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Issue Article

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ANTI-INFLAMMATORY POTENTIAL OF ENDOPHYTES ASPERGILLUS SYDOWII STRAIN AV5L AND PENICILLIUM VERRUCOSUM AV6L ISOLATED FROM MEDICINAL PLANT ALOE VERA

***Abstract**: In this study we evaluated the anti-inflammatory activity of ethyl acetate extracts of the endophytic fungi Aspergillus sydowii strain AV5L and Penicillium verrucosum AV6L using inhibiting thermally denatured proteins (egg albumin (EA) and bovine serum albumin (BSA)) methods and membrane stabilizing assay. The result demonstrated that increasing the concentration of the extract up to 500 µg/ml led a significant enhancement of their inhibitory activity. The crude extract of A.sydowii strain AV5L inhibited the BSA denaturation by the 67 % while for P.verrucosum AV6L this figure reached 71 %, which was comparable with the activity of acetylsalicylic acid activity (78%). In the erythrocyte membrane stabilization assay P.verrucosum AV6L extract demonstrated the most pronounced membrane-stabilizing activity, providing hemolysis inhibition at a level of 70.4% at concentration of 600 µg/ml which is comparable to the standard acetylsalicylic acid (72.56%). In the EA denaturation assay, the IC_{50} value for P.verrucosum was 455.1 µg/ml whereas for A.sydowii strain AV5L that value was 676.3 µg/ml and 376,9 µg/ml for acetylsalicylic acid. In the BSA system, the lowest IC_{50} value was also recorded for P.verrucosum AV6L (358.4 µg/ml) which was lower than that of A.sydowii strain AV5L (365,1 µg/ml) and acetylsalicylic acid (393,7 µg/ml). These results demonstrate the high ability of the extract to prevent thermal denaturation of proteins and to stabilize their structure. In the test of red blood cells membrane stabilization, the IC_{50} value was 388,7 µg/ml for P.verrucosum AV6L which was close to the standard value (388,4 µg/ml). Extract of A.sydowii strain AV5L demonstrated a little higher IC_{50} value (404,9 µg/ml) indicating moderate membrane stabilizing activity. In summary, the obtained data confirm the pronounced anti-inflammatory potential of the evaluated endophytes and further investigation of their secondary metabolites as a of novel anti-inflammatory compounds.*

***Key words**: endophytic fungi, anti-inflammatory activity, inhibition of protein denaturation, red blood membrane stabilization, bioactive compounds, IC_{50} value.*

***Language**: English*
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Introduction

Inflammation is a complex, protective reaction of the body against infection agents, toxic compounds and tissue damage. Despite physiological role of inflammatory process, its chronic disease course is the key factor in the development of many pathological conditions, including gastrointestinal diseases, autoimmune and metabolic disorders (Netea M.G., 2025, Soares et.al.,2023). Nowadays, inflammation associated by microbial enzymes, particularly urease, has become an important subject of interest.

Urease is a nickel-dependent that catalyzes the hydrolyses of urea to form ammonia and carbon dioxide. This enzyme is widespread among pathogenic microorganisms and plays a key role in their virulence. Specifically, *Helocobacter pylori* urease ensures the microorganism survival in the acidic environment by locally increasing pH which promotes colonization of the mucosa and the development of inflammatory gastric diseases (Mobley et.al 1995, Mazzei et.al 2020).

Although synthetic urease inhibitors, such as Acetohydroxamic acid, are currently available, their clinical application is limited due to significant side effects and toxicity. Similarly, the use of synthetic anti-inflammatory drugs is also accompanied by the development of adverse effects, which increased interest in natural sources of bioactive compounds (Seyedan et.al 2019, De la Garza et.al.2018). Endophytic microorganisms that asymptomatically colonize the internal tissues of plants are considered as a promising source of biologically active secondary metabolites. According to a review by Strobel and Daisy (2003), endophytes are able to synthesize a wide range of compounds with antimicrobial, antioxidant, and anti-inflammatory activity. Later, Kusari et al. (2012) showed that many endophytic fungi produce metabolites identical or structurally similar to those of their host plant compounds, which highlighting their importance for pharmacological and biotechnological research.

Previously, in our studies, we found that ethyl acetate extracts of the endophytic fungi *Aspergillus sydowii* strain AV5L and *Penicillium verrucosum* AV6L have pronounced urease-inhibiting activity (Ruzieva et.al 2025). A qualitative phytochemical analysis revealed the presence of flavonoids, terpenoids, tannins, cardiac glycosides and peptides (Vakhabova et.al 2025), which justifies the expediency of further study of their biological activity. Taking into account the established phytochemical composition of extracts and their pronounced ability to inhibit urease, the present research evaluates the anti-inflammatory activity of secondary metabolites of these endophytes using in vitro models of inhibition of protein denaturation and stabilization of cell membranes. These methods are widely used for the primary assessment of the anti-inflammatory potential of natural compounds.

Materials and methods

Isolation and cultivation of endophytic fungi. The endophytic fungi *Aspergillus sydowii* strain AV5L and *Penicillium verrucosum* AV6L were isolated from healthy *Aloe vera* leaves collected in Tashkent, Uzbekistan. The isolates were preliminarily identified by molecular methods and deposited in the NCBI database. Endophytic fungi were grown by immersion fermentation in 500 ml flasks containing 250 ml of Potato dextrose liquid medium for 7 days at 28 °C on a rotary shaker (Kumar et.al.2013).

Extraction of secondary metabolites 5 g of biomass of the endophytic fungus was homogenized and transferred to a conical flask containing 50 ml of ethyl acetate, and left for 48 hours on a shaker at room temperature. The mixture was then filtered through a filter paper (Whatman #1), after which the extract was dried on a rotary evaporator and dissolved in 1 ml of ethyl acetate, which was used as a starting solution and stored at 4 °C until further use. To evaluate the anti-inflammatory activity of the extracts, egg (EA), bovine serum (BSA) albumins and a membrane stabilization assays were used to confirm the universality and reproducibility of the inhibitory effect of the extracts on various proteins. The method was implemented in accordance with Mizushima Y Kobayashi M (1968) with modifications proposed by C.Sree Kumari et.al (2015).

Inhibition of egg albumin denaturation. The reaction mixture with a volume of 5 ml consisted of 0.2 ml of egg albumin, 2.8 ml of phosphate buffer (pH 6.4) and 2 ml of extracts of various concentrations. A similar volume of distilled water served as a control. The mixture was incubated at 370 ° C for 15 minutes, and then heated at 70^0 C for 5 minutes. After cooling, the adsorption was measured at 660 nm. Acetylsalicylic acid was used as the standard, from which a control was prepared in a concentration similar to the experimental extract. The denaturation of protein (X) inhibition by the extract and the standard was expressed as a percentage and calculated using the formula: $X = \frac{A0 - At}{A0} \times 100$, where A0 is the optical density of the control; At is the optical density of the test sample.

Inhibition of bovine serum albumin denaturation. The reaction mixture (0.5ml) consisted of 0.45 ml of BSA (5% aqueous solution) and 0.05 ml of endophyte fungi extract of various concentrations. The samples were incubated at 37^0C for 30 minutes. After cooling, 2.5 ml of phosphate-salt buffer (pH 6.3) was added to each sample. The adsorption was measured at 660 nm. 0.05 ml of distilled water was used as a control instead of the extract. The denaturation of protein (X) inhibition by the extract and the standard was expressed as a percentage and calculated using the formula: $X = \frac{A0 - At}{A0} \times 100$, where A0 is the optical density of the control; At is the optical density of the test sample.

Membrane-stabilizing activity. Preparation of erythrocyte suspension. Fresh whole human blood (10 ml) was collected and transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 minutes and rinsed three times with an equal volume of saline solution. Blood volume was measured and a 10% (v/v) suspension in saline solution was prepared.

Hemolysis induced by heating. The reaction mixture (2 ml) consisted of 1 ml of the test sample and 1 ml of a 10% suspension of erythrocytes. Saline solution was added to the control tube instead of samples. Acetylsalicylic acid was used as the standard. The tubes were incubated in a water bath at 56^0C for 30 minutes. Then they were cooled under running water. After that, the mixture was centrifuged at 2500 rpm for 5 minutes, and the optical density of the supernatant was measured at 560 nm. The experiment was carried out in three repetitions. The percentage of membrane stabilization was calculated using the formula: $X = \frac{A0 - At}{A0} \times 100$,, where A0 is the optical density of the control; At is the optical density of the test sample.

Results and discussion

The anti-inflammatory activity of secondary metabolites of endophytic fungi *Aspergillus sydowii* strain AV5L and *Penicillium verrucosum* AV6L was evaluated using in vitro models of protein denaturation inhibition and a membrane-stabilizing test. These assays enable the reproduction of key mechanisms involved in the inflammatory process, including damage to protein structures and disruption of the cell membrane integrity. In the EA denaturation inhibition test, *P. verrucosum* AV6L extract demonstrated higher activity compared to *A. sydowii* strain AV5L in almost all studied concentrations. The maximum inhibitory activity of *P. verrucosum* AV6L extract was 70% at a concentration of 500 µg/ml, which was comparable to the activity of the acetylsalicylic acid standard drug (72%). Extract of *A. sydowii* AV5L at a similar concentration inhibited albumin denaturation by 64% (Figure 1).

With an increase in concentration to 600 µg/ml, a slight decrease in the activity of both extracts was observed: the degree of inhibition was 59% for *A. sydowii* strain AV5L and 60% for *P. verrucosum* AV6L. A decrease in activity at higher concentrations may be due to saturation of the active components or a change in the nature of their interaction with protein structures.

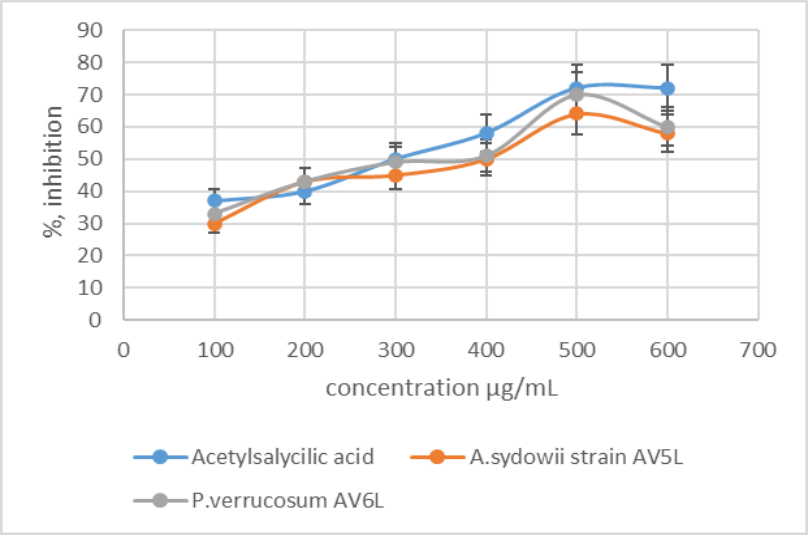

Figure 1. Anti-inflammatory activity of A. sydowii AV5L and P. verrucosum AV6L on inhibition of EA denaturation

Evaluation of the anti-inflammatory activity of ethyl acetate extracts of endophytic fungi *A. sydowii* strain AV5L and *P. verrucosum* AV6L using the BSA denaturation inhibition model demonstrated pronounced dose-dependent activity in the concentration range of 100-600 µg/ml (Figure 2). When the concentration was increased to 500 µg/ml, a significant increase in the inhibitory activity of both

extracts was observed. The extract of *A. sydowii* strain AV5L inhibited BSA denaturation by 67%, whereas for *P. verrucosum* AV6L this indicator reached 71%, which was comparable with the activity of acetylsalicylic acid (78%). With a further increase in concentration to 600 µg/ml, a decrease in the activity of the studied extracts was noted: the degree of inhibition was 65% for *A. sydowii* strainAV5L and 63% for *P. verrucosum* AV6L. This trend may be due to saturation of the active components or a change in their interaction with protein molecules at high concentrations.

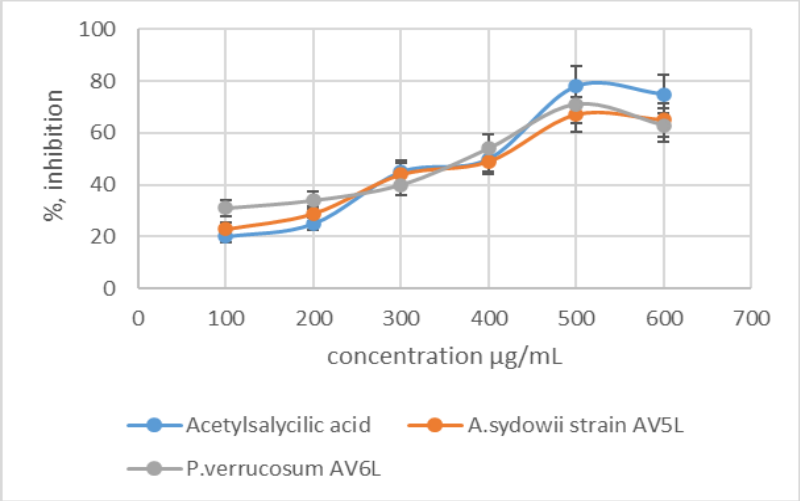

Figure 2. Anti-inflammatory activity of *A. sydowii* strain AV5L and *P. verrucosum* AV6L on inhibition of BSA denaturation

In general, the obtained results confirm the pronounced anti-inflammatory potential of both endophytic strains with the highest activity in the range of 500 µg/ml and a subsequent tendency to decrease the effect with a further increase in concentration. Thus, the use of two different protein systems- BSA and EA increases the reliability and versatility of the obtained results. To further study the mechanism of the anti-inflammatory effect of ethyl acetate extracts *A. sydowii* AV5L and *P. verrucosum* AV6L have been studied for their effect on the stabilization of red blood cell membranes (RBC). This method is widely used to evaluate the anti-inflammatory activity of natural compounds, since its ability to prevent thermally induced hemolysis correlates with the membrane-stabilizing effect and potential anti-inflammatory activity.

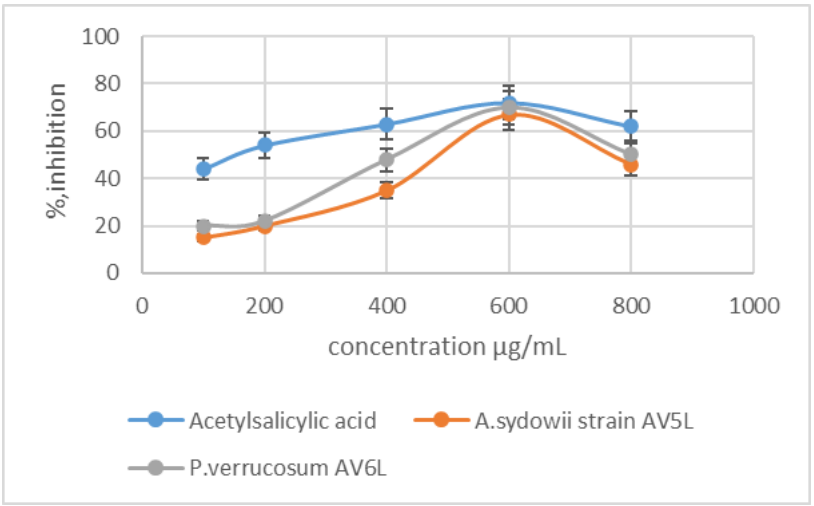

Figure 3. Anti-inflammatory activity of *A. sydowii* strain AV5L and *P. verrucosum* AV6L on inhibition of protein denaturation in a membrane stabilizing test

P. verrucosum AV6L extract showed the most pronounced membrane-stabilizing activity, providing hemolysis inhibition at the level of 70.4% at a concentration of 600 µg/ml, which was comparable to the activity of the standard drug acetylsalicylic acid (72.56%) at a similar concentration. Extract of *A.*

sydowii strain AV5L at the same concentration showed slightly lower activity — 67.9% inhibition of hemolysis (Fig. 3). The results indicate that the ability of the studied extracts to stabilize the membranes of RBC may be one of the mechanisms of their anti-inflammatory effect. The stabilization of cell membranes helps to prevent damage to cellular structures and protein denaturation under the influence of inflammatory factors, which confirms the potential effectiveness of the studied extracts as anti-inflammatory agents.

Figure 4 demonstrates the values of IC_{50} extracts of *A. sydowii* strain AV5L, *P. verrucosum* AV6L and acetylsalicylic acid in assays of inhibition of denaturation of EA, BSA and membrane stabilization test. In the EA system, the lowest IC_{50} value was recorded for acetylsalicylic acid (376.9 µg/ml), while the IC_{50} of *P. verrucosum AV6L* extract was 455.1 µg/ml, indicating a higher anti-inflammatory activity compared with *A. sydowii* strain AV5L (676.3 µg/ml).

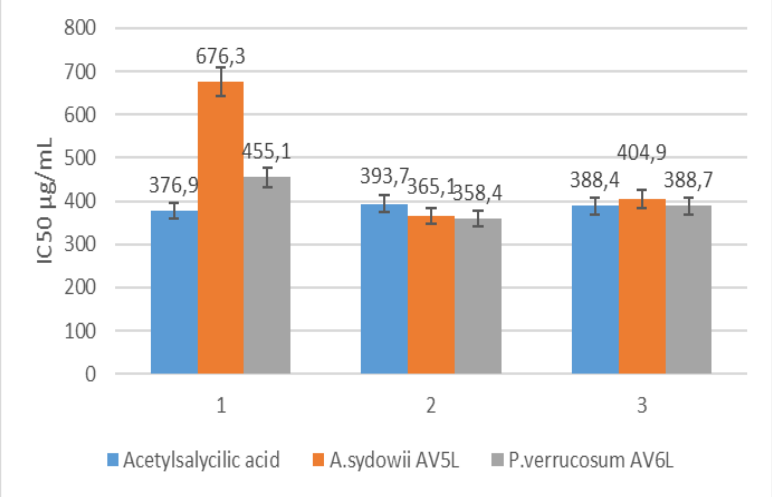

Figure 4. IC values of ethyl acetate extracts of endophytic fungi *A. sydowii* strainAV5L and *P. verrucosum* AV6L in assay of inhibition of denaturation of EA (1), BSA (2) and membrane stabilization test (3)

In the BSA model, both extracts showed relatively low activity (365.1 and 358.4 µg/ml), however, the lowest IC_{50} value was recorded for *P. verrucosum* AV6L (358.4 µg/ml), which indicates its higher ability to stabilize the protein structure during thermal denaturation. In the membrane-stabilizing IC_{50} test, *P. verrucosum* AV6L (388.7 µg/ml) practically did not differ from acetylsalicylic acid (388.4 µg/ml), while *A. sydowii* strain AV5L demonstrated a slightly higher IC_{50} value (404.9 µg/ml), reflecting a moderate difference in the membrane-stabilizing effect. The results are consistent with literature data confirming the pronounced anti-inflammatory activity of secondary metabolites of endophytic fungi of the genus *Penicillium*, which are able to inhibit protein denaturation, stabilize biological membranes, and suppress the production of inflammatory mediators at a level comparable to nonsteroidal anti-inflammatory drugs (Govindappa et al., 2011; Kharwar et al., 2019; Zhang et al., 2020). Similar data on moderate anti-inflammatory activity were obtained for representatives of the genus *Aspergillus*, whose metabolites inhibit thermal denaturation of albumin and hemolysis of erythrocytes in a concentration-dependent form (Mishra et al., 2017; El-Sayed et al., 2021) (Figure 4).

CONCLUSION

Thus, both endophytic fungi studied, *A. sydowii* strain AV5L and *P. verrucosum* AV6L, exhibit the ability to inhibit protein denaturation and stabilize erythrocyte membranes, which confirms their anti-inflammatory properties. *P. verrucosum* AV6L showed higher efficacy in the model with egg, bovine albumin and membrane-stabilizing test, whereas *A. sydowii* strain AV5L showed a moderate difference in the membrane-stabilizing test. These differences may be associated with variations in the spatial structure of protein substrates and differences in the mechanisms of interaction of bioactive components of extracts with proteins. The data confirm the presence of biologically active metabolites in the studied endophytic fungi with the potential for anti-inflammatory effects comparable to the effect of a classic nonsteroidal anti-inflammatory drug.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations:
EA-egg albumin
BSA- bovine serum albumin
RBC- red blood cells

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