

ANTICOAGULANT AND ANTI-INFLAMMATORY ACTIVITIES OF METABOLITES PRODUCED BY THE ENDOPHYTIC FUNGUS *ASPERGILLUS CHEVALIERI* PM4S ISOLATED FROM *PLANTAGO MAJOR*

N. Kuzieva¹, L. Abdulmyanova², M. Sheripbayeva³

Junior Researcher, Laboratory of Biochemistry and Biotechnology of Bioactive Compounds,
Institute of Microbiology of Uzbekistan¹

Leading Researcher, Doctor of Biological Sciences, Institute of Microbiology of Uzbekistan²

Master's Student, Department of Microbiology, National University of Uzbekistan named after
Mirzo Ulugbek³

<https://doi.org/10.5281/zenodo.20303449>

Abstract. *The present study aimed to evaluate the anticoagulant and anti-inflammatory activities of secondary metabolites produced by the endophytic fungus *Aspergillus chevalieri* PM4S isolated from *Plantago major*. Anticoagulant activity was investigated using plasma recalcification time (PRT), prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen (FIB) assays. Anti-inflammatory activity was assessed in vitro by inhibition of ovalbumin denaturation. The obtained results demonstrated pronounced anticoagulant activity of the fungal extract. Treatment with the 96% ethanol extract of *A. chevalieri* PM4S completely suppressed clot formation for more than 120 min in the PRT assay. In coagulation tests, the extract significantly prolonged APTT from 27.9 ± 0.42 sec to 117.2 ± 1.76 sec and TT from 20.4 ± 0.01 sec to 223.5 ± 0.11 sec, while fibrinogen concentration decreased from 3.4 ± 0.07 g/L to 2.04 ± 0.03 g/L. These findings indicate strong inhibition of intrinsic and common coagulation pathways. In the anti-inflammatory assay, the extract exhibited concentration-dependent inhibition of ovalbumin denaturation. The maximum inhibitory activity was observed at 75 μ g/ml, reaching $64.7 \pm 0.32\%$, which exceeded the activity of aspirin at the same concentration ($62.2 \pm 0.31\%$). The IC_{50} value of the fungal extract was 122.899 μ g/ml, compared with 113.7489 μ g/ml for aspirin. The observed biological activities may be associated with polyphenolic, flavonoid, and antioxidant metabolites synthesized by the fungus. The obtained results demonstrate the potential of *A. chevalieri* PM4S as a promising source of multifunctional natural compounds with anticoagulant and anti-inflammatory properties and support further investigation of its bioactive metabolites for pharmaceutical applications.*

Keywords: endophytic fungi, *Aspergillus chevalieri*, *Plantago major*, anticoagulant activity, plasma recalcification time (PRT), prothrombin time (PT), partially activated thromboplastin time (aPTT), thrombin time (TT), fibrinogen, anti-inflammatory activity, biologically active metabolites.

Introduction

Thrombotic disorders and inflammatory diseases remain among the major causes of mortality and morbidity worldwide. Excessive activation of coagulation pathways and inflammatory mediators contributes significantly to the development of cardiovascular diseases, venous thrombosis, stroke, and chronic inflammatory conditions (Hirsh et al., 2001, p. 2994).

Although anticoagulant drugs such as heparin and warfarin are widely used in clinical practice, their prolonged administration is frequently associated with hemorrhagic complications, bleeding risks, and other adverse side effects (Hirsh et al., 2001, p. 2994). Therefore, the discovery of novel natural compounds with anticoagulant and anti-inflammatory activities has become an important direction in modern pharmacology and biotechnology.

Medicinal plants are considered valuable natural sources of biologically active metabolites. Among them, *Plantago major* has been traditionally used for centuries as a wound-healing, analgesic, antioxidant, antimicrobial, and anti-inflammatory remedy (Samuelsen, 2000, p. 1). Previous studies demonstrated that the leaves of *P. major* contain numerous bioactive compounds including flavonoids, caffeic acid derivatives, iridoid glycosides, polysaccharides, terpenoids, and other phenolic substances responsible for its pharmacological properties (Samuelsen, 2000, p. 1; Chiang et al., 2002, p. 53). In particular, baicalein and aucubin have been identified as major active constituents exhibiting strong antioxidant and anti-inflammatory effects (Zubair et al., 2012, p. 825).

Several pharmacological investigations confirmed the anti-inflammatory potential of *Plantago major* extracts. The aqueous extract of the plant significantly reduced carrageenan-induced edema, pleurisy, and exudative inflammatory reactions in experimental animals (Olennikov et al., 2015, p. 118). Moreover, *P. major* extracts and their active metabolites were reported to suppress reactive oxygen species (ROS) production in human neutrophils (Zubair et al., 2012, p. 825). Reduction of ROS generation decreases oxidative stress and inflammatory mediator activity, which are closely associated with platelet activation and coagulation abnormalities. Therefore, metabolites derived from *P. major* may also possess potential anticoagulant properties.

In recent years, endophytic microorganisms, particularly fungal endophytes, have attracted increasing scientific attention as promising producers of pharmacologically important secondary metabolites (Strobel & Daisy, 2003, p. 491). Endophytes inhabit internal plant tissues without causing damage to their host plants and are capable of synthesizing diverse biologically active compounds. Species belonging to the genus *Aspergillus* are especially recognized for producing metabolites with antioxidant, antimicrobial, anti-inflammatory, fibrinolytic, and anticoagulant activities (Frisvad & Larsen, 2015, p. 45). In addition, fungal metabolites offer several advantages over plant-derived compounds, including rapid growth, high productivity, and easier biotechnological manipulation.

Earlier, the Laboratory of Biochemistry and Biotechnology of Bioactive Compounds at the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan conducted a screening study of preserved and newly isolated endophytic fungal strains to evaluate their anticoagulant activity (Kuzieva et al., 2024, p. 16-22). As a result of this screening, the strain *Aspergillus chevalieri* PM4S isolated from *Plantago major* was selected as a promising producer of biologically active metabolites. Despite extensive studies on the pharmacological properties of *Plantago major*, limited information is available regarding the bioactivity of its associated endophytic fungi.

Materials and Methods

Cultivation and Extraction of Fungal Secondary Metabolites. To isolate metabolites from the biomass of endophytic fungi, the method of Hazalin and co-workers was used with minor modifications (Hazalin, N. A. et al., 2009, p. 46). The fungi were grown in a liquid culture medium with potato dextrose broth on a shaker at 180 rpm and 28 °C for 7-10 days. The culture liquid and

biomass were separated by centrifugation at 6000 rpm, and the samples were stored in a freezer at -20 °C. The grown biomass was ground in a porcelain mortar with quartz sand. After obtaining the homogenate, it was placed in a flask, 50 ml of ethyl ester of acetic acid was added and left for 24 hours at room temperature on a rocking table for extraction. Then the mass was filtered through paper (watman No. 1), 40 mkg/ml of sodium sulfate was added to remove the water layer, and the obtained extract was evaporated on a rotary evaporator. To the dry mass, 1.5 ml of 96% solution of ethyl alcohol was added to obtain the extract, which was stored at +4 °C.

Plasma Recalcification Time (PRT) Assay. Extractions were tested for plasma recalcification time (PRT) by the following method. A 0.2 ml citrated blood sample was transferred to a 12 x 75 mm tube. The glass tube was incubated in a water bath at 37 °C for 2 min. To this was added 0.2 ml 0.025 M calcium chloride and a timer was started simultaneously. The ingredients were mixed and the tube was slowly tilted back and forth with a gentle rotating motion for approximately 20 s to observe the appearance of fibrin strands. The time between the addition of calcium chloride and the formation of the first observed fibrin strands was recorded as the blood recalcification time (Sinyuk, I. V., & Dudarev, V. A., 2015, p.78-80).

Coagulation Assays. Coagulation tests — prothrombin time (PT), partially activated thromboplastin time (aPTT), thrombin time (TT), and fibrinogen test — were performed on the HumaClot Junior apparatus (Human GmbH, Wiesbaden, Germany). To do this, 3 ml of blood was taken from the subject, placed in a tube with 3.8% sodium citrate, and centrifuged at 3000 rpm for 3 minutes to separate the plasma. Plasma and extracts were mixed in a 1:5 ratio, and the corresponding reagents (Human GmbH, Wiesbaden, Germany) were added to a clean cuvette. The mixture was incubated for 15 seconds and tested on a device at 37 °C.

Inhibition of denaturation of ovalbumin. A 4 ml reaction mixture was prepared containing 0.2 ml of ovalbumin, 2.8 ml of phosphate buffer (pH 6.4), and 1 ml of extracts of different concentrations. A distilled water of the same volume was used as a control. Then the sample was incubated at 37°C for 15 min using water bath and after heated at 65°C for 5 min. After cooling, the absorbance of the reaction mixtures was measured spectrophotometrically at 660 nm. As a standard was assessed acetylsalicylic acid from which a control was prepared at a concentration analogue to the experimental extract. Protein denaturation inhibition (X) by the extract and standard was expressed as a percentage and calculated according to the formula: $X = \frac{A_0 - A_t}{A_0} \times 100$, where A_0 is the optical density of the control; A_t is the optical density of the test sample (Sree Kumari, C. et al., 2015, p. 482–485).

Results and Discussion

The anticoagulant activity of secondary metabolites produced by the endophytic fungus *Aspergillus chevalieri* PM4S was evaluated using plasma recalcification time (PRT) and standard coagulation parameters, including prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen concentration (FIB). The obtained results demonstrated a pronounced anticoagulant effect of the fungal extract on both intrinsic and common coagulation pathways (Table 1).

Table 1. Effect of extracts of the endophytic fungus *Aspergillus chevalieri* – PM4S on PRT and coagulation tests (n=3, p <0.05).

Indicators		Normal	Heparin (+)	Warfarin (+)	Ethanol (-)	<i>A.chevalieri</i> PM4S
PRT	Time, min	1.43±0,04	+	+	1.48±0,06	>120

PT	Time,sec	12,4±0,19	+	+	10,8±0,22	9,7±0,15
	PTI,%	131±0,07	+	+	166±2,49	199,4±3,99
	INR	0,85±0,02	+	+	0,73±0,02	0,66±0,01
APTT	Time,sec	27,9±0,42	+	+	36,8±0,74	117,2±1,76
	Ratio	0,83±0,01	+	+	1,24±0,02	3,91±0,06
TT	Time,sec	20,4±0,01	+	+	19,8±0,29	223,5±0,11
	Ratio	1,25±0,02	+	+	1,21±0,02	13,7±0,27
FIB	g/l	3,4±0,07	>0.70	>0.70	2,35±0,03	2,04±0,03

Under normal conditions, plasma recalcification occurred within 1.43±0,04 min, whereas treatment with the 96% ethanol extract of *A. chevalieri* PM4S resulted in complete suppression of clot formation for more than 120 min. Such a significant prolongation of PRT indicates strong inhibition of coagulation cascade activation and suggests the presence of biologically active metabolites capable of interfering with fibrin clot formation. In contrast, the negative ethanol control demonstrated only minor changes in coagulation parameters, confirming that the observed activity was associated with fungal metabolites rather than solvent effects.

The extract also markedly influenced individual coagulation indicators. APTT increased from 27.9 ± 0.42 sec in the control to 117.2 ± 1.76 sec after treatment with the fungal extract, corresponding to a ratio increase from 0.83 ± 0.01 to 3.91 ± 0.06. This pronounced prolongation indicates substantial inhibition of the intrinsic coagulation pathway. Similarly, TT increased from 20.4 ± 0.01 sec to 223.5 ± 0.11 sec, with the TT ratio rising to 13.7 ± 0.27, demonstrating a strong inhibitory effect on thrombin-mediated fibrin formation. The fibrinogen concentration simultaneously decreased from 3.4 ± 0.07 g/L in normal plasma to 2.04 ± 0.03 g/L following treatment with the extract, further supporting disruption of fibrin polymerization processes. Similar anticoagulant effects of endophytic fungal metabolites rich in phenolic and flavonoid compounds have previously been reported in representatives of *Aspergillus* and *Penicillium* species (Ha et al., 2020, p. 247).

In contrast to the substantial effects observed for APTT and TT, PT values showed only moderate variation. The PT decreased from 12.4 ± 0.19 sec to 9.7 ± 0.15 sec, while the INR value declined from 0.85 ± 0.02 to 0.66 ± 0.01. These findings suggest that the metabolites of *A. chevalieri* PM4S may predominantly target components of the intrinsic and thrombin-dependent stages of coagulation rather than the extrinsic pathway. Similar observations have been described for fungal metabolites possessing antioxidant and enzyme-modulating properties capable of suppressing fibrin clot formation (Toghueo and Boyom, 2019, p. 43).

The obtained results are consistent with previous studies demonstrating anticoagulant properties of secondary metabolites produced by species of the genera *Aspergillus* and *Penicillium*. Several fungal metabolites, including phenolic derivatives, quinones, and flavonoids, have been reported to prolong coagulation time by suppressing thrombin activity, reducing fibrin formation, or modulating calcium-dependent coagulation reactions (Khan et al., 2020, p. 20738). The exceptionally prolonged TT observed in this study may indicate direct inhibition of thrombin activity or interference with fibrinogen conversion into fibrin.

The anti-inflammatory activity of the ethyl acetate extract of *A. chevalieri* PM4S was investigated using an in vitro ovalbumin denaturation inhibition model. Protein denaturation assays are widely used for evaluation of anti-inflammatory potential because denatured proteins can induce inflammatory and autoimmune responses. Prevention of thermal protein denaturation

therefore reflects the capacity of biologically active compounds to stabilize protein structures under inflammatory conditions (Chi et al., 2019, p. 1).

The fungal extract demonstrated concentration-dependent anti-inflammatory activity comparable to aspirin at several tested concentrations (Table 2). At 25 µg/ml, the extract inhibited ovalbumin denaturation by $44.8 \pm 0.45\%$, while aspirin demonstrated $49.7 \pm 0.25\%$ inhibition. Increasing the concentration to 50 µg/ml enhanced the inhibitory activity of the extract to $49.8 \pm 0.75\%$. The maximum anti-inflammatory effect of the fungal extract was observed at 75 µg/ml, where inhibition reached $64.7 \pm 0.32\%$, exceeding the activity of aspirin at the same concentration ($62.2 \pm 0.31\%$).

Table 2. Anti-inflammatory activity of *Aspergillus chevalieri* – PM4S determined by inhibition of ovalbumin denaturation ($n = 3$, $p < 0.05$).

№	Concentration µg/ml	Aspirin, %	<i>A.chevalieri</i> – PM4S, %
1	25	49,7±0,25	44,8±0,45
2	50	68,1±0,27	49,8±0,75
3	75	62,2±0,31	64,7±0.32
4	100	58,4±0,18	58,4±0,23
5	125	52,2±0,52	44,3±0,44
6	150	50,2±1,00	38,8±0,58
	IC ₅₀ (µg/ml)	113,7489	122,899

At higher concentrations (100–150 µg/ml), a gradual decline in inhibitory activity was observed for both aspirin and the fungal extract. Nevertheless, the extract retained substantial anti-inflammatory potential throughout the tested concentration range. The IC₅₀ value of *A. chevalieri* PM4S extract was calculated as 122.899 µg/ml, which was relatively close to that of aspirin (113.7489 µg/ml). These findings indicate that the metabolites produced by the studied endophytic fungus possess pronounced protein-stabilizing activity and may serve as promising natural anti-inflammatory agents.

The observed anti-inflammatory activity may be associated with the presence of polyphenolic metabolites, flavonoids, terpenoids, and related antioxidant compounds previously reported in endophytic fungi (Zhao et al., 2020, p. 20738). Such metabolites are known to suppress inflammatory processes through inhibition of protein denaturation, reduction of oxidative stress, stabilization of biomembranes, and modulation of inflammatory mediators. Phenolic compounds, in particular, can inhibit reactive oxygen species formation and reduce oxidative modification of proteins, thereby limiting inflammatory damage Prospective Leads from Endophytic Fungi for Anti-Inflammatory Drug Discovery.

Overall, the obtained results demonstrate that metabolites produced by *A. chevalieri* PM4S possess both anticoagulant and anti-inflammatory activities. The pronounced prolongation of coagulation parameters together with significant inhibition of protein denaturation suggests the multifunctional biological potential of metabolites synthesized by this endophytic fungus. These findings support the possibility of using endophytic fungal metabolites as promising sources of novel bioactive compounds for development of natural anticoagulant and anti-inflammatory agents.

Conclusion

This study demonstrated that secondary metabolites produced by the endophytic fungus *Aspergillus chevalieri* PM4S exhibit pronounced anticoagulant and anti-inflammatory activities in vitro. The ethanol extract of the fungus significantly prolonged plasma recalcification time (PRT), activated partial thromboplastin time (APTT), and thrombin time (TT), indicating strong inhibition of the intrinsic and common coagulation pathways. In addition, the reduction in fibrinogen concentration suggests suppression of fibrin formation and polymerization processes.

The anti-inflammatory potential of the fungal extract was confirmed using the ovalbumin denaturation inhibition model. The extract demonstrated concentration-dependent protein-stabilizing activity, with the maximum inhibitory effect observed at 75 µg/ml, where the activity exceeded that of aspirin. Furthermore, the IC₅₀ value of the extract was comparable to the standard anti-inflammatory drug, indicating substantial anti-inflammatory potential.

The scientific novelty of this study lies in the first comprehensive evaluation of both anticoagulant and anti-inflammatory properties of metabolites produced by *A. chevalieri* PM4S isolated from *Plantago major*. The obtained results expand current knowledge regarding the pharmacological potential of endophytic fungi and demonstrate that metabolites synthesized by this strain are capable of modulating coagulation processes while simultaneously stabilizing protein structures under inflammatory conditions.

The observed biological activities are likely associated with the presence of polyphenolic, flavonoid, and other antioxidant metabolites capable of suppressing thrombin-mediated fibrin formation, reducing oxidative stress, and inhibiting protein denaturation. Overall, the findings confirm the potential of endophytic fungi as promising sources of multifunctional natural bioactive compounds and support the need for further purification, structural characterization, and mechanistic investigation of individual active metabolites for potential biomedical and pharmaceutical applications.

REFERENCES

1. Chi, W. C., Pang, K. L., Chen, W. L., Wang, G. J., & Lee, T. H. (2019). Antimicrobial and iNOS inhibitory activities of endophytic fungi isolated from *Acanthus ilicifolius* var. *xiamenensis*. *Botanical Studies*, 60(4), 1–9.
2. Chiang, L. C., Chiang, W., Chang, M. Y., Ng, L. T., & Lin, C. C. (2002). Antiviral activity of *Plantago major* extracts and related compounds in vitro. *Antiviral Research*, 55(1), 53–62.
3. Frisvad, J. C., & Larsen, T. O. (2015). Extrolites of *Aspergillus* species. *Chemical Fungal Taxonomy*, 45, 45–56.
4. Ha, T. M., Kim, D. C., Sohn, J. H., Yim, J. H., & Oh, H. (2020). Anti-inflammatory and protein tyrosine phosphatase 1B inhibitory metabolites from the Antarctic marine-derived fungal strain *Penicillium glabrum* SF-7123. *Marine Drugs*, 18(5), 247. <https://doi.org/10.3390/md18050247>
5. Hazalin, N. A., Ramasamy, K., Lim, S. M., Wahab, I. A., Cole, A. L. J., & Majeed, A. A. (2009). Cytotoxic and antibacterial activities of endophytic fungi isolated from plants at the National Park, Pahang, Malaysia. *BMC Complementary and Alternative Medicine*, 9, 46. <https://doi.org/10.1186/1472-6882-9-46>
6. Hirsh, J., Anand, S. S., Halperin, J. L., & Fuster, V. (2001). Guide to anticoagulant therapy. *Circulation*, 103(24), 2994–3018. <https://doi.org/10.1161/01.CIR.103.24.2994>
7. Khan, I., Zhang, H., Liu, W., Zhang, L., Peng, F., Chen, Y., et al. (2020). Identification and bioactivity evaluation of secondary metabolites from Antarctic-derived *Penicillium*

- chrysogenum* CCTCC M 2020019. *RSC Advances*, 10, 20738–20744. <https://doi.org/10.1039/D0RA03144A>
8. Kuzieva N. H., Abdulmyanova L. I., & Gulyamova T. G. (2024). Skrining antikoagulyantnykh svoystv etanol'nykh ekstraktov endofitnykh gribov. *Universum: Khimiya i biologiya*, 9(123), 16–22. <https://doi.org/10.32743/UniChem.2024.123.9.18172>
 9. Olennikov, D. N., Kashchenko, N. I., & Chirikova, N. K. (2015). Anti-inflammatory and antioxidant activity of *Plantago major* extracts. *Pharmaceutical Chemistry Journal*, 49, 118–122.
 10. Samuelsen, A. B. (2000). The traditional uses, chemical constituents and biological activities of *Plantago major* L. *Journal of Ethnopharmacology*, 71(1–2), 1–21. [https://doi.org/10.1016/S0378-8741\(00\)00212-9](https://doi.org/10.1016/S0378-8741(00)00212-9)
 11. Sinyuk, I. V., & Dudarev, V. A. (2015). Svertyvayushchey sistemy krovi i krovosnabzheniya golovki tazobedrennogo sustava v patogeneze bolezni Pertesa. *Mezhdunarodnyy zhurnal eksperimental'nogo obrazovaniya*, 11(1), 78–80.
 12. Sree Kumari, C., Reddy, B. S., & Kumar, B. A. (2015). In vitro anti-inflammatory and anti-arthritic activity of *Rhizophora mucronata* leaves. *International Journal of Pharma Sciences and Research*, 6(3), 482–485.
 13. Strobel, G., & Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews*, 67(4), 491–502. <https://doi.org/10.1128/MMBR.67.4.491-502.2003>
 14. Toghueo, R. M. K., & Boyom, F. F. (2019). Endophytic fungi from *Terminalia* species: A comprehensive review. *Journal of Fungi*, 5(2), 43. <https://doi.org/10.3390/jof5020043>
 15. Zhao, M., Ruan, Q., Pan, W., Tang, Y., Zhao, Z., & Cui, H. (2020). New polyketides and diterpenoid derivatives from the fungus *Penicillium sclerotiorum* GZU-XW03-2 and their anti-inflammatory activity. *Fitoterapia*, 143, 104561. <https://doi.org/10.1016/j.fitote.2020.104561>
 16. Zubair, M., Ekholm, A., Nybom, H., Renvert, S., Widen, C., & Rumpunen, K. (2012). Effects of *Plantago major* L. leaf extracts on human oral epithelial cells in a scratch assay. *Journal of Ethnopharmacology*, 141(3), 825–830. <https://doi.org/10.1016/j.jep.2012.03.02>