ISSN: 2279 - 0594

Journal of Biomedical and Pharmaceutical Research

Available Online at www.jbpr.in

CODEN: - JBPRAU (Source: - American Chemical Society)

Index Copernicus Value: 63.24

PubMed (National Library of Medicine): ID: (101671502)

Volume 6, Issue 2: March-April: 2017, 128-135

Research Article

# ANALYSIS OF PRIMARY METABOLITES FROM *VERTICILLIUMLECANII* AND STUDIED ITS IMMUNOLOGICAL ASPECTS OF CRUDE PROTEASE AGAINST RAPITHER-AB

**Pradnya P Raskar[1], Amit Gupta,[1, 2]*Nilima Pendharkar,[1] Bharat Shinde[1, 2]**

[1]Department of Microbiology, Vidya Pratishthan's, Arts, Science and Commerce College, Baramati, Maharashtra, India

[2]Department of Immunology and Virology, Vidya Pratishthan's, School of Biotechnology, Arts, Science and Commerce College, Baramati, Maharashtra, India

Received09March 2017; Accepted 21April 2017

**ABSTRACT**

**Objective-** The objective of our study is to examine its primary metabolites (protein content) from dried powder of *Verticilliumlecanii*. In addition, immunological studies were conducted in order to analyzing the protease content against Rapither-AB using crude enzyme of *Verticilliumlecanii* in virally infected human whole blood samples.

**Methods-** For these studies, quantifying the protein content from *Verticilliumlecanii* using Nanodrop method. In contrast, crude protease isolated from crude enzyme against Rapither-AB in order to analyze its immunological activity against infected blood samples for estimating its proliferation and total cellular content. In addition, Elisa experiment were performed using protein of *Verticilliumlecanii* as coating antigen and measure its antibody profile of various medicinal plants against this protein antigen.

**Results-**The results of these studies showed production of protein at a very low concentration of dried powder, *Verticilliumlecanii*and also showed the presence of protease content against crude enzyme of *Verticilliumlecanii* containing Rapither AB and claimed its antimicrobial properties at higher doses with respect to decline in proliferation assay and total cellular content in case of virally infected blood samples. In contrast, few of these medicinal plants are able to produce antibody production against this fungal strain i.e. *Verticilliumlecanii*.

**Conclusion**- Overall these studies indicate its antimicrobial properties of protease extracted from crude enzyme of *Verticilliumlecanii* against Rapither AB containing arteether.

**Keywords**: *Verticilliumlecanii*; protein; protease; Rapither AB; arteether

## INTRODUCTION:

Most of synthetic chemicals that are available in the market which are expensive and destructive to the environment and also showed toxic effect on humans, animals and other non-target organisms [1, 2]. In addition, these chemicals also showed some harmful effect to other beneficial organisms as well. Some of the insecticides act like as carcinogenic agents and are even carried through food chain which in turn affects the non-target organism. Therefore alternative vector control strategies for controlling these harmful effects especially effective and low cost are extremely imperative [3, 4].So, these microorganisms are kept under consideration and number of biocontrol agents against insects are available. Recently, researchers more emphasis on *Verticilliumlecanii* because of its qualities like shelf life, mode of action, non-toxic effects to environment, high host specificity and non-persistence [5, 6].

Most of the predators, parasitoids including fungi and other beneficial organisms that are used for the biocontrol of insect pests. This fungus i.e.*Verticilliumlecanii* is one of the member of*Deuteromycetes*species that can be used for crop protection. In addition, genus *Verticillium* encompasses a cosmopolitan group of ascomycete fungi and is considered to be nonpathogenic

*Corresponding author: Dr Amit Gupta| E-mail:amitvsbt@gmail.com; amitgupta@vsbt.res.in

inhumans [7, 8]. *Verticillium* produces one of the antifungal compound i.e.*vergosin a*nd showed antitumor properties and also used in the form of antibiotic, as well as a wide variety of additional compounds used by various industries.As per the literature, one of the most potent and useful aspect of biological control organism that is present within the soilinhabiting fungus *Verticilliumlecanii*. This fungus (firstly described in year 1861) also called as *Lecanicilliumlecanii* (approved name, entomopathogenic fungus species) and is widely reported as well as distributed all over the world [7-11]. As per the literature, this fungus is totally antagonistic to insects (Phylum *Arthropoda*) and other fungi. In addition, this fungus is available commercially for controlling insect control which is responsible for causing various infectious diseases. Lot of research work is still going on related to this fungi and considered as biocontrol agent for *heterodera glycines*[12]. In contrast, mycelium of this fungus produces a toxin called ascyclodepsipeptide (bassianolide) and also produces other insecticidal toxins i.e. dipicolinic acid which generally infect aphids, whiteflies, rust fungi, scale insects and lead to death the host [13, 14]. In other words, the effectiveness of this bio-control agent i.e. *Verticilliumlecanii*has been proved also in soil in order to eliminate the burden of various infectious agent.

Arteether, fast acting blood schizonticide specifically indicated for the treatment of chloroquine-resistant *Plasmodium falciparum* malaria and cerebral malaria cases [15]. It is a semi-synthetic derivative of artemisinin, natural product of the Chinese plant *Artemisia annua*. It is currently only used as a second line drug in severe cases of malaria [16].In this regard, we worked on protein extracted from fungi and then isolated its protease content of crude enzyme against Rapither AB in order to determined its immunological activity in virally infected human whole blood samples.

## MATERIALS AND METHODS

### Collection and identification of *V. lecanii*

Pure culture of fungal species i.e.*Verticilliumlecanii*(**Fig.1**) was collected from KrishiVigyan Kendra, Sharda Nagar, Baramati, Maharashtra, India. These samples were loaded or inoculated in potato dextrose agar(PDA; potato 200g, dextrose 20g, agar 15 g, distilled water 1 litre; pH 5.7) mediumby multiple tube dilution technique. All the plates were incubated at 26°Cfor 4 days. The fungal colonies which werepicked up and purified by streaking andincubated at 26°C for 7-8 days. Green conidiaforming fungal bodies were selected andmicroscopic observation was identified to be*Verticilliumlecanii*. The culture was maintained on PDA slants.

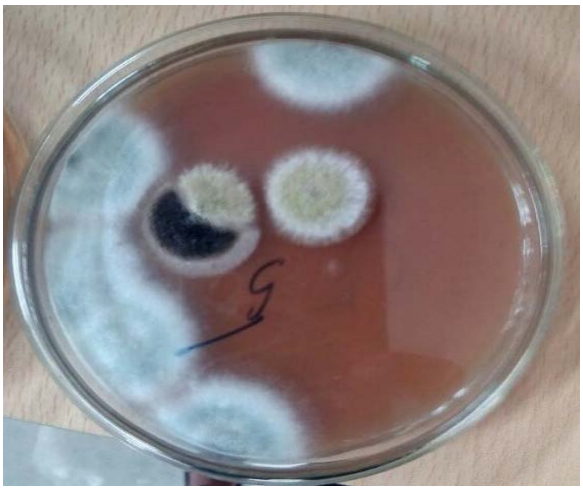

**Figure 1: *Verticilliumlecanii***

### Extraction of protein

In this study, *Verticilliumlecanii*powder (2 g) were added in extraction buffer (i.e. 20 mM Tris HCl) which is dissolved in PBS, pH 7.4. Incubate fungal powder along with extraction buffer for 5-10 minutes at room temperature. Centrifuging at 6000 rpm; 10 minutes at 4°C and then supernatant was collected and then add similar volume of ice cold acetone. Incubate (10-15 min) the solution at room temperature and then centrifuged. Collect the pellet and washed with ice cold acetone to remove the pigments including lipids as well. Finally, protein concentration of *Verticilliumlecanii* was determined by using Nano drop method [17].

### Crude Protease production and enzyme assay

After getting the protein from dried powder of*Verticilliumlecanii*that are dissolved in PBS buffer. For crude enzyme collection, centrifuging the protein content (10000 rpm at 4$^{o}$C for 30 minutes) of *Verticilliumlecanii* and then estimate its protease content against Rapither-ABusing NanoDrop method.

Calorimetric assay was performed for protease estimation using Rapither-AB as substrate. For these studies, add equal volume of Rapither AB and crude enzyme extract of *Verticilliumlecanii* in 15 ml tube. Incubate it for another 2-3 h at room temperature. After incubation, add trichloroacetic acid (TCA) solution in order to cease its enzymatic reaction. Centrifuging (6000 rpm, 10 min) the samples and supernatant was collected and then add similar volume of sodium hydroxide (NaOH) solution in comparison with trichloroacetic acid (TCA) solution. Incubate it another 20 minutes at room temperature. Thereafter, addition of FolinsColins reagent (500 µl) and the intensity of blue colour was measured at 700 nm within half an hour using spectrophotometer [17, 18].

**Antimicrobial assay**

In this study, lysed virally infected human whole blood (100 µl) were cultured with variable doses of proteases (18.94 – 75.78 mg/ml, 50 µl) extracted from *Verticilliumlecanii* containing Rapither AB. Incubate the 96-well plates for 24 h at 37 °C. Trypsin (0.5 mg/ml; 50 µl) used as standard for these immunopharmacological studies. Centrifuging (4500 rpm for 8 minutesat 4 °C) the plates and then add fresh complete medium was added into the 96-well plates. Again, incubating 96 well plates for another 4 h along with MTT (5 mg/ml, 10 µl) continued. After incubation, the plateswere suddenlycentrifuged with discarding the supernatant, collecting the pellet and finally dispersing in dimethyl sulphoxide (DMSO) solution. The optical density was measured at 570 nm [17-19].

**Estimation of total cellular content**

Lysed virally infected human whole blood (n =6; $10^5$ cells/well; 100 µl) were collected and cultured in 96 well flat bottom tissue culture plate for 48 h incubation along with variable doses of proteases(18.94 – 75.78 mg/ml, 50 µl). Collect and transfer the samples from culture plate into 3 ml falcon tube. Centrifuge the samples at 6000 rpm at 4ºC and then washed with PBS pertaining to observe the total cellular content. Finally samples were analyzed through UV visible spectrophotometer at 570 nm [17-19].

**ELISA**

Indirect Elisa was performed in 96 well plate, himedia for estimating antibody production against protein (*Verticilliumlecanii*, 100 µg/well) using various medicinal plants (10 mg/ml; 50 µl). In this study, protein of *Verticilliumlecanii* used as coating antigen and incubate its plate for overnight at 4ºC. After incubation, first of all block this plate with 1% bovine serum albumin (BSA). Incubate the plate for one hour at room temperature and then wash the plate with PBS (2-3 times). Thereafter, add fixed concentration of various medicinal plants i.e. *Strychnosnuxvomica, Ficus religiosa* and *Azadirachta indica*(10 mg/ml; in 96 well plate). Incubate the plate for another 4h incubation at carbon dioxide incubator. Afterwards, again wash the plate with PBS (2-3 times) and then add secondary antibody (horse antiserum; 1:10000 dilution). Incubate the plate for another 1h at carbon dioxide incubator. After incubation, wash the plate with PBS and then add substrate, TMB. Incubate the plate for another 10-15 minutes in dark at room temperature. Afterwards, stop solution was added and optical density was measured at 450 nm [19, 20].

**Statistical analysis**

The difference between control and treated group of proteases extracted from crude enzyme of *Verticilliumlecanii*containing Rapither AB is determined by one way ANOVA test (Bonferroni multiple comparison test). *P <0.05; *P<0.01; ***P<0.001

**RESULTS**

**Estimation of protein content**

The results of *Verticilliumlecanii* related to protein content as shown in **Fig.2**. The results showed the presence of protein content at a very low concentration (4.907 mg/ml; 10 µl) which is determined through Nanodrop method**.**

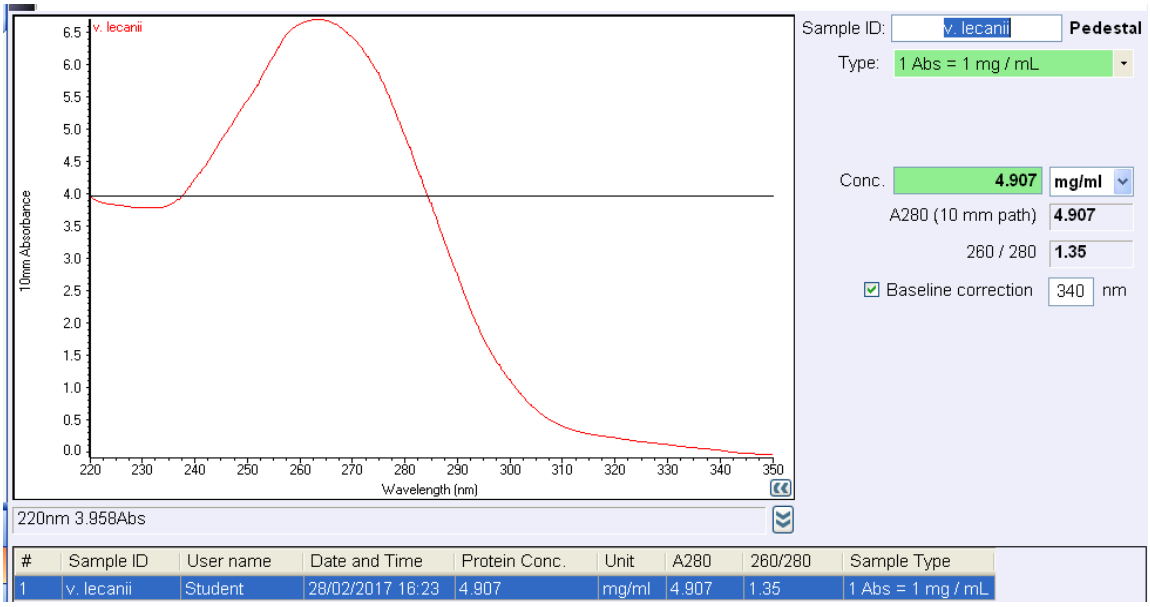


**Figure 2: Estimation of protein content in *Verticilliumlecanii.***

## Estimation of protease content

In an effort to determine the effect of protease content extracted from crude enzyme of *Verticilliumlecanii*containing Rapither AB as shown in **Fig.3**. The results showed the presence of protease content (i.e. 75.78 mg/ml; 10 µl) which is confirmed through Folincolins reagent and further studies will be taken into further consideration for determining its immunological properties.

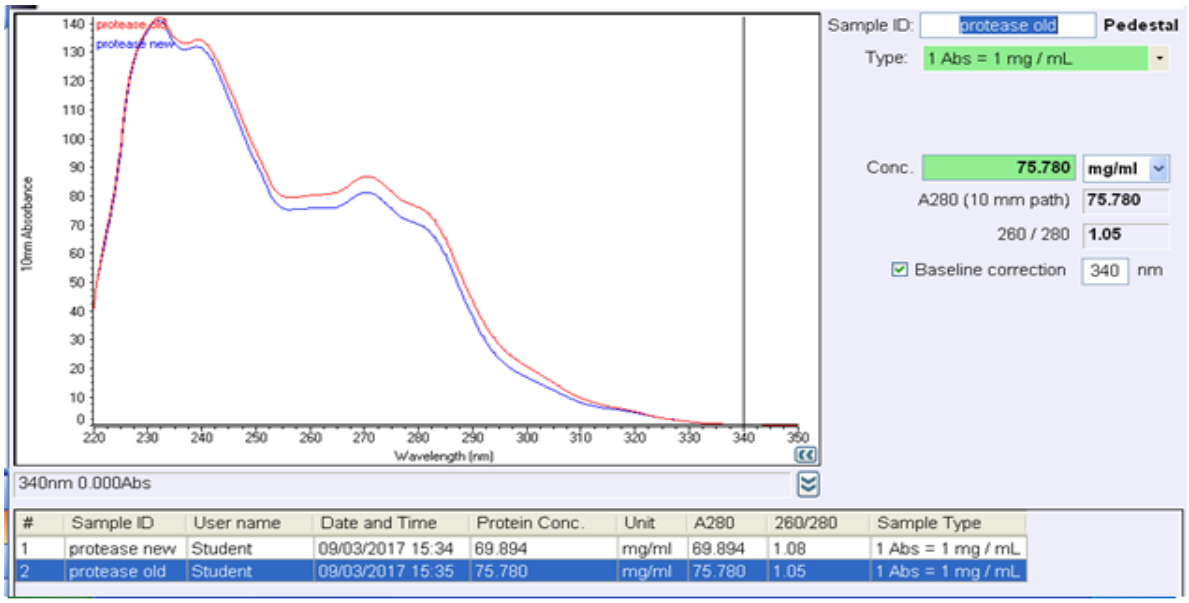


**Figure 3: Estimation of protease content in crude enzyme of *Verticilliumlecanii*containing Rapither AB containing arteether.**

## Antimicrobial activity

The effect of variable doses of protease content in virally infected whole blood samples as shown in **Fig.4**. The results showed that protease at higher concentration (i.e. 75.58 mg/ml; 50 µl) inhibit proliferation rate in virally infected human whole blood samples. In short, protease from*Verticilliumlecanii*containing Rapither AB showed antimicrobial activity.

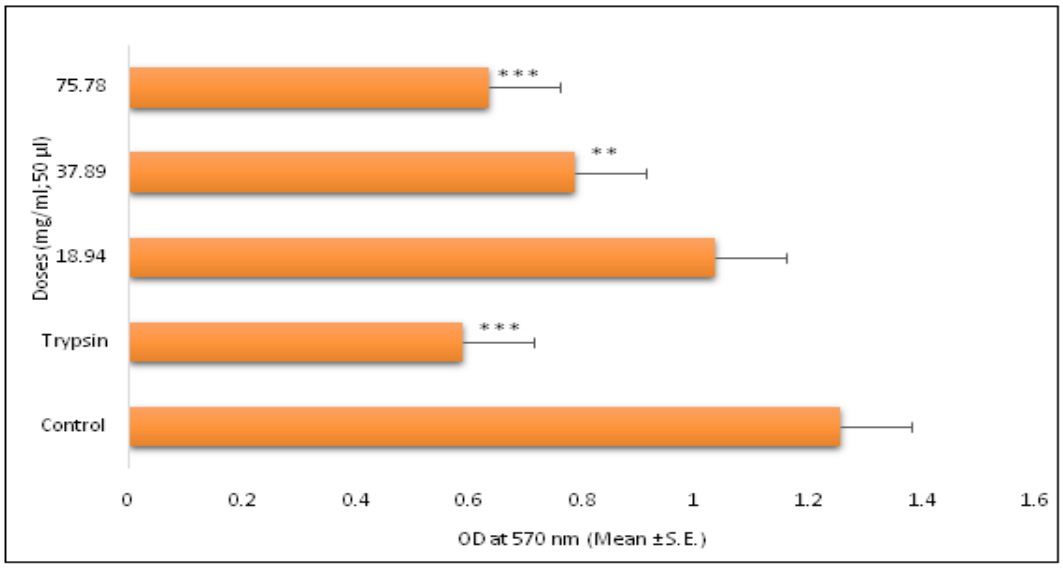


**Figure 4: Antimicrobial activity**

To determine the effect of variable doses of proteases (18.94 – 75.78 mg/ml, 50 µl) extracted from crude enzyme of *Verticilliumlecanii*containing Rapither AB on virally infected lysed human whole blood. Values are expressed as Mean ± S.E. The difference between the controls versus variable doses of protease is determined by one way ANOVA test (Bonferroni multiple comparison test).).*P<0.05; **P<0.01, ***P<0.001

**Total cellular content**

In order to measure total cellular content in virally infected human whole blood samples as shown in **Fig.5**. The results showed that protease at high concentration declined in total cellular content as compared to control.

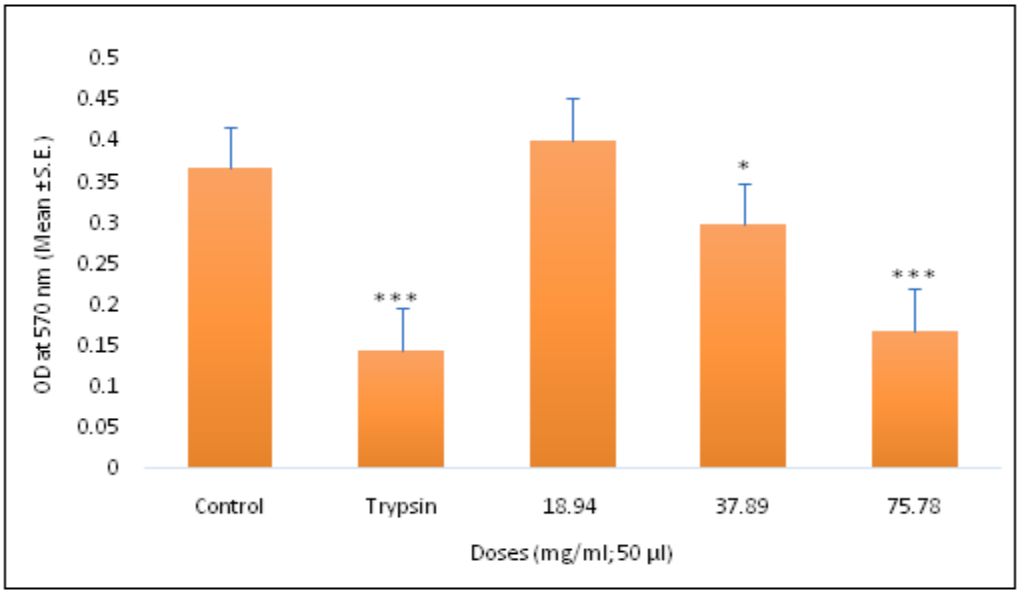


**Figure 5: Estimation of total cellular content.**

To determine the effect of variable doses of proteases (18.94 – 75.78 mg/ml, 50 µl) extracted from crude enzyme of *Verticilliumlecanii*containing Rapither AB on total cellular content in virally infected lysed human whole blood. Values are expressed as Mean ± S.E. The difference between the controls versus variable doses of proteases is determined by one way ANOVA test (Bonferroni multiple comparison test).).*P<0.05; **P<0.01, ***P<0.001

**Elisa**

Indirect Elisa was performed using various medicinal plants as shown in **Fig.6**. Out of these, only *Azadirachta indica* will be able to produce antibody production against protein extracted from *Verticilliumlecanii.*

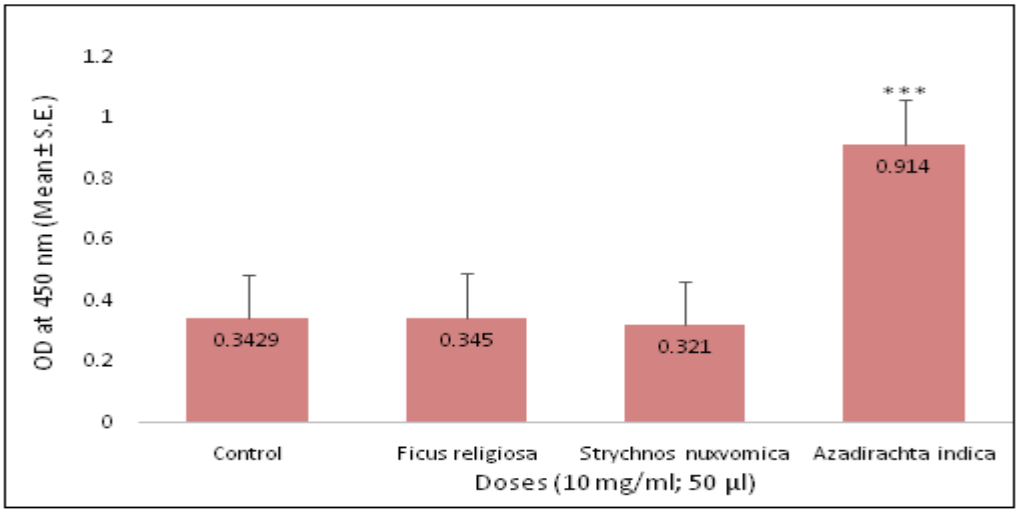


**Figure 6: ELISA assay.**

To determine antibody production against protein antigen of *Verticilliumlecanii*using various medicinal plants i.e. *Ficus religiosa, Strychnosnuxvomica*and*Azadirachta indica*. Horse anti-serum used as secondary antibody and the optical density was measured at 450 nm. Values are expressed as Mean ± S.E. The difference between the controls versus variable doses of medicinal plants against protein antigen of *Verticilliumlecanii*is determined by one way ANOVA test (Bonferroni multiple comparison test).).*P<0.05; **P<0.01, ***P<0.001

## DISCUSSION

As per the literature related to various contagious diseases especially Malaria that is transmitted to humans by the bite of infected female mosquitoes. More than 30 species of anopheles species have been identified.Globally in year 2012, 207 million malaria cases are identified, out of these 600,000 deaths are reported. According to WHO, more than 75 % cases of Malaria are identified as well as reported in South-East Asia [21]. In view of this, researchers start focusing on biopesticides especially entomopathogenic fungi i.e. *Beauveriabassiana* and *Metarhiziumanisopliae* that have been used in order to eliminate this infectious disease in all over the world [22]. These fungi including some isolates are identified and showed only few of them have some capability to reduce the burden of malaria. As per the literature, various isolates of entomopathogenic fungi were screened and showed potential biological control agents of *Ae. aegypti, An. Stephensi* etc. [23]In this regard, we focused on species of*Verticillium*and determined its protein and also estimate the protease content against Rapither AB. Recently, One of the ethyl ether derivatives of artemisinin i.e. Arteether (alpha, beta), schizontocidal drug isgenerally used for the treatment of *falciparum malaria* and is readily available in the market in the form of Rapither AB [6]. In the present study, we discuss about crude enzyme of *Verticilliumlecanii*and tested against Rapither AB containing arteether in order to estimate its protease content and then analyzing its antimicrobial activity in virally infected human whole blood samples.

In an effort to reduce the burden of infectious disease i.e. malaria, fungi protein and isolated its protease content against Rapither AB containing arteetherand demonstrated that these proteases that played an important role in various infectious diseases including malaria.Till now, there is no correlation between proteases and malaria because of upregulation of protease activity represents a cause or consequence of immune disorders. In view of this, we focused on those proteases extracted from *Verticilliumlecanii*against

Rapither AB containing arteetherusing PBS buffer, pH 7.4 on virally infected human whole blood samples and determined its immunological activity pertaining to lymphocyte proliferation assay and total cellular content which provides novel therapeutic approach for treating or controlling infectious diseases especially malaria. In this study, proteases are optimally active in the neutral environment and some of them may not be retained with in the environment and mediate their potent antimicrobial activity.

The results of these studies showed that these proteases showed decline in proliferation rate in virally infected lysed human whole blood samples as compared to control. In other words, lymphocytes activation in case of virally infected blood samples is generally due to the expression of co-stimulatory molecules that are present on the cell membrane and also through the action of several cytokines. So, cytokines depending upon lymphocyte proliferation that play a crucial role in controlling the expansion of T cells during immune response to pathogenic antigens [24, 25]. Similarly, these proteases showed decline in total cellular content in viral infected samples as compared to control. In addition, medicinal plants especially Azadirachta indica are able to produce antibody production against protein antigen of *Verticilliumlecanii.* In short, overall these studies claimed that these proteases from *Verticilliumlecanii* against Rapither AB containing arteether showed antimicrobial activity.

## CONCLUSION

Immunological finding of these proteases from *Verticilliumlecanii* against Rapither AB containing arteetherwas very interesting with respect to decline in proliferation rate, total cellular content and haemolytic activity which is observed in virally infected human whole blood samples. The percentage of cell population in case of viral infected blood counts return to normal baselinemuch faster. In conclusion, protease from*Verticilliumlecanii* against Rapither AB containing arteether demonstrated potent protease inhibitory activity. Further studies are required pertaining to isolation, characterization and elucidation of structures of these proteases.

## AUTHORS CONTRIBUTION

This work was carried out in collaboration between three authors. Dr Amit Gupta and Miss Pradnya P Raskar designed the study, wrote the protocol and interpreted the data where P anchored the field study, gathered the initial data related to her M.Sc. Microbiology dissertation work under Dr Amit Gupta guidance and performed preliminary data analysis. Dr Amit Gupta, Miss Pradnya P Raskar, Mrs Nilima Pendharkar and Dr Bharat Shinde managed the literature searches whereas Dr Amit Gupta along with Miss Pradnya P Raskar produced the initial draft. The final manuscript has been read and approved by all authors.

## ACKNOWLEDGEMENT

All the authors are highly thankful to our organization i.e. Vidya Pratishthan, Arts, Science and Commerce College, Baramati, District Pune, Maharashtra. In addition, we are highly thankful to Mangal pathology lab, Baramati for providing virally infected human blood samples for our studies.

## REFERENCES

1. Abdel-Mallek AY, Moharram AM, Abdel-Kader MI, Omar SA. Effect of soil treatment with the organophosphorus insecticide profenofos on the fungal flora and some microbial activities. Microbiological Research 1994;149:167-171.
2. Andrea MM, Peres TB, Luchini LC, Pettinelli Junior A. Impact of long-term pesticide application on some soil biological parameters. Journal of Environmental Science and Health B 2000;35:297-307.
3. KilicA,Akay MT. A three generation study with genetically modified Bt corn in rats: Biochemical and histopathological investigation. Food and Chemical Toxicology 2008; 46 (3): 1164–1170.
4. Molyneux DH. Common themes in changing vector-borne disease scenarios, Transactions of the Royal Society of Tropical Medicine and Parasitology 2003; 97: 129–132.
5. Jin YL, Seong WK, Cheol SY, Jeong JK, Dong RC, Seung WK. *Verticilliumlecanii* Spore Formulation Using UV Protectant and Wetting Agent and the Biocontrol of Cotton Aphids. Biotechnology Letters 2006; 28 (13): 1041-1045.

6. Jeong JK, Goettel MS, Gillespie DR. Potential of Lecanicillium species for dual microbial control of aphids and the cucumber powdery mildew fungus, *Sphaerothecafuliginea* . Biological Control 2007; 40 (3): 327–332.
7. Askary H, Benhamou N, Brodeur J. Ultrastructural and Cytochemical Investigations of the Antagonistic Effect of *Verticilliumlecanii* on Cucumber Powdery Mildew. Phytopathology 1997; 87:359-368.
8. Askary H, Carriere Y, Belanger RR, Brodeur J (1998) Pathogenicity of the Fungus *Verticilliumlecanii* to Aphids and Powdery Mildew. Biocontrol Science and Technology 8: 23- 32.
9. Barson G. Laboratory studies on the fungus *Verticilliumlecanii*, a larval pathogen of the large elm bark beetle (Scolytusscolytus). Annals of Applied Biology 1976; 83: 207-214.
10. Beyer PU, Hirte WF, Sermann H. The behaviour of the entomopathogenic fungus *Verticillium*lecanii (Zimm.)Viegas in soil; I. Viability in soil at different ecological conditions. ZeitschriftfürPflanzenkrankheiten und Pflanzenschutz 1997; 104 (1): 54-64.
11. Chandler D, Heale JB, Gillespie AT. Competitive interaction between strains of *Verticilliumlecanii* on two insect hosts. Annals of Applied Biology 1993; 122: 435-440.
12. Niblack TL, Lambert KN, Tylka GL. A model plant pathogen from the kingdom animalia: Heterodera glycines, the soybean cyst nematode. Annu Rev Phytopathol 2006; 44:283–303.
13. Sreenivasa MY, González-Jaén MT, Dass RS, Rharith Raj AP, Janardhana GR. A PCR-based assay for the detection and differentiation of potential fumonisin- producing Fusarium verticillioides isolated from Indian maize Kernels. Food Biotechnol 2008; 22:160–170.
14. Carrion G, Romero A, Rico Vary V. Use of *Verticilliumlecanii* as a biocontrol agent against bean rust (*Uromycesappendiculatus*). Fitopathologia 1999; 34:214–219.
15. Van Vugt M, Brockman A, Gemperli B, Luxemburger C, Gathmann I, Royce C, Slight T, Looareesuwan S, White N J, Nosten F. A randomised comparison of artemether-benflumetol and artesunate-mefloquine in the treatment of multidrug-resistant falciparum malaria. Antimicrob Agents Chemother 1998; 42:135–139.
16. Douglas NM, Anstey NM, Angus BJ, Nosten F, Price RN. Artemisinin combination therapy for vivax malaria. Lancet Infect Dis2010; 10 (6): 405–416.
17. Gupta A, Shah AP,Chaphalkar SR. Immunopharmacological exploration of proteases from *Catharanthus roseus* on virally infected human whole blood. Journal of Disease and Global Health 2016; 6 (1): 36 – 42.
18. Gupta A, Chaphalkar SR. Analytical studies of protease extracted from *Azadirachta indica*. World Journal of Pharmaceutical research 2015; 4 (11):1391 – 1398.
19. Mane V, Gupta A, Pendharkar N, Shinde B. Exploration of primary metabolites from *Lemna minor* and determined its immunomodulatory and anti-inflammatory activity. European Journal of Pharmaceutical and Medical research 2017; 4(4): 384-388.
20. Gupta A, Chaphalkar SR. Immunoadjuvant potential of *Azadirachta indica* against rabies, hepatitis and DPT vaccine antigen.International Journal of Medical and pharmaceutical sciences 2015; 5(7): 1 – 5.
21. Gupta A, Chaphalkar SR. Current approaches and problems in malaria vaccine development. European Journal of Biological research 2016; 6(1): 14 – 20.
22. Erler F, Ates AO.Potential of two entomopathogenic fungi, *Beauveriabassiana* and *Metarhiziumanis opliae* (Coleoptera: *Scarabaeidae*), as biological control agents against the June beetle. J Insect Sci. 2015; 15(1): 44.
23. Huang YJS, Higgs S, Vanlandingham DL. Biological Control Strategies for Mosquito Vectors of Arboviruses. Insects 2017; 8, 21-25.
24. *Trinchieri G, Kubin M, Bellone G, Cassatella MA. Cytokine cross-talk between phagocytic cells and lymphocytes: relevance for differentiation/activation of phagocytic cells and regulation of adaptive immunity. J Cell Biochem. 1993; 53: 301–308.*
25. *Ramanathan S, Gagnon J, Ilangumaran S. Antigen-nonspecific activation of CD8+ T lymphocytes by cytokines: relevance to immunity, autoimmunity, and cancer. Arch ImmunolTherExp (Warsz). 2008; 56:311–23.*