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EXTRACTION OF PROTEASES FROM LEAVES OF *MIMUSOPS ELENGI* AND ITS IMMUNOPHARMACOLOGICAL APPLICATIONS

Amit Gupta[1*], Ankit P Shah[2], AR Chabukswar[2], Sushama R Chaphalkar[1]

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

[2]MAEER's Maharashtra Institute of Pharmacy (MIT), Pune, India

Abstract:

As per Ayurveda, Mimusops elengi, medicinal plant leaves are widely used as traditional medicine for human diseases. Protease, a potential candidate extracted from the leaves of Mimusops elengi using phosphate buffered saline (PBS) of different pH values (3 to 9) and determined its effect in virally infected human whole blood is not so for studied. So an experimental attempt was made to determine the protease activity of leaves of Mimusops elengi. Buffers of different pH range were used for extraction of the leaves to identify the best buffer for extraction of protease (acid, basic and neutral). Total protein (pH 4, 5, 8 and 9) content and protease (pH 4, 8 and 9) activity were determined in the leaves of different pH value. Mimusops elengi leaves showed higher protease activity when the extraction was carried out at pH 4.0. The results of the present study indicate that protease activity of leaves of Mimusops elengi on virally infected human whole blood and it may be responsible for returning to its normal blood profile. Similar studies also reported in animal model studies i.e. Swiss mice were immunized on day 0 and day 7 with 10^9 cells of virally infected (symptoms such as cough, sneezing etc) lysed human whole blood along with or without diluted and concentrated form of protease extracted from Mimusops elengi against BSA in a final volume of 1 ml. on day 10, EDTA blood was collected from retro-orbital plexus for the estimation of cell surface markers (CD3/CD4/CD8) in mice. The results showed that protease from Mimusops elengi returned to its normal blood profile and continued to play an essential role or showed its necessity for human health care.

***Key words**: Mimusops elengi; protein; protease; BSA; virally infected; mice.*

Corresponding author

Dr Amit Gupta

Assistant Professor/Scientist, VSBT

E-mail address: - amitvsbt@gmail.com; amitgupta@vsbt.res.in

QR code

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INTRODUCTION:

Proteases are omnipresent enzymes found in all plants, animals and micro-organisms. For more than one hundred years, immunobiological studies have been supported on their well-known property to catalyze the hydrolysis of certain peptides in target proteins [1]. Later, more authentication of its significance in many immunobiological processes has been supported in all living organisms. Thus, proteases synchronize the fate, localization and activity of many substrates, create new biologically immunoactive molecules, contribute to process cellular information, transduce and amplify molecular signals and regulate protein/protein/peptide interactions [2, 3]. In view of this, these proteases are being vital to their host cells, they may also be potentially damaging when over expressed or present in higher concentrations e.g. activation during cancer, cardiovascular (diabetes) diseases etc. For these reasons, function of this group of enzymes i.e. proteases needs to be strictly regulated and supervised [4]. In another words, these proteases that hydrolyze peptide bonds and can therefore degrade proteins and peptides. The opportunistic human pathogens i.e. bacteria, virus, fungi etc has an arsenal of impressively efficient proteases that helps to preserve an infection and thereby controlling and modifying the environment according to the needs of these pathogens within the host tissue [5].

As per the classification of proteases (e.g. glutamic acid, threonine etc) operate under acidic, basic and neutral conditions so they are called as acidic, basic and neutral proteases [6]. The examples of these proteases are acidic (Aspartate, Glutamate), basic (serine, cysteine) and neutral (Zn-metalloendopeptidase). These proteases (acidic, basic and neutral) are extensively used as well as applied in various microbiology labs and pharmaceutical industries especially for cell culture including tissue dissociation, peptide synthesis, recombinant antibody fragments, purification of nucleic acids (undesirable proteins digestion), peptide sequencing and proteolytic digestion of proteins in proteomics etc [7]. The main feature of proteases in our immune system which showed more effectiveness against number of pathogens. There are number of protease inhibitors e.g. HIV 1 Protease (saquinavir, ritonavir, amprenavir, nelfinavir) which is already reported and also widely distributed in the plant and animal kingdom e.g. serine, aspartic, Cysteine etc. To further explore the knowledge of proteases, structure activity relationship (SAR) studies have been developed to create new products or medicine against various diseases. For instance, HIV protease enzyme would help us to prevent HIV virus from spreading throughout the body [8, 9].

Mimusops elengi (common name called as bakul; family *Sapotaceae*) mostly found in Northwestern Himalayas, Eastern Ghats, Western Ghats, Central Deccan Plateau, East Coast, West Coast and Indo-gangetic Plain [10, 11]. The bark, flowers, fruits, and seeds are used in Ayurvedic medicine in which it is purported to be astringent, cooling, anthelmintic, tonic, and febrifuge. It is mainly used for dental ailments such as bleeding gums, caries and loose teeth [10, 11, 12]. In the present study, our group focused on those proteases (acid, basic and neutral) from *Mimusops elengi* and determined its effect in virally infected human whole blood and also observed its effect in mice model studies.

MATERIALS AND METHODS:

Chemical Reagents

Sodium Chloride (NaCl), Potassium dihydrogen phosphate (KH_2PO_4), Potassium chloride (KCl), Sodium bicarbonate ($NaHCO_3$), Disodium hydrogen phosphate (Na_2HPO_4), Hydrochloric acid (HCl), Sodium hydroxide (NaOH), Tris HCl, Acetone (CH_3COCH_3), Ethylene diamine Tetracetic Acid (EDTA), Ammonium chloride (NH_4Cl), Citric acid ($C_6H_8O_7$) and Monosodium citrate ($C_6H_7NaO_7$) were purchased from Sisco research laboratories private limited, Andheri Mumbai, India and Himedia Laboratories, Mumbai, India. CD4 PE, CD3FITC and CD8 FITC anti-mouse monoclonal antibodies procured from BD Pharmingen, India.

Collection of Plant Material

Fresh plant leaves of *Mimusops elengi were* collected or gathered from the udyan of Vidya Pratishthan's School of Biotechnology, Baramati, Maharashtra, India.

Preparation of Plant Material

All these leaves were macerated in mortar and pestle with liquid nitrogen (- 196 °C) to make a powder. The powder (dried extract) was stored at 4 °C for extraction, scrutinize and determine its protein content. This protein content especially crude enzyme was used for the investigation of protease based assay (acid, basic and neutral) against specific protein antigen (BSA). The immunopharmacological studies of *Mimusops elengi* were carried out at 0 - 5°C.

Extraction of Protein

For protein estimation, weigh 8 g of leaves powder macerated in double amount of extraction buffer (i.e. 20 mM Tris HCl) in PBS of different pH concentration ranging from 3 to 9 using 1N NaOH or 1N HCl. Afterwards, incubate the test candidate's

powder along with extraction buffer for 5 -7 minutes at room temperature and then centrifuged at 5500 rpm for 10 minutes at 4°C. Collect the supernatant after centrifuging and then add similar proportion of ice cold acetone. Incubate these samples (test candidates) for 8-10 minutes at room temperature and then centrifugation at corresponding speed and time as mentioned above [13]. After centrifuging, collect the pellet and washed with ice cold acetone to expel the pigments as well as lipids. Finally, the protein concentration was resolved through Nano drop method.

Crude Protease Production and Enzyme Assay

The extraction of protein (using Tris HCl and ice cold acetone) from non-identical concentration of PBS buffer (pH 3 to 9) using fresh plant leaves. Finally, the protein was dispersed again in different pH values of PBS buffer (3 to 9). Centrifuged the total protein content at 10000 rpm at 4°C for 30 minutes and the supernatant (crude enzyme extract) was pipette out and used for analyzing and estimate its protease content against specific protein antigen, bovine serum albumin (BSA).

Protease activity was determined by calorimetric assay using BSA as substrate. In this study, crude enzyme extract of total protein content (extracted from plant leaves) was assayed by using 1% BSA dissolved in citrate buffer (pH 7). For these studies, equal proportion of BSA and crude enzyme extract of test candidates in 50 ml conical flask and allowed to stand for 2h at room temperature. Afterwards, addition of TCA solution pertaining to cease the enzymatic reaction and then centrifuging (10000 rpm, 30 minutes at 4 °C). The supernatant was collected and add equal proportion of NaOH solution in correlation with TCA solution. Incubate these samples i.e. plant test candidates for 30 minutes at room temperature [13]. Afterwards, addition of Folins reagent (0.5 ml) pertaining to examine its intensity with respect to the appearance of blue color was measured at 650 - 700 nm within half an hour using spectrophotometer.

Analytical Studies (pH and Temperature) of Crude Protease

For these analytical studies, different pH (3 to 9) values of buffer were formulated. Sequentially dilution of non-identical series of crude enzyme extract of test candidates was mixed with homogenous bulk of BSA dispersed in PBS buffer of different pH values. BSA used as standard for these studies. After incubation (1 h) at room temperature and optical density (OD) was measured after with UV-visible spectrophotometer at 595 nm.

Moreover, different temperatures were specified (0°C, 4°C, 18°C, 45°C and 90°C) in order to determine its protease activity of test candidates against specific protein antigen, BSA. Similar quantity of crude enzyme extract (test candidates) and BSA solution dissolved in PBS of different pH values. Again, performed Lowry test and OD was measured at 595 nm using UV-visible spectrophotometer [13]. For these analytical studies, two controls were taken i.e. enzyme control and the next one is BSA.

Clinical and Preclinical Studies

Flow cytometry analysis of human whole blood including analysis of anti-coagulant blood samples of Swiss mice for counting and examine the cells count suspended in a stream of fluid. In flow cytometry (using forward, FSC and side scatter, SSC), gating applied for data acquisition of events (/10000) of cell populations representing different phenotypes (lymphocytes, monocytes and granulocytes including surface markers) analyzed using cell quest software.

Flow Cytometric Estimation on Virally Infected Human Blood Samples Pertaining To Human Blood Counts

In order to determine or examined the effect of proteases from *Mimusops elengi* using variable doses on virally infected human blood sample for analyzing its blood counts (lymphocytes, monocytes and granulocytes count) using flow cytometry.

For the estimation of blood counts, virally infected positive and negative anti-coagulant (EDTA) blood samples of human were collected from pathology lab and estimate the lymphocytes, monocytes and granulocytes count. In this study, human whole blood (100 µl) was taken and then stained with or without CD14 FITC monocyte surface marker including addition of variable concentration of test candidates Incubate the samples for 2 h at 37°C, 5% carbon dioxide incubator. Afterwards, add FACS lysing solution (2-3 ml) was taken and incubate it for 8 minutes. Centrifuging and washing the samples with buffer PBS (two times). Collect the pellet and dissolved in PBS. Afterwards, the samples were processed through flow cytometer (FACS Calibur) [14, 15].

Estimation of CD3/CD4/CD8 in Swiss Mice

For preclinical studies, Swiss mice were immunized intraperitoneally on day 0 and day 7 with 10^9 cells of virally infected (symptoms such as cough, sneezing etc) lysed human whole blood along with or without diluted and concentrated form of protease extracted from *Mimusops elengi* against BSA in a final volume of 1 ml. on day 10, EDTA blood was collected from

retro-orbital plexus for the estimation of cell surface markers (CD3/CD4/CD8) in mice.

In this experiment, 100 µl of whole blood was taken in each tube. Mouse monoclonal antibodies i.e. FITC labeled CD8 (CTL response), CD3 (total T cell count) and PE labeled CD4 (helper T cells) were added directly to whole blood (50 µl). Falcon tubes containing treated (*Mimusops elengi*) blood samples of mice were incubated in dark for 30 -45 min at room temperature. Subsequently, addition of FACS lysis solution (1x) was added at room temperature with gentle mixing pursued by incubation for 10 min. Non-treated (control) and treated samples of *Mimusops elengi* were spinned (2500 rpm, 10 min at 4 °C) and the supernatant was aspirated and washed with phosphate buffered saline. After centrifugation, pellet dispersed in PBS and scrutinizes the cells through flow cytometer [16].

Statistical Analysis

Readings are expressed as Mean ± S.E. The difference between the control and treated groups of *Mimusops elengi* which is determined through one way ANOVA test (Boniferroni multiple comparison test).

RESULTS:

Estimation of Protein Content

To determine the protein content extracted from fresh plant leaves of *Mimusops elengi* through NanoDrop using pH buffer (PBS, 3 to 9) of different concentrations as shown in **Table** 1 For isolation of protein using Tris HCL and ice cold acetone, BSA used as a standard for these studies. The results showed that *Mimusops elengi* showed moderate amount of protein content (pH 3 to 9) ranging from 1.906 to 15.682 mg/ml

Estimation of Crude Protease Production

The effect of variable pH concentration of PBS buffer (3 to 9) dissolved in protein containing citrate buffer extracted from the leaves of *Mimusops elengi* against BSA for the estimation of protease which is determined through spectrophotometer as shown in **Table 1**. The results showed that *Mimusops elengi* showed moderate amount of protease content (pH 3 to 9) ranging from 0.142 to 1.666 mg/ml.

Effect of pH and Temperature on Crude Protease

The effect of pH (3 to 9) and temperature (0°C, 4 °C, 18 °C, 45 °C and 90 °C) on protease concentration extracted from *Mimusops elengi* against BSA as shown in **Table 2.** The results showed that the protease was found to be active in *Mimusops elengi* at 45ºC but its activity decline at 90ºC.

Estimation of Lymphocytes, Monocytes and Granulocytes Count on Infected Human Whole Blood Containing Proteases Extracted From Mimusops Elengi Using Flow Cytometry

The effect of variable doses of proteases on lymphocytes, monocytes and granulocytes count on infected human whole blood (symptoms such as cough, fever etc) using flow cytometry as shown in **Fig 1**. In *Mimusops elengi*, the results showed that the maximum effect of acid and basic proteases was observed at 1.109 mg/ml (pH 4), 1.159 mg/ml (pH 8) and 1.326 mg/ml (pH 9). Both these proteases (acid and basic) showed enhancement of lymphocytes, monocytes and granulocytes count but slightly decline in monocytes count in case of basic protease (pH 9). Similarly, forward and side scatter showed enhancement after treatment with acid and basic proteases extracted from *Mimusops elengi.* Overall, the results showed that these proteases from *Mimusops elengi* showed significant effect against infected human whole blood and tried to return its normal blood profile.

Estimation of CD3/CD4/CD8 Surface Markers in Mice

The effect of proteases from Mimusops elengi on CD3, CD4 and CD8 surface marker using virally infected human whole blood as specific antigen in mice (**Fig.2**). The results showed that these proteases especially pH 9 returned its normal blood profile after exposure of virally infected lysed human whole blood on day 0 and 7. Similar results were reported in case of pH4 and pH8 (data not shown).

DISCUSSION:

Medicinal plants have become an important source of our daily life requirements despite the progress or achievement in the field of medical and pharmaceutical sciences. There are more than 3000 medicinal plants that are known to have medicinal properties i.e. anti-inflammatory, anti-viral, anti-bacterial, anti-diabetic, immunomodulatory etc in all over the world [11, 12]. Most of the medicinal plants involving various proteases that are present in various plant products in order to develop new drug against various diseases. Most of them need to be tested in more detail, especially in animal models and if successful, in clinical trials. While the physicochemical properties of these protease inhibitors have been broadly scrutinized, their immunobiological effects, e.g. immunostimulatory/ immunosuppressive/ immunoadjuvant effect, remain relatively unexplored [13]. Normally, these medicinal plants especially test candidates (leaves and flowers) are promising source of proteases involved in various immunopathological conditions or diseases such as

cardiovascular diseases (Diabetes) and neurodegenerative disorders (Alzheimer's disease, brain cancer, encephalitis, epilepsy, Parkinson's disease etc).

For the last 10 years, immunopharmacologists focused on those proteases (acidic, basic and neutral) extracted from various medicinal plants including microorganisms against specific protein antigen which are major source and used as a raw material for pharmaceutical industry as potential drug target for number of human diseases. In addition, most of infectious/contagious micro-organisms including viral pathogens necessitate proteases for replication or use proteases as virulence factors or protease target remedies for diverse type of human diseases e.g. AIDS. Finally, proteases are also play a key role in plant proteins (processing, expansion and destruction) and also played an important role in biotechnological industry because of their usefulness as biochemical reagents or in the manufacture of numerous products. Therefore, attempt was made to determine the protein content of *Mimusops elengi* which dissolved in PBS buffers of different pH range (3 to 9) and then measured its protease content against specific protein antigen, BSA.

In order to estimate the total protein content from the leaves of *Mimusops elengi* showed higher variation in response to different pH (ranging from 3 to 9) of the buffer that was used for extraction using Tris HCl and ice cold acetone. The results showed that increase in protein content was observed in *Mimusops elengi* (pH 3 to 9; ranging from 1.906 to 7.209) showed moderate amount of protein as compared to blank. Similarly, in proteases extracted from *Mimusops elengi* against BSA showed maximum activity was obtained at pH *4*, *8* and pH 9 only using buffer for extraction was PBS. In addition, the optimum temperature of proteolytic enzymes of leaves of *Mimusops elengi* was measured by incubating with BSA along with different concentration of proteases stored at different temperatures. The results showed that the protease was still active at 45 °C afterwards the protease activity still declined at higher temperature i.e. 90°C.

After extraction of protease from leaves and flowers of *Mimusops elengi* and examined its immunopharmacological activity in infected human whole blood (common cold, fever, and cough) samples using flow cytometry (FACS Calibur). This instrument is used for examine the properties of the cells (using FSC and SSC) suspended in a stream of fluid and its generally used for determining cell cycle analysis, immunophenotyping (CD3/CD4/CD8/CD14), blood counts, cytokine estimation etc. Recently, this instrument is consistently applied in medical research or immunopharmacological studies (preclinical as well as clinical research) for determining the immunophenotyping as well as the assessment of immune status of healthy animals and human. Thus, it provides indispensable data for evaluating the dominance, orientation and result of the immune response to antigens, as well as for therapy in specific cases and prevention in cases of exposure to pathogens. The identification of cells (lymphocytes, monocytes and granulocytes) in flow cytometer analyzed through light scattered properties (coherent light source, 488 nm, blue); i.e. Forward scattered light (measure shape and size of cells; small angles scattering between 0.5 to 5°C) and side scatter (measure the granularity of cells; large angles scattering between 15 to 150°C). In flow cytometry analysis, measure the live and dead cells population in the form of forward and side scatter respectively [17, 18]. As per the principle of flow cytometry, dead cells have higher side scatter and lower forward scatter and vice versa. In this study, focused on proteases (acidic, basic and neutral) extracted from leaves and flowers of test candidates on infected human whole blood using flow cytometry for measuring blood counts and then analyzing its dead versus live cell population in the form of forward and side scatter respectively. In addition these proteases also examine in animal model studies especially Swiss mice using infected human whole blood as antigen pertaining to examine its surface markers (CD3/CD4/CD8) in mice.

Human positive blood samples of virally infected diseases were collected from Mangal Pathology laboratory, Baramati, Maharashtra, India at different time intervals. These virally infected samples were collected on the basis of hematological studies i.e. lymphocytes, monocytes and granulocytes count which is determined through flow cytometric analysis. For these studies, immunobiologically active compounds extracted from various natural sources have always been of great interest to scientists working on infectious diseases or to improve immune function. To achieve this objective, focused on proteases from medicinal plants especially *Mimusops elengi* for determined its immunopharmacological activity on infected human whole blood samples. In order to validate the potential use of *Mimusops elengi* as a tool in screening for immunopharmacological activity on human whole blood using flow cytometry. This technique is

quick, time consuming and produced a reproducible data in human whole blood after treatment with proteases extracted from *Mimusops elengi* in order to analyzed the number of blood counts i.e.

lymphocytes, monocytes and granulocytes count. The results showed that these proteases showed dose dependent decrease or increased in lymphocytes, monocytes and granulocytes count as already mentioned in results section. Due to the declining or enhancement of lymphocytes, monocytes and granulocytes count are generally due to exposure of these proteases elicits various immunopharmacological activities which was confirmed through flow cytometry. To further confirm the immunopharmacological activity of proteases extracted from test candidates using forward and side scatter. Analysis of these proteases from various medicinal plants especially *Mimusops elengi* on virally infected human whole blood demonstrated that these proteases (acid and basic) showed rapid recovery in case of monocytes and granulocytes count and revealed its expression into normal blood profile. These observations provide further evidence that the safety profile of these proteases from *Mimusops elengi* in animal model studies against virally infected human whole blood.

In an attempt to inject intraperitoneally virally infected lysed (10^9 cells/ml) human whole blood samples on day 0 and day 7 with or without proteases in Swiss mice in order to determine the cell surface markers i.e. CD3/CD4/CD8 through flow cytometer and tried to evaluate and quantify the interactions and activation of these proteases in case of virally infected samples. The *in vivo* experimental studies in mice were further authenticating with *in vitro* experiments using lymphocytes, monocytes and granulocytes count unaccompanied from virally infected individuals. This humanized mouse model based studies may be used to model the human immune system in scenarios of health and its pathological studies and should be further appraisal of therapeutic candidates in an *in vivo* setting applicable to human physiology.

While the immune response to virally infected samples is classified in terms of CD3/CD4/CD8 population in mice. The activation of immunoregulatory T lymphocyte subsets (CD3 count) has been observed in viral infection, being more evident in mice than in human [16]. There are however, as yet no well defined surface markers to determine the viral infection will develop severe complications during the early stage of disease. Number of studies was already performed pertaining to cellular status in mice pertaining the value of these surface markers after the challenging stage of the disease. The results showed that challenging immunization with virally infected blood samples showed increase in CD3 count but after exposure of these proteases (acid and basic), there is rapid decline in CD3 count. In addition, cell mediated immunity which is mediated by T lymphocytes which played important role to fight against viral infections. Among the T lymphocytes, T helper cells induce B lymphocytes to secrete antibodies and cytotoxic T lymphocytes help phagocytes to destroy infections induced by pathogen and to kill intracellular pathogen or microbes [16]. In this study, virally infected samples of human along with protease (acid and basic) in mice and showed rapid decreased in CD4 and CD8 count after challenging dose as compared to virally infected control samples. Since mononuclear cells are over activated during acute viral infection, it is expected that increased in activation of T cell marker such as soluble CD4, CD8 and CD3 surface markers. The data so far suggests that proteases extracted from *Mimusops elengi* showed remarkable decline in surface markers and has the ability to return its normal blood profile after exposure of these proteases in virally infected human blood samples.

CONCLUSION:

The use of proteases as medicine is also popular as the study of proteases has helped us better understands inflammatory conditions and immune regulation. If a protein (part of living cell) component including other necessary things that are needed for the cell to carry out various interactions with other immune cells then proteases will not be able to cleave it because normal living cells also contain an inhibitor mechanism that stops the cleaving process. Generally, protease insufficiency or shortage can cause many health related problems e.g. acidity create in the stomach through digestion of protein material; if there are not enough proteases then this acidic equilibrium is disconnected causing an enlargement in alkaline disposition in the blood which could lead to insomnia or perturbation. There are number of proteases that are available or still under investigation or in clinical trials. Recently, there has been considerable attention to those proteases derived from various micro-organisms including medicinal plant products that has been given to screening for possible anti-viral, anti-diabetic activity etc. In addition, researchers focused on number of medicinal plant products especially leaves and flowers that have formed the basis of traditional medicine systems and continued to play an essential role or showed its necessity for human health care.

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Table 1: Estimation of protein content using PBS of different concentrations pH 3 to 9 extracted from *Mimusops elengi*

Name of the plant	Protein(NanoDrop) (mg/ml)							Proteases (UV spectrophotometer) (mg/ml)						
pH	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Mimusops elengi	1.906	5.199	5.709	4.213	6.16	7.209	5.952	-	0.422	0.152	-	-	0.598	0.423

For protein estimation, leaves powder macerated in extraction buffer using PBS of different pH concentration ranging from 3 to 9. Afterwards, incubate, centrifuged and then collect the supernatant and add similar proportion of ice cold acetone. Incubate these samples at room temperature and then centrifugation. Finally, collect the pellet and washed with ice cold acetone to expel the pigments as well as lipids. Finally, the protein concentration was resolved through Nano drop method.

Protease activity was determined by calorimetric assay using BSA as substrate. All these details are mentioned in materials and methods section.

Table 2: Effect of pH and temperature on crude protease extracted from *Mimusops elengi*

Acid protease (pH 3- 6)

Plant material	Temperature (Mean ± S.E)				
	0ºC	**4ºC**	**18 ºC**	**45 ºC**	**90 ºC**
Mimusops elengi	0.512 ± 0.008	0.536 ± 0.06	0.524 ± 0.12	0.529 ± 0.10	0.041 ± 0.008

Neutral protease (pH 7-7.2)

Plant material	Temperature (Mean ± S.E)				
	0ºC	**4ºC**	**18 ºC**	**45 ºC**	**90 ºC**
Mimusops elengi	0.368 ± 0.12	0.312 ± 0.08	0.314 ± 0.10	0.298 ± 0.08	0.072 ± 0.010

Basic protease (pH 7-7.2)

Plant material	Temperature (Mean ± S.E)				
	0ºC	**4ºC**	**18 ºC**	**45 ºC**	**90 ºC**
Mimusops elengi	0.488 ± 0.08	0.502 ± 0.02	0.488 ± 0.07	0.312 ± 0.02	0.096 ± 0.013

pH

Plant material	pH (Mean ± S.E)				
	pH 3	**pH 4-5**	**pH 6**	**pH 7-8**	**pH 9**
Mimusops elengi	0.512 ± 0.13	0.544 ± 0.14	0.432 ± 0.11	0.448 ± 0.08	0.397 ± 0.02

For these analytical studies, different temperatures and pH (3 to 9) values of buffer were formulated. Sequentially dilution of non-identical series of crude enzyme extract of test candidates was mixed with homogenous bulk of BSA dispersed in PBS buffer of different pH values. BSA used as standard for these studies. After incubation (1 h) at room temperature and optical density (OD) was measured after with UV-visible spectrophotometer at 595 nm

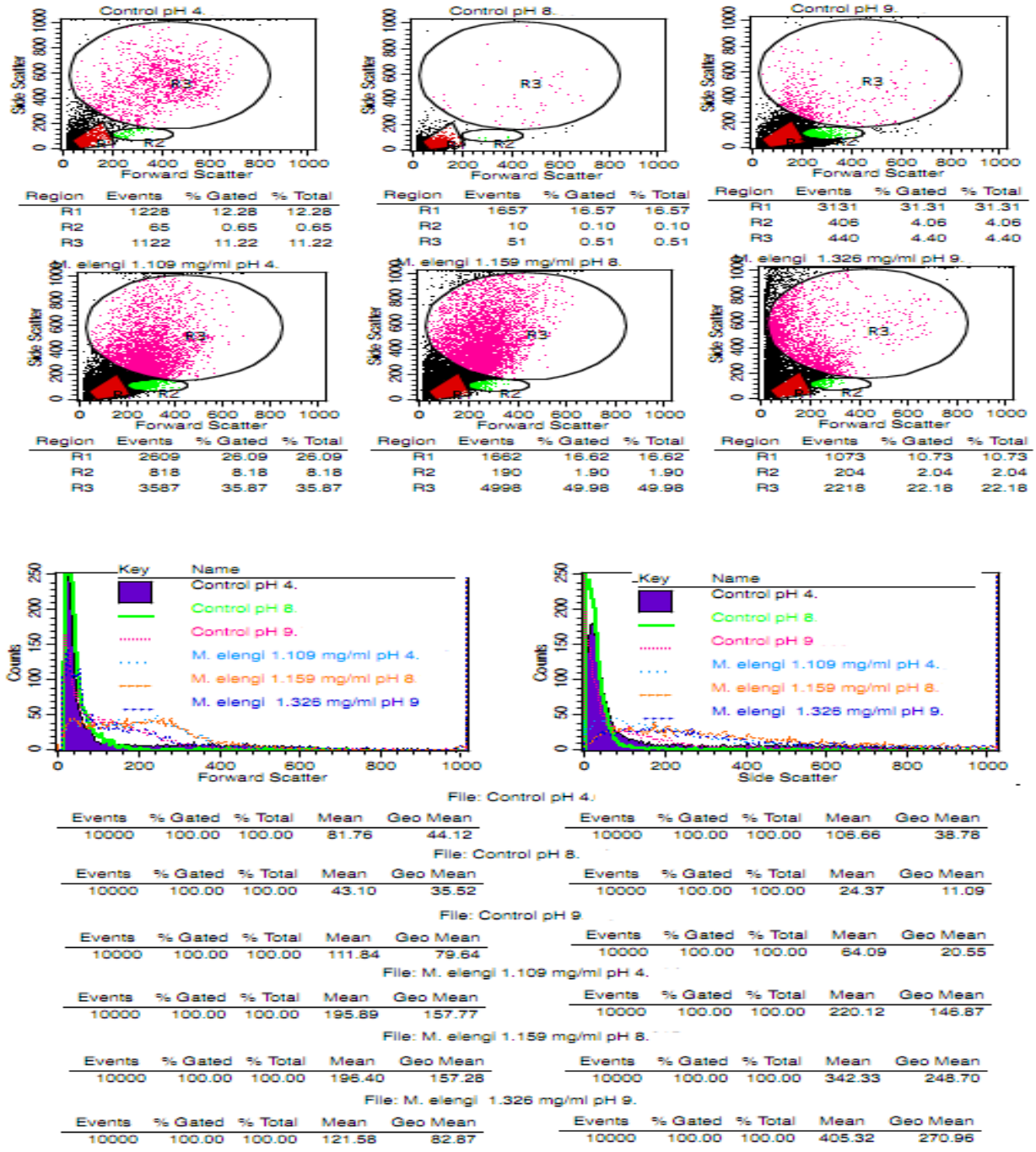

Fig 1: Effect of acid (pH4), and basic (pH8, 9) protease extracted from the leaves of *M. elengi* on lymphocytes, monocytes and granulocytes count in infected human whole blood.

Lysed human whole blood were cultured with protease of particular pH value and then observed its blood count using flow cytometer (FACS Calibur).

a) First graph represents blood counts i.e. R1- Lymphocytes; R2- Monocytes and R3- Granulocytes.

b) Second graph represents total blood counts.

FSC- represents Forward scatter and SSC- Side scatter

Events- 10,000; Software- Cell Quest; Flow Cytometer- FACS Calibur; Company- BD India

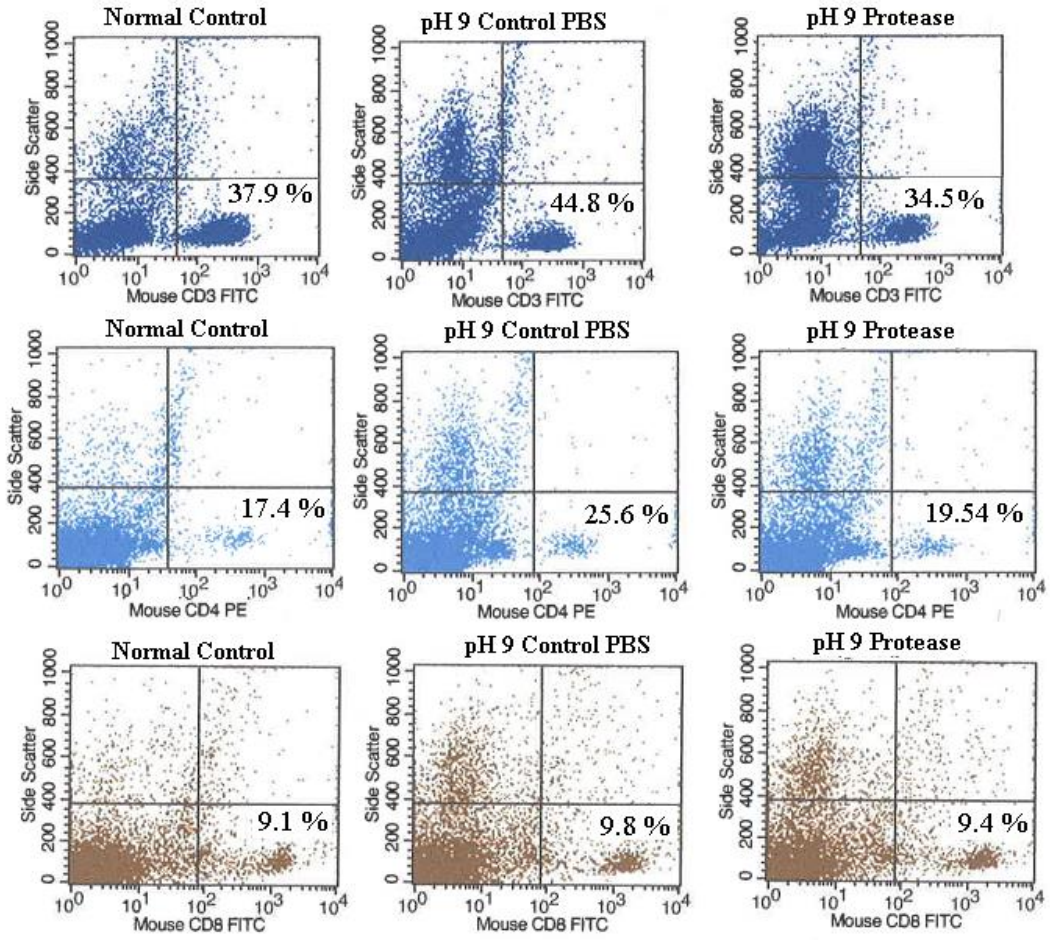

Fig 2: Effect of basic protease (pH 9) extracted from the leaves of Mimusops elengi on CD3, CD4, CD8 count in Swiss mice using virally infected lysed human whole blood as specific antigen .

Lysed human whole blood (10^9 cells) were immunized intraperitoneally with or without protease on day 0 and 7. On day 10, blood was collected from the retro-orbital plexus of mice for the estimation of surface markers i.e. CD3, CD4 and CD8 surface marker and then observed through flow cytometer (FACS Calibur). In this experiment, 100 µl of whole blood was taken in each tube. Mouse monoclonal antibodies i.e. FITC labeled CD8 (CTL response), CD3 (total T cell count) and PE labeled CD4 (helper T cells) were added directly to whole blood (50 µl). Falcon tubes containing treated (test candidates) blood samples of mice were incubated in dark for 30 -45 min at room temperature. Subsequently, addition of FACS lysis solution (1x) was added at room temperature with gentle mixing followed by incubation for 10 min. The samples were spinned (2500 rpm, 10 min at 4 °C) and the supernatant was aspirated and washed two times with phosphate buffered saline. After centrifugation, pellet dispersed in PBS and scrutinizes the cells through flow cytometer

Events- 10,000

Software- Cell Quest

Flow Cytometer- FACS Calibur; Company- BD India