3	Sadhana S Raut	F	35	Sample 29	Kumbhar Radhe Shyam Wamanrau	M	50
Sample 4	Buvasaheb R Jagtap	M	73	Sample 30	Vatre arjun Khandu	M	60
Sample 5	Neelam B Vharkate	F	66	Sample 31	Kale Bapu Baban	M	62
Sample 6	Kunda V Hulbe	F	61	Sample 32	Nare Narayan Maruti	M	60
Sample 7	Bhunrav A Sawant	M	45	Sample 33	Ghorpade Namdev Shankar Rav	M	75
Sample 8	Sanjay B Salunke	M	42	Sample 34	Virkar Mohan Digambar	M	58
Sample 9	Pawar Patil Buva	M	65	Sample 35	Bhosale Mivrutti Waghu	M	60
Sample 10	Manisha S Khandale	F	35	Sample 36	Deakate Janabai Buvaji	F	55
Sample 11	Ramchandra I Nadgouda	M	53	Sample 37	Sawant Kanchan Gorakh	F	24
Sample 12	More Naresh Narayan	M	46	Sample 38	Bhosale Pragati Vijay	F	19
Sample 13	Sarjerao B Kale	M	58	Sample 39	Bankar Shobha Rajendra	F	46
Sample 14	Khandare Mrudula Jawahar	F	56	Sample 40	Choudhar Dheeraj Wadaso	M	17
Sample 15	Dashi Rajkumar J	M	47	Sample 41	Pawar Sonali Sudhir	F	28
Sample 16	Shaikh Jamila A	F	57	Sample 42	Surya Vamshi Bharat Maruti	M	57
Sample 17	Bagav Alka Ramdas	F	45	Sample 43	Khindare Anil Suryakant	M	40
Sample 18	Shende Puspawati Babanrav	F	59	Sample 44	Gophane Bhagwat Dinkar	M	45
Sample 19	Anaspure Monda Gajanan	F	67	Sample 45	Chindhe Bhagwan Pandurang	M	43
Sample 20	Khaire Janabhai Hanumant	F	40	Sample 46	Mahamuni Gajanan Dnyaneshwar	M	38
Sample 21	Shah Manoj Sharad Kumar	M	46	Sample 47	Sonar Surendra Kisan	M	41
Sample 22	Joshi Shashikala Vinayak	F	72	Sample 48	Nimbalkar Shivajirav Bhikulal	M	84
Sample 23	Wagh Subash Vishwanath	M	37	Sample 49	Bhise Nivrutti Vithal	M	43
Sample 24	Vaidya Taranandan R Chandra	M	52	Sample 50	Kulkarni Vijayashree Madan	F	58
Sample 25	Jadhav Chhaya Sharma V	F	62	Sample 51	Galande Shamkant Manohar	M	44
Sample 26	Mehta Harshvardhan Hukumchand	M	58	Sample 52	Barge Shailendra Namdev	M	46

DISCUSSION

Medicinal plants are composed of many constituents; hence it is very important to obtain reliable as well as accurate chromatographic fingerprints that represent immunopharmacologically active components of the natural product. HPTLC fingerprinting profile is very important parameter of aqueous extract standardization for the proper identification of medicinal plants. To understand the actual chemical constituents responsible for the immunopharmacological activities of the plant detailed chromatographic studies are needed to be carried. The present study is to determine the composition of aqueous extract of *Syzygium cumini* using the HPTLC technique. In this study, aqueous extract showed number of multiple peaks corresponds to glycosides, flavonoids, terpenoids and phenolics. The data of HPTLC fingerprint profile could be used as a valuable analytical tool in the routine quality control and standardization. The objective of our study is to bring about the information of *Syzygium cumini* used in various parts of world for the treatment of diabetes [17, 18]. Number of human diabetes blood samples has been collected for their use as anti-diabetes. Although different types of Huminsulin of different ratios are available in the market which is widely used to treat diabetes. The development of new anti-diabetic from the highly active natural product i.e. *Syzygium cumini*, which have been discovered, is crucial as well as important in order to overcome the increasing resistance of sugar level to available anti-diabetic drugs [19-21]. Therefore, there is a need to advance the work on medicinal plants especially *Syzygium cumini* which have been shown to have anti-diabetic activity in human blood samples through further *in vivo* testing in non diabetic animal models to observe the CD3/CD4/CD8 count in mice.

In case of human diabetic blood samples, granulocytes which played an important role in the host inflammatory response against infection [22]. Several studies have shown that the reduction of granulocytes contributes to the incidence of infections in diabetes. Number of research papers which are published and reported in diabetic rats and mice also showed a decreased neutrophil migration, phagocytosis capacity and hydrogen peroxide production [23]. Furthermore, the reduction of blood glucose levels by insulin treatment of diabetic patients has been reported to be significantly correlated with improvement of neutrophil phagocytosis capacity [23].

Flow cytometry is a contemporary analytical method for the assessment of qualitative as well as quantitative information of biological and physical characteristics of prokaryotic and eukaryotic cells [24]. Today in immunopharmacology, flow cytometry is routinely used in clinical laboratories for the assessment of the immune status of healthy animals and human [25]. However, the main application of this method is important in immunopharmacology such as hematology, toxicology etc [24, 25]. Furthermore, this flow cytometric method is very useful in immunopharmacology for the evaluation of disease and measures its prevention, diagnosis and therapy. Meanwhile, in this flow cytometric based assay, the results showed that the formulation at a very low concentration increased in the number of granulocytes as compared to control and huminsulin 50/50. Similar studies are observed in case of monocytes, the formulation also showed similar reduction in the level of monocytes as compared to huminsulin 50/50 where as Huminsulin 50/50 showed increased in the level of lymphocytes but in case of this formulation retained the level of lymphocytes as compared to control. After treating the formulation and huminsulin 50/50 with human diabetic blood plasma samples, there we get transparent solution of plasma content but at a very high concentration of formulation, the protein as well as ionic molecules content gets settled at the bottom. The results showed that the formulation at a very high concentration there is increased in the level of forward scatter (shape and size) and side scatter (granularity) of the cell as compared to control. Overall, the anti-diabetic activity of formulation was affected in dose dependant fashion i.e. lower doses of the formulation were more effective than that of higher doses.

In animal model studies especially mice to determine the toxicity of formulation, the variable concentration of formulation immunized intraperitoneally to observe the CD3/CD4/CD8 count in mice. The results showed that the formulation showed increase in the level of CD3 and CD4/CD8 count in mice as compared to control and huminsulin 50/50. At a very low concentration of formulation, there is increased in the number of CD4 count as compared to control. In this study, it gives clear indication that our formulation showed no toxic effect in animal model studies. However, further studies including controlled clinical trials are necessary before specific traditional remedies can be recommended on a large scale.

CONCLUSION

The diabetes mellitus also called as silent killer. To control this disease with huminsulin 50/50 could be possibly achieved through the formulation of *Syzygium cumini.* The results showed that enhancement of granulocytes, forward scatter, side scatter and reduction of monocytes due to *Syzygium cumini* which played an important role in the host inflammatory response against infection. On the basis of this objective, it can be concluded that the formulation could be a potent anti-diabetic agent for next generation.

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