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IN VITRO INVESTIGATION OF ANTIOXIDANT AND ANTICOAGULANT PROPERTIES OF *Calocybe indica* (MILKY MUSHROOM)

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The growing demand for natural therapeutic compounds has intensified the search for bioactive molecules with diverse therapeutic properties, including antioxidant and anticoagulant activities. *Calocybe indica* (milky mushroom), an edible mushroom widely cultivated on a large scale in South Asia, is known for its richness in phytochemicals. However, research on *C. indica* is relatively less, particularly regarding its bioactivity and the majority of the studies were carried out on its nutrient content. Therefore, this study was conducted to evaluate and compare the antioxidant and anticoagulant activities of aqueous, methanol, dichloromethane (DCM), and hexane extracts of *C. indica* fruiting bodies. Authenticated fruiting bodies of *C. indica* were freeze-dried, cold macerated, and concentrated. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method, while antioxidant activity was measured using the 2,2-diphenyl-1 picrylhydrazyl (DPPH) radical scavenging assay. Among the extracts, the methanol and aqueous extracts exhibited the highest antioxidant activity, with the lowest IC_{50} values of 561.7 mg/mL and 549.8 mg/mL, respectively, indicating strong free radical scavenging potential. These were followed by the hexane (788.3 mg/mL) and DCM (961.6 mg/mL) extracts. A similar trend was observed in TPC values, further confirming these activities, with the aqueous extract showing the highest TPC (176.96 mg GAE/g), demonstrating a strong correlation between phenolic content and antioxidant activity. Anticoagulant activity was evaluated by measuring the prolongation of prothrombin time (PT). IBM-SPSS version 26 was used for statistical analysis. The bar chart analysis indicated that all four extracts, at varying concentrations, caused a significant increase in PT values compared to the control, confirming anticoagulant potential. All extracts contributed to prolonged coagulation time, highlighting the potential of *C. indica* as a natural anticoagulant. This study highlights the promising antioxidant and anticoagulant potential of *C. indica*, supported by its high phenolic content and ability to prolong prothrombin time. These findings are consistent with previous studies that report the therapeutic effects of mushroom-derived compounds such as polysaccharides and phenolics. Further research is needed to isolate the active constituents and confirm their efficacy and safety through in vivo studies. *C. indica* shows strong potential for development as a natural therapeutic agent against oxidative stress and thrombotic disorders.

Keywords: *Calocybe indica*, milky mushroom, antioxidant, anticoagulant activity

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INTRODUCTION

Calocybe indica, a mushroom native to tropical Asia and widely cultivated in Sri Lanka, has long been appreciated for its nutritional and medicinal values (Miles & Chang, 2004). It is rich in bioactive compounds such as polysaccharides, flavonoids, phenolics, and terpenoids, which are associated with notable antioxidant and anticoagulant properties (Shashikant et al., 2022). Antioxidants play a critical role in scavenging free radicals and preventing oxidative stress-induced cellular damage. Anticoagulant agents help regulate blood coagulation pathways, thereby reducing the risk of thrombotic disorders. Despite its growing use in nutraceutical formulations, *C. indica* remains relatively understudied, particularly in terms of *in vitro* pharmacological evaluations that consider solvent-specific bioactivity (Datta et al., 2024), this study aimed to investigate the functional properties of *Calocybe indica* through *in vitro* assays by evaluating the antioxidant and anticoagulant activities of its aqueous, methanol, dichloromethane (DCM), and hexane extracts.

METHODOLOGY

Fresh fruiting bodies of *Calocybe indica* were collected from a commercial mushroom cultivation facility located in Piliyandala, Sri Lanka. The specimens were authenticated based on morphological characteristics before being processed, which involved dehydration, grinding into a fine powder, and subsequent solvent extraction. Extractions were carried out using four different solvents: water, methanol, DCM, and hexane, at a 1:10 weight-to-volume (w/v) ratio to ensure a broad spectrum of bioactive constituents. The bioactivity of the resulting extracts was assessed through a series of *in vitro* assays.

Antioxidant potential was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and supported by the Total Phenolic Content (TPC) assay, with gallic acid employed as the standard reference compound (Dominguez-López et al., 2024; Munteanu & Apetrei, 2021). Anticoagulant activity was evaluated by measuring Prothrombin Time (PT) using human plasma samples against a control (Nataraj et al., 2022). All experimental data were subjected to statistical analysis using IBM SPSS version 26 and GraphPad Prism software, where IC_{50} values, correlation coefficients (R^2), and p-values were calculated to assess the potency and statistical significance of the observed effects.

RESULTS AND DISCUSSION

Antioxidant Activity

The aqueous and methanol extracts of *Calocybe indica* demonstrated notable antioxidant potential *in vitro*. As mentioned in the table 1, among the tested extracts, the aqueous extract showed the highest total phenolic content (TPC), measured at 176.96 mg gallic acid equivalents per gram (mg GAE/g), followed by the methanol extract at 102.94 mg GAE/g. In the DPPH radical scavenging assay, both extracts exhibited strong antioxidant activity, as reflected by their comparatively low IC_{50} values, which indicate greater potency. Trolox, used as the standard antioxidant, showed an IC_{50} value of 106.5 mg/mL, providing a reference point for the evaluation of extract efficacy. The pronounced antioxidant effects observed are likely due to the presence of bioactive compounds such as flavonoids and phenolic compounds, which are known for their ability to neutralize free radicals and mitigate oxidative stress.

Table 22. TPC and IC_{50} values from DPPH radical scavenging assay of aqueous, methanol, dichloromethane (DCM), and hexane extracts of *Calocybe indica* fruiting bodies.

Extract	TPC (mg GAE/g)	DPPH IC50 (mg/mL)
Aqueous	176.96	549.8
Methanol	102.94	561.7
DCM	57.55	961.6
Hexane	87.25	788.3

Anticoagulant Activity

All solvent extracts of *Calocybe indica* exhibited anticoagulant effects by prolonging coagulation time in a dose-dependent manner, as observed in Prothrombin Time (PT) assays (Figure 1). Among them, the methanol and aqueous extracts showed the most pronounced activity, indicating their potential effectiveness in interfering with thrombin formation and thereby extending clotting time. These findings highlight the potential of *C. indica* as a natural source of anticoagulant agents, with possible applications in promoting cardiovascular health and preventing thrombotic conditions.

Collectively, the findings demonstrated the functional versatility of *Calocybe indica*, particularly in terms of its antioxidant and anticoagulant properties. Among the tested extracts, the aqueous and methanol extracts consistently demonstrated the highest levels of bioactivity, highlighting their potential as effective sources of natural therapeutic agents. These results not only support the traditional and emerging use of *C. indica* as a functional dietary component but also provide a strong foundation for future investigations focused on the isolation, characterization, and formulation of its active constituents. Such research could further enhance its applicability in the development of nutraceuticals aimed at combating oxidative stress and preventing thrombotic disorders.

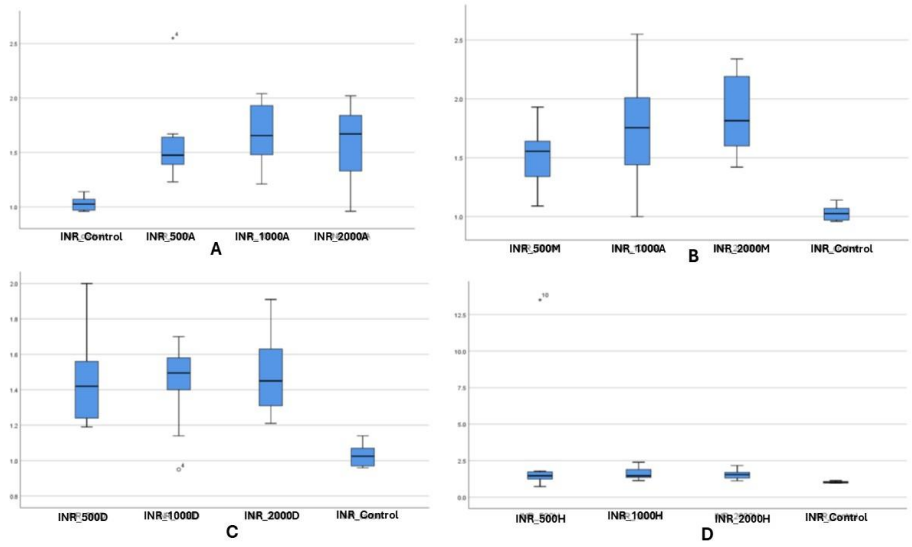

Figure 31. Box plots represent the distribution of INR values across replicates. Effect of different concentrations (500, 1000, and 2000 µg/mL) of *Calocybe indica* extracts on prothrombin time (PT), represented by INR values. (A) Aqueous (B) Methanol (C) Dichloromethane (DCM) (D) Hexane extracts.

CONCLUSIONS/RECOMMENDATIONS

This study presents the first comparative evaluation of *Calocybe indica* extracts prepared using different solvents with a particular focus on both antioxidant and anticoagulant activities. Among the extracts tested, methanol and aqueous extracts exhibited the most promising antioxidant properties, while also demonstrating moderate anticoagulant effects. These bioactivities, coupled with the mushroom's natural origin and low toxicity, support its potential as a valuable ingredient in functional food formulations and herbal pharmaceutical preparations.

Based on the outcomes of this study, further investigations are recommended to isolate and characterize the specific bioactive compounds responsible for the observed functional properties. In addition, *in vivo* assays and comprehensive toxicity evaluations should be conducted to assess safety and therapeutic efficacy in biological systems, thereby enhancing clinical relevance. Exploring synergistic combinations with other natural products or antibiotics may also reveal enhanced or broadened pharmacological effects, potentially leading to the development of more effective nutraceutical or therapeutic formulations. These future directions are essential to unlock the full medicinal potential of the mushroom *Calocybe indica*.

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