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Tissue Inhibitors Of Metalloproteinases (TIMPs) From Aspergillus Flavus- A New Dimention For The Treatment Of Rheumatoid Arthritis

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Abstract:
Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that principally attacks synovial joints. In the present investigation, we have focused on the isolation of TIMPs from soil fungus. Several TIMP producing fungi were isolated from soil using glycerol agar plates and were confirmed using casein agar media. The optimization of TIMP was carried out by varying the parameters like pH, temperature and the concentration of metal ions. The effect of TIMP on thermolysin (MMP-2) was assayed by carrying out the anti-inflammatory activity using standard procedures. The results showed that crude sample from five different organisms inhibited the MMP-2 activity. Among these strains, the best results were seen in Aspergillus flavus sample. The antioxidant activity was also tested by using the DPPH method. The crude sample was purified using ammonium sulphate precipitation method. Gelatin zymography was conducted to confirm the activity of TIMP containing sample on thermolysin and Aspergillus flavus has confirmed the production of TIMP.

Key words*: Aspergillus flavus, TIMP, thermolysin, anti-inflammatory, gelatin zymography.*

1.Introduction

About 0.3%-1% of the world's population is afflicted by rheumatoid arthritis (Silman et al. 1993), women being three times more common than men (WHO, 2000). Various treatments are available, that include non-pharmacological treatments, like physical therapy, occupational therapy and nutritional therapy which do not really stop the progression of joint destruction. Analgesia (painkillers) and anti-inflammatory drugs, including steroids, are used to suppress the symptoms, while disease-modifying antirheumatic drugs (DMARDs) are required to inhibit or halt the underlying immune process and prevent long-term damage.

2.Materials And Methods:

2.1.Isolation Of Microorganisms

Isolation of microorganisms from soil was carried out using the method explained by Murao et al. (1982). Samples of soil were collected from different places and were incubated at 30°C until distinct colonies were developed. The lyophilized form of Matrix Metalloproteinase (MMP-2) of Bacillus thermoproteolyticus (thermolysin) was obtained from Sigma Aldrich, Bangalore. This enzyme was dissolved in 0.1 m Tris-HCl buffer, pH 7.5, containing 20mM calcium acetate. The microorganisms thus isolated were cultured on a medium containing the thermolysin solution in order to assure the growth of only those microorganisms that could produce inhibitors for this MMP-2. Each colony was now cut cylindrically and placed carefully on a plain agar layer. A casein agar solution containing thermolysin was then added to it, and incubated for 15h at 30°C. After treatment with 0.44 m tricloroacetic acid, the white zone colony around the cylindrical agar was transferred into a slant of medium B. The isolated microorganisms were then cultured at 30°C for 2~3 days on broth form medium B. The supernatant of the culture was used for further assays.

2.2.TIMP Assay

The activity of TIMPs present in the supernatant was assayed using the ninhydrin test. Here we use gelatin as the substrate for thermolysin. Thermolysin degrades gelatin to release free amino acids. The amino acid liberated is allowed to react with ninhydrin reagent and incubated in boiling water bath for 15mins. The purple colour developed in the reaction tube was measured at 570nm. Similar steps were carried out with culture supernatant, thermolysin and gelatin. The decrease in the amount of amino acids liberated was then measured which tells about the inhibitory effect of TIMPs. This was carried out on the 2^{nd}, 4^{th} and 6^{th} day. The activity was checked at three different incubation periods, i.e., at half an hour, one hour and two hours at varying parameters, such as varying pH and temperature and also in the presence of chelating metal ions.

2.3.Optimization Of Activity Of TIMPS

Various process parameters influencing TIMP activity were optimized. The effect of temperature (15 to 40°C), pH (pH 2 to 12), presence of metal chelating ions on TIMPs activity were determined by incubating thermolysin, gelatin and culture filtrate containing TIMPs at varying parameters.

- The effect of varying the pH:
  The activity of TIMPs was tested at five different values of pH on every alternate day (2, 4, 7, 9 and 12).
- Effect of temperature variation:
  The activity of TIMPs was tested at four different values of temperature (15^oC, 25^oC, 30^oC and 40^oC) on 2^{nd}, 4^{th} & 6^{th} day.
- Effect of metal chelating ions:
  The chelating metal ions were used to test their effect on the activity of the TIMPs by adding different concentrations of $MgCl_2$ and $ZnSO_4$ that were allowed to react with the culture supernatant for different time intervals.

2.4.Testing For In Vitro Anti-Inflammatory Activity

2.4.1.Inhibition Of Albumin Denaturation

Method of Mizushima Y, Kobayashi M (1968) & Sakat et al. (2010) was followed with minor modifications. The reaction mixture consisted of test extracts and 1% aqueous solution of bovine albumin fraction. pH of the reaction mixture was adjusted using small amounts of HCl at 37^0C. The sample extracts were incubated at 37^0C for 20 min and then heated to 51^0C for 20 min. After cooling the samples, turbidity was measured spectrophotometrically at 660nm. The experiment was performed in triplicates. Percent inhibition of protein denaturation was calculated as follows:

Percent inhibition = $[Abs_{control} - Abs_{sample}] \times 100/Abs_{control}$

2.4.2.Membrane Stabilization Tests

- Preparation of Red Blood Cells (RBCs) suspension
  Fresh whole human blood (10 ml) was collected and transferred to the centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of normal saline. The volume of blood was measured and reconstituted as 10% v/v suspension with normal saline (Govindappa et al., 2011).
- Heat induced hemolytic test
  The reaction mixture (2 ml) consisted of 1 ml of the test sample solution and 1 ml of 10% RBCs suspension, instead of test sample only saline was added to the controller test tube. Aspirin was taken as a standard drug. All the centrifuge tubes containing reaction mixture were incubated in a water bath at 56°C for 30 min. At the end of the incubation the tubes were cooled under running tap water. The reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatants was taken at 560 nm. The experiment was performed in triplicates for all the test samples. Percent membrane stabilization activity was calculated by the formula mentioned above (Govindappa et al., 2011).
- Antioxidant activity
  This test was performed using the method of Liyana-Pathirana and Shahidi (2005). The free radical scavenging activities of the extracts were measured by using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH). The capability to scavenge the DPPH radical was calculated using the following equation:
  DPPH scavenging effect (%) = $[\{A0 - A1\} A0] \times 100$,
  Where, A_0 is the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated and obtained by interpolation from linear regression analysis.
- Ammonium sulfate precipitation method
  The culture supernatant was purified using ammonium sulfate precipitation method and the sample at which maximum precipitation was obtained, was used to carry out the confirmatory assay using gelatin Zymography. Absorbance was measured at 660 nm.
- Confirmatory assay by Gelatin Zymography
  The sample with maximum precipitation was used to carry out the confirmatory assay using gelatin Zymography. Gelatin Zymography was carried out by the modified method of Hrabec et al. (2002). Activated MMP-2 (thermolysin) was also added to one of the wells. After electrophoresis, the gel was washed with 2.5% (w/v) Triton X-100 twice for 15 min each to remove SDS, and then rinsed briefly with a reaction buffer (50 mM Tris-HCl, 3.8 mM $CaCl_2$, pH8.0). The gel was then incubated at

37°C for 16 h in the reaction buffer and was stained with 0.1% Comassive brilliant blue R-250 for image analysis. The MMP-2 activities were detected as clear bands against the blue background.

3.Results & Discussion

3.1.Isolation Of TIMP Producing Microorganisms

On culturing the organisms on casein agar medium containing thermolysin solution, significant difference was observed in the growth characteristics of the organisms in control (casein agar medium - thermolysin) & those grown on test media (casein agar medium + thermolysin). (Figure 1)

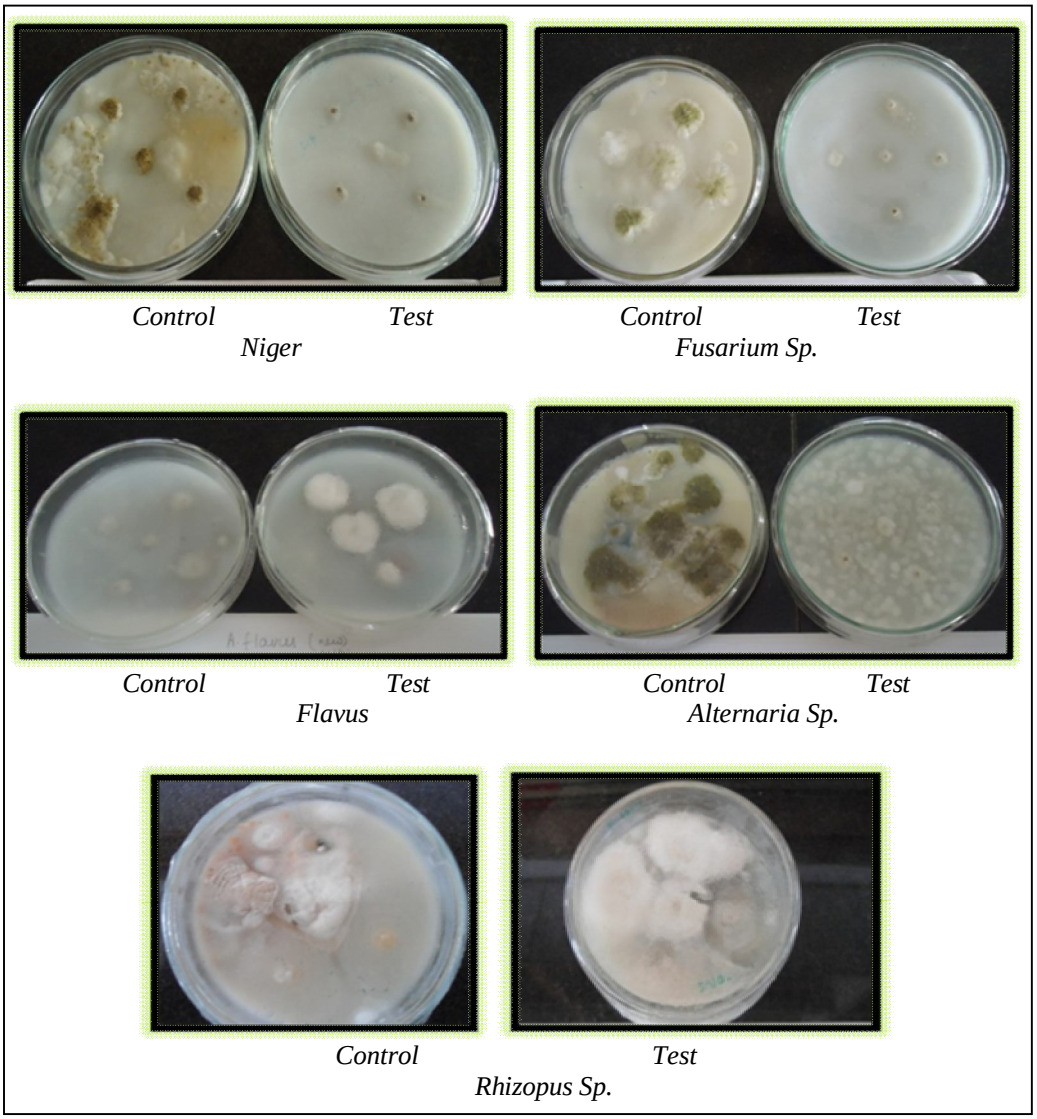

Figure 1: Organisms Grown On Casein Agar Media

Here we observed that, the organisms that could produce inhibitors against Thermolysin grew as cottony, white colored colonies irrespective of their original colony characteristics.

3.2.TIMP Assay

The TIMP assay was carried out using the ninhydrin test on the 2nd, 4th and 6th day The activity was checked at three different incubation periods, i.e., at half an hour, one hour and two hours intervals at varying parameters. (Figure 2.)

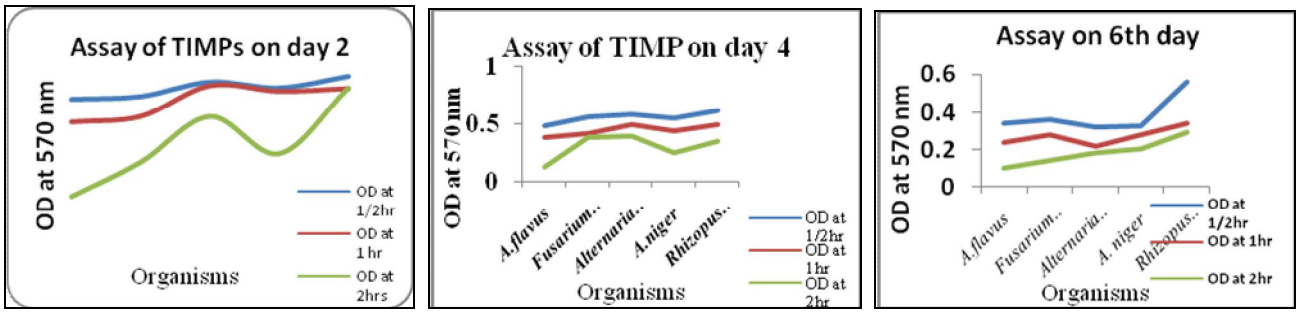

Figure 2: Assay Of TIMPS On Day 2, Day 4 & Day 6

The graphs here represent the results of assay to study the activity of TIMPs on Thermolysin. Here, least activity of thermolysin is seen at the A. flavus sample. Hence we can say that maximum activity of TIMPs was seen in A. flavus sample.

- Effect of varying pH
  The activity of TIMPs was tested at five different values of pH on the 2nd, 4th and 6th day at pH values 2, 4, 7, 9 and 12. (Figure 3)

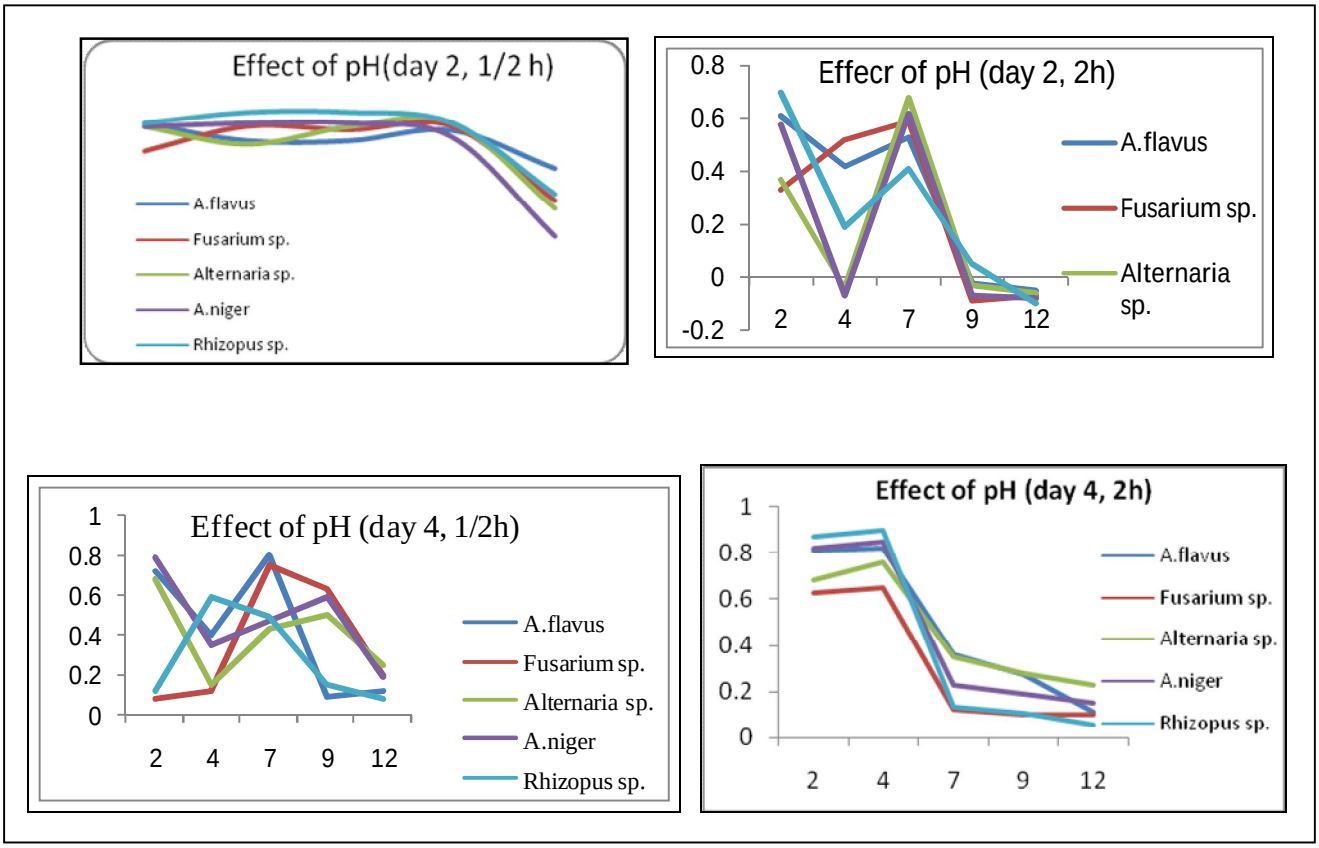

Figure 3: Assay Of Timps On Day 2 At ½ Hour & 1 Hour & Assay Of Timps On Day 4 At ½ Hour & 2 Hour:

By analyzing the results for the activity of TIMPs on Thermolysin, at various values of pH we can conclude that maximum activity of TIMPs was seen at pH 9 and 12 in A. flavus and Fusarium sp. Sample supernatants.

- Effect of temperature variations on TIMP activity
  The activity of TIMPs in each culture sample was studied on the 2nd, 4th and 6th day at different temperatures (15°C, 25°C, 30°C and 40°C), in order to see if varying temperatures lead to variations in the activity of the TIMPs. (Figure 4.)

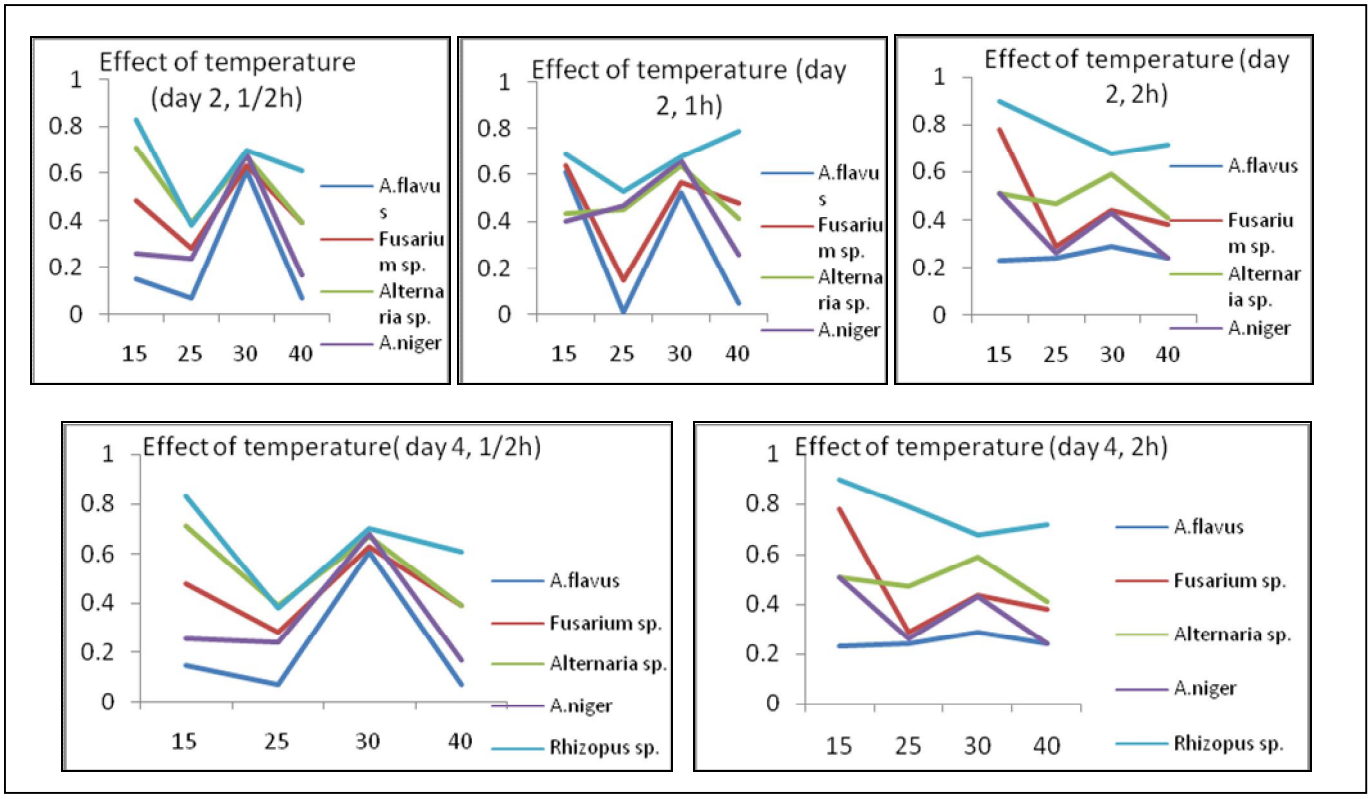

Figure 4: Assay Of Timps At Different Temperatures On Day 2 At ½ Hour, 1 Hour & 2 Hour Incubation
&
Assay Of Timps At Different Temperatures On Day 4, At ½ Hour, 1 Hour & 2 Hour Incubation

By comparing all the values of absorbance at different temperatures, we can infer that the maximum activity of TIMP was seen at 25°C in A. flavus sample supernatant.

- Effect of Metal chelating ions; $MgCl_2$ & $ZnSO_4$
  The chelating metal ions were used to test their effect on the activity of the TIMPs by adding different concentrations (0.2mM & 0.5mM) of $MgCl_2$ and $ZnSO_4$ that were allowed to react with the culture supernatant for different time intervals. (Figure 5)

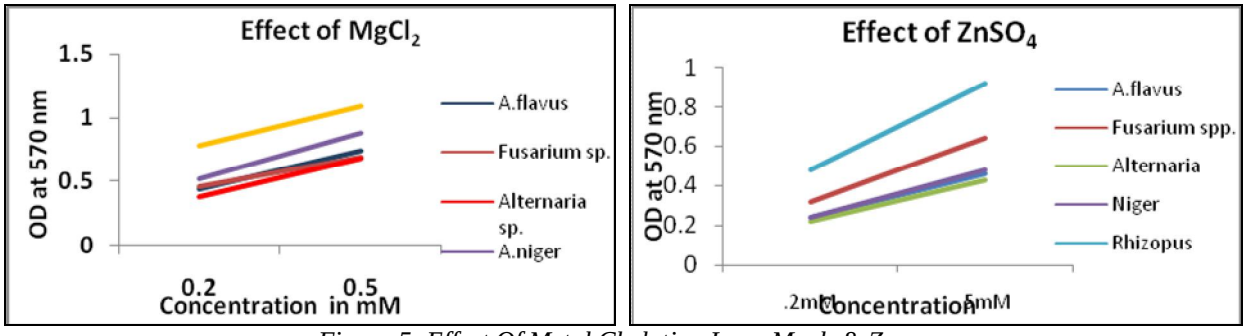

Figure 5: Effect Of Metal Chelating Ions; $Mgcl_2$ & $Znso_4$

By comparing the values of effect of presence of metal chelating ions on the activity of TIMPs on Thermolysin, it was seen that Alternaria sp. and Fusarium sp. showed maximum resistance to such metal ions. We can also conclude that the TIMPs are more resistant to $MgCl_2$ than to $ZnSO_4$.

3.3.Testing For In Vitro Anti-Inflammatory Activity

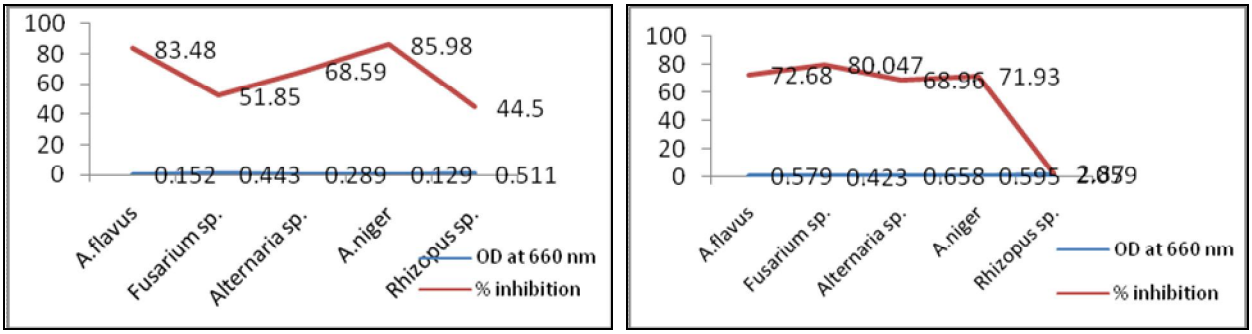

Figure 6: Inhibition Of Albumin Denaturation & Heat Induced Hemolytic Activity

Both, the inhibition of albumin denaturation test as well as the heat induced hemolytic activity highest anti-inflammatory activity was seen in Fusarium sp. & in A. Flavus.the activity was least in Rhizopus sp.

3.4.Test For Anti-Oxidant Activity

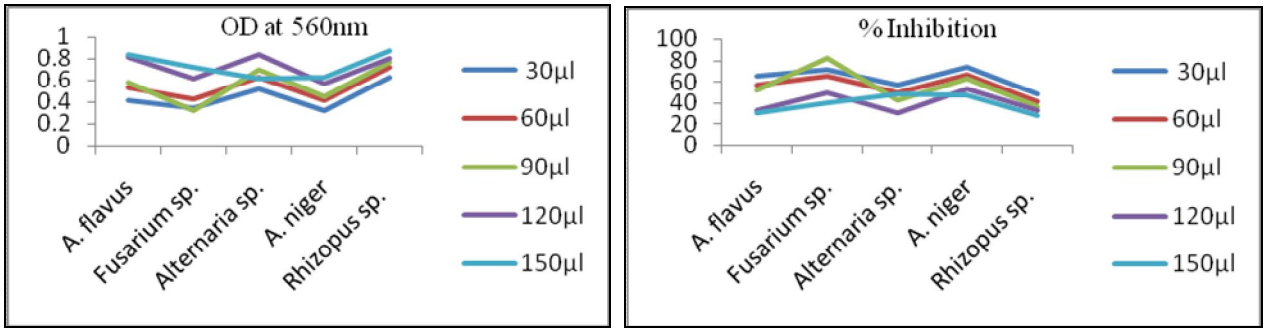

Figure 7: Anti-Oxidant Activity, OD At 560 Nm & % Inhibition

Here the maximum anti-oxidant activity was shown by the Fusarium sp. and the A. niger sample at a concentration of 90µl.

3.5.Ammonium Sulfate Precipitation

Ammonium sulfate precipitation method was used to purify the culture supernatant, as a prerequisite for performing gelatin zymography. (Figure 8)

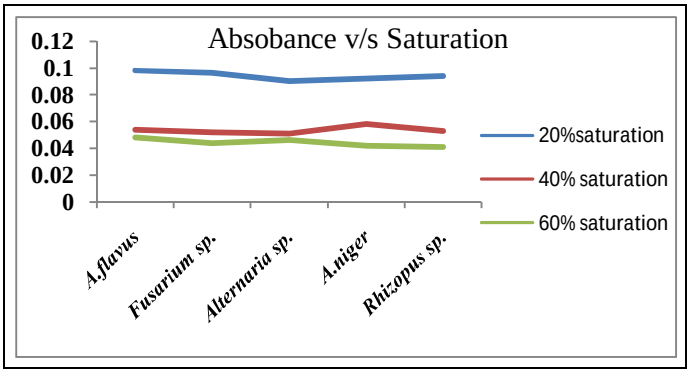

Figure 8: Absorbance Versus Saturation In Ammonium Sulfate Precipitation

From the graph we can clearly conclude that maximum precipitation of the proteins has occurred at 20% saturation, hence that sample was taken for confirmatory assay by Gelatine Zymography.

3.6.Confirmatory Analysis; Gelatin Zymography

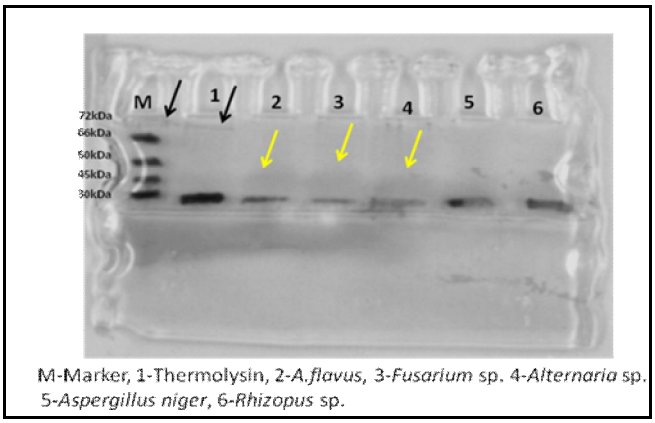

Figure 9: Gelatin Zymography

Here the black colour arrows represent the presence of our thermolysin near 72kDa. The arrows in yellow color represent that our sample supernatant contained TIMPs specific to Thermolysin (MMP2) that inhibited the Thermolysin from degrading the gelatin.

4.Conclusion

From the above studies, we can conclude that Aspergillus flavus supernatant shows the maximum decrease on the activity of MMP-2 (Thermolysin), and thus showing maximum inhibition. Also, maximum inhibition of MMP activity was seen in Aspergillus flavus sample in both, the anti-inflammatory activity & anti-oxidant test. However, a standard protocol for the extraction of TIMPs has to be developed so that the efficacy of the purified proteins can be tested on animal models for further confirmation.

5.References

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