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Analysis of secondary metabolites, antioxidant properties, HPLC analysis, and protein content of *Calostoma insigne* in Cambodia

Lina Ly[1], Visessakseth So[1], Sokunvary Oeung[2], Huykhim Ung[3], Philip Poul[1], Kimchhay Mao[3], Sreyroth Norng[3], Vottey Nara[3], Mengky Keun[3], Mengkhim Heim[4], Sin Chea[4]*

[1]Division of Pharmacognosy, Faculty of Pharmacy, University of Puthisastra, Phnom Penh, Cambodia
[2]Division of Toxicology, Faculty of Pharmacy, University of Puthisastra, Phnom Penh, Cambodia
[3]Division of Basic Pharmaceutical Sciences, Faculty of Pharmacy, University of Puthisastra, Phnom Penh, Cambodia
[4]Department of Pharmacy, Faculty of Pharmacy, University of Puthisastra, Phnom Penh, Cambodia

*Corresponding author: Sin Chea, csin1@puthisastra.edu.kh

ABSTRACT

Background: *Calostoma insigne* (*C. insigne*) is a wild edible mushroom commonly found in the forests of Southeast Asia. In Cambodia, it is traditionally consumed as a dessert, with locals believing in its health benefits. However, there is a significant lack of research investigating its therapeutic properties and chemical compounds, particularly within the Cambodian context.

Objectives: This study aimed to explore the secondary metabolites, protein content and antioxidant activities in *C. insigne.*

Methods: Methanolic extract of *C. insigne* was utilized to define the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activities was screened using ferric reducing antioxidation power assay (FRAP) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging in microplate spectrophotometers. Protein content was performed using Kjeldahl method. HPLC detected gallic acid, chlorogenic acid, and luteolin in the crude extraction.

Results: The analysis showed significant levels of secondary metabolites with TPC (9.28 ± 0.51 mg GAE/g of CE), TFC (242.41 ± 23.82 mg quercetin equivalent/g of crude extract). Antioxidant activities of DPPH radical revealed a concentration dependent manner ($P < 0.05$), and HPLC detected luteolin (15.83 ± 0.01 mg/g per crude extraction). TFC showed a strong statistically significantly negative correlation with FRAP value explained $r = -0.879$, $P < 0.05$. TFC could be the biomarker in extraction with high-reducing antioxidant power.

Conclusion: This study confirmed the presence of secondary metabolites, their potential as antioxidant activities, and protein content for further development in food products, pharmaceutical and natural health products.

Keywords: Antioxidant, HPLC, Mushroom, Protein, Secondary metabolites

1. Introduction

Cambodia is recognized for its rich biodiversity, encompassing a variety of species including animals, insects, reptiles, plants, and fungi. In the Centre Cardamon Protected Forest (CCPF), researchers have identified 48 genera of mushrooms, contributing to the understanding of their diversity [1]. Mushrooms are significant as both food and medicine, being associated with health-boosting properties, cancer risk reduction, blood sugar regulation, and detoxification mechanisms [2]. Edible mushrooms are rich in protein, vitamins, carbohydrates, fibre, antioxidant properties, including polysaccharides, which contribute to their numerous health benefits, such as antifungal and antimicrobial activities. These properties make them valuable in the nutraceutical and pharmaceutical industries [3].

The phenolic content and flavonoids in mushrooms are secondary metabolites which are particularly noteworthy due to their substantial health benefits. Phenolic compounds are recognized for their potent antioxidant properties, which play a critical role in neutralizing free radicals and reducing oxidative stress, a key factor in the development of chronic diseases such as cancer and cardiovascular diseases [4]. Studies indicated that the antioxidant activity of mushrooms correlates closely with their total phenolic and flavonoid content, suggesting that these compounds significantly contribute to the health-promoting effects of edible mushrooms [5, 6]. Furthermore, flavonoids are known to exhibit anti-inflammatory and anti-cancer properties, enhancing the overall therapeutic potential of mushrooms [7].

One edible wild mushroom favoured for consumption as a dessert is *Calostoma insigne*, which belongs to *the Calostomataceae* family and *Calostoma* genus [8]. This species has a distinctive appearance, characterized by a jelly-like fruit body surrounding a powdery spore layer, resembling a bulbous formation akin to a golf ball [9]. It can be found in Southeast Asian countries with tropical rainforests on the hills, thriving in decayed leaves and soil during the rainy season; however, it is listed as an endangered species due to deforestation and agricultural activities [10]. It has a globose white gelatinous fruit body and powdery spores inside [11]. This mushroom is popular among the Cambodian population for being consumed raw with milk and ice as a sweet treat. It is believed to have therapeutic

properties that can alleviate stomach aches and reduce fever [12].

Despite the known benefits, research on *C. insigne* remains limited. While some studies in Thailand have explored its antimicrobial, antimicrobial, anti-glucosidase, and anticancer properties [13]. Regarding its bioactive compound, antioxidant activities, protein, and HPLC analysis, still lack of scientific evidence exploring and claims for its health benefit in Cambodia.

Therefore, this study aimed to investigate the secondary metabolites, antioxidant activities, and protein content in *C. insigne* to assess its potential benefits for human health.

2. Methods

2.1 Study Area

C. insigne was collected in August 2023 during rainy season in Tbaeng mountain, which is in Tbaeng Meanchey district, Preah Vihear Province, Cambodia (latitude 13.6824770º N and longitude 104.9535380º W) (Figure 1).

Figure 1: Cultivated location of *C. insigne* in Tbaeng mountain, Preah Vihear Province. These images adapted from google earth

2.2 Study Design

This study utilized a descriptive experimental design aimed at investigating the secondary metabolites, antioxidant properties, protein content, and HPLC analysis of *Calostoma insigne*. The study was conducted between August and December 2023, focusing on samples collected from the Tbaeng mountain region in Preah Vihear Province, Cambodia. The design comprised laboratory-based analyses, including quantitative and qualitative assessments of secondary metabolites and antioxidant activity through

spectrophotometric and chromatographic methods. The experimental framework included the following phases:

- Sample Collection: Wild *C. insigne* mushrooms were collected during the rainy season from a tropical forest.
- Preparation and Extraction: Samples underwent methanolic extraction to isolate crude extracts.
- Laboratory Analysis: The extracts were subjected to phenolic and flavonoid content determination, antioxidant assays, protein quantification using the Kjeldahl method, and HPLC profiling for phytochemical analysis.

The study's design emphasized precision in measurements and reproducibility through the use of validated methodologies and multiple replicates.

2.3 Sample size and sampling

A purposive sampling method was employed to ensure the inclusion of high-quality *C. insigne* specimens for analysis. The samples were collected from a defined area in Tbaeng mountain, geographically identified as latitude 13.6824770°N and longitude 104.9535380°W. The sampling strategy accounted for the following:

- Inclusion Criteria: Only mature *C. insigne* specimens with intact gelatinous and powdery spore structures were collected to ensure consistency in chemical composition.
- Exclusion Criteria: Damaged or prematurely harvested mushrooms were excluded to minimize variability in metabolite concentrations.
- Sample Size Determination: Approximately 1 kilogram of fresh mushrooms was harvested, yielding sufficient powdered spore material for analytical replication (n=5 per assay). The sample size was based on prior studies that determined optimal quantities for extraction and subsequent assays.

2.4 Data Collection

2.4.1 Materials and reagents

Chlorogenic acid was bought from AK Scientific (Union, CA, USA). Folin reagent, aluminium chloride, ferric chloride, ferrous sulphate, sodium hydroxide, sodium carbonate, acetic acid, sulfuric acid, hydrochloric acid and methanol in analytical

grade were bought from Merck KGaA (Darmstadt, Germany). Methanol and acetonitrile in HPLC grade were bought from RCI Labscan V.S. CHEM HOUSE (Bangkok Thailand). Gallic acid was bought from Acros Organics BV (Geel, Belgium). 2,2-Diphenyl-1-picrylhydrazyl was bought from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was bought from Thermo Fisher Scientific (Fair Lawn, NJ, US). Quercetin and boric acid were bought from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Luteolin was bought from LGC Labor GmbH (Augsburg, Germany). VAT catalyst tablets were bought from VELP Scientific Srl, (Usmate (MB) - Italy). Dimethyl sulfoxide (DMSO) was bought from VWR International (Fontenay-sous-Bois, Franch).

2.4.2 Mushroom Extraction

Firstly, mushrooms were cleaned with Distilled Water (DW) and cut to isolate powdery spores inside the gelatinous layer. Secondly, the powdery spores 20 grams (g) were transferred to 10 millilitres (ml) of hydrochloride acid (HCl) in methanol and subjected to ultrasonicated for 30 minutes (min) at 30 degrees Celsius (°C) to extract compounds. Rotary evaporator (IKA RV10, Selangor, Malaysia) was operated to filter mixture and separated solvent to collect crude extraction at room temperature. Crude extraction was calculated to find percentage of extraction yield following formula [14]:

Extraction yield (%) = (Mass of crude extraction × 100)/ Mass of powdery spores (1)

Lastly, crude extraction was stored in a fridge at 4 °C for further analysis.

2.4.3 Total Phenolic Content (TPC) by Folin-Ciocalteu method

The crude extraction was dissolved with 1 ml of dimethyl sulfoxide (DMSO). TPC was detected by *Folin-Ciocalteu* method [15, 16]. The 120 microlite (µl) was added with 15 µl of DMSO for blank in in 96 well microplates. Then, gallic acid standard was utilized for the positive control that dissolved in DMSO to prepare different concentrations from 5 to 15 µg/ml. For assay, 15 µl of crude extraction was mixed with Folin reagent (120 µl) in a microplate as the sample and covered with aluminium paper for 5 min away from light at room temperature. After that, sodium carbonate (Na_2CO_3) buffer was added in both sample and blank and kept for another 90 min at room temperature. The absorbance of blue solution contained molybdenum (V) in Folin reagent was adjusted at wavelength 725

nanometre (nm) with a microplate spectrophotometer (Thermo Scientific™ Multiskan™ FC, Boston, MA, USA) to create a curved from plot between absorbance against gallic acid concentration [17]. TPC was estimated from this gallic acid standard curve (y = 0.0593x - 0.0693, R^2 = 0.9982). This experiment was finalized with five replicates of each blank, sample, and standard.

2.4.4 Total Flavonoid Content (TFC) by aluminium chloride buffer

TFC was estimated by using aluminium chloride ($AlCl_3$) [15, 16]. The 100 µl of DMSO was added with 2% of $AlCl_3$ buffer for blank in a microplate. Then quercetin was dissolved in DMSO and prepared different concentrations from 20 to 60 µg/ml as a standardized compound. After that, crude extract was mixed with $AlCl_3$ as 1:1 ratio a microplate and kept for 60 min at temperature room. The experiment was finalized with five replicates of blank, sample, and standard. Test solution of absorbance was estimated at the wavelength of 400 nm in microplate spectrophotometers (Thermo Scientific™ Multiskan™ FC, Boston, MA, USA) [17]. TFC was estimated from this quercetin standard curve (y = 0.018x – 0.1733, R^2 = 0.9938).

2.4.5 Antioxidant Activities

Ferric Reducing Antioxidant Power Assay

Ferrous sulfate ($FeSO_4$) and quercetin were dissolved in DMSO. FRAP reagent was prepare by mixing of acetate buffer (pH 3.6), ferric chloride ($FeCl_3$), and 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) solution in a 10:1:1 ratio [18, 19]. The mixture was added into each well of a microplate in 1:1 ratio with each five replications, and the plate was covered with aluminium paper for 30 min in room temperature. The TPTZ absorbance complex colour was adjusted at 593 nm with a microplate spectrophotometer (Thermo Scientific™ Multiskan™ FC, Boston, MA, USA) with DMSO blank [20]. FRAP was calculated at the different concentrations from the standard curve of $FeSO_4$ (y= 0.0463x – 0.1845, R^2= 0.9915). Quercetin was used as a positive control in this test (y= 0.0404x – 0.922, R^2= 0.9966).

DPPH Radical Scavenging Assay

Different concentrations of crude extraction were dissolved in DMSO from 100 to 1,000 µg/ml and DPPH reagent was added in 1:1 ratio of the microplate for five replications and keep for 30 min in the dark place at the room temperature. Absorbance loss of DPPH

radical was measured at 515 nm by using the microplate spectrophotometer (Thermo Scientific™ Multiskan™ FC, Boston, MA, USA) [21]. Gallic acid with different concentration was plotted as a standard curve ($y = 10.493x + 41.785$, $R^2 = 0.9978$) [22, 23]. DPPH radical scavenging quantity was presented as DPPH radical inhibition percentages at 50% (IC50). Percentages (%) of scavenging effect of DPPH were estimated according to equations [24]:

%Percentages of DPPH radical scavenging effect= (Absorbance of control – Absorbance of crude extraction)/ (Absorbance of control) × 100 (2)

- Absorbance of control= absorbance or a reaction combination in absence of sample antioxidant.
- Absorbance of crude extraction= absorbance of reaction mixture in crude extraction present.

2.4.6 Protein Content by Kjeldahl Method

Fresh mushrooms were washed and blended into a homogenous mixture. Approximately 1 g of the sample was accurately weighed and transferred into a digestion tube. Two catalyst tablets of VST (code A00000277) and 15 ml of concentrated sulfuric acid (H_2SO_4) were also added and mixed well. The digestion process was performed using a VELP DK 8 Digestion unit (VELP Scientifica Srl, Usmate (MB) - Italy) at 420ºC for 60 mins. After digestion, the tubes were allowed to cold from 50 to 60 °C. The distillation was performed using a VELP UDK129 Distillation unit (VELP Scientifica Srl, Usmate (MB) - Italy), where a 1:1 ratio of water and sodium hydroxide (NaOH) was used. The distilled nitrogen was collected in 4% of 30 ml of boric acid. Boric acid solution and then titrated with 0.2 N of hydrochloric acid. The blank was carried out in underwent the same process without mushrooms sample. The protein content was calculated by [25]:

- Protein (%) = $[(V_{sample} - V_{blank}) \times 14.007 \times 0.2 \times 6.25]$ / Mass of sample (3)

Where V_{sample} was the ml of HCl used for titrated of the sample, V_{blank} was the ml of HCl used for titrated of the blank, 14.007 was the atomic mass of nitrogen, 0.2 in the normality of HCl, and 6.25 was the conversion factor for nitrogen to protein.

2.4.7 High Performance Liquid Chromatography (HPLC)

The crude extraction of *C. insigne* was determined by HPLC. The analysis was

implemented using an LC-2030C3D quaternary pump (Shimadzu, Kyoto, Japan) equipped with a diode array detector (DAD). The crude extraction was dissolved in DMSO, filtered through a 0.45 μm membrane filter, and injected into the GIST C18 shim-pack column (4.6 × 250 mm, 5 μm) (KTA Technologies Corporation, Tokyo, Japan) maintained at the temperature of 38 °C. An autosampler injected 20 μl of the sample with the mobile phase flowing at 0.8 ml/min. The mobile phase contained of 1% acetic acid in purified water (A) and acetonitrile (B). The gradient elution was employed as stated: from 0 to 5 min, a linear gradient from 5 to 9% of acetonitrile; from 5 to 15 min, 9 to 11% of acetonitrile; from 15 to 22 min, linear gradient from 11 to 15% of acetonitrile; from 22 to 30 min, linear gradient from 15 to 18% of acetonitrile; and from 30 to 38 min; from 38 to 43 min, linear gradient from 23 to 80% of acetonitrile; from 43 to 46 min, linear gradient from 80 to 80% acetonitrile; from 46 to 55 min, isocratic at 90 to 95% acetonitrile; from 55 to 60 min, linear decreased from 95 to 5% acetonitrile; with a 10 min re-equilibration period of 5% of acetonitrile used among the runs. The detector wavelengths were set to 280 nm, 380 nm, and 450 nm. Compounds were detected by comparing their retention time and UV spectra standards. The phenolic and flavonoid standards which included gallic acid (1), chlorogenic acid (2), luteolin (3).

2.5 Data Analysis

The result was described as mean ± standard deviation (SD) of five replications (n=5). Correlation coefficient analysis was performed using Statistical Package for the Social Science (SPSS) Version 20, SPSS Inc, Armonk, NY, USA. ANOVA post-hoc analysis with Turkey corrected at $P < 0.05$ was used to compare the percent of inhibition of DPPH radical of *C. insigne* extraction

2.6 Ethical Clearance

This study was approved by Committee in Research at University of Puthisastra (No. 22IR24)

3. Results

Mushroom Characteristics

C. insigne that was collected from Tbaeng mountain, located in Preh Vihea province, Cambodia, had a round shape and a striking bright white-brown color (Figure 2.a). Tbaeng mountain is tropical rainforest where the mushroom was collected. It grows on humid soil with decomposition leaves, and people can harvest it only once per year in rainy season from forest. The size of this

mushroom varies with maximum between 2.5 millimeter (mm) to 4 mm while the minimum size was between 0.5 mm to 1 mm (Figure 2.b). The gelatinous layer is wet and sloppy while the spore is dry powdery (Figure 2.c).

Figure 2: (a) *Calostoma insigne* mushroom growing on soil and dead leave in the forest, (b) the mushroom's lengths, and (c) gelatinous fruitbody (white arrows) and powdery spore (black arrows)

Secondary metabolic and protein content

The percentage (%) yield of *C. insigne* by methanol extraction was 0.25% and was obtained in this study. The secondary metabolic results of TPC were described standard gallic acid equivalent (GAE) in milligrams per gram of crude extraction (CE). *C. insigne* had total phenolic content of 9.28 ± 0.51 mg GAE/g of CE (Table 1). Flavonoid content contained 242.41 ± 23.82 mg QE/g of CE (Table 1). The result of protein content was found to be 4.38 ± 0.00 mg/g of CE (0.44%) which provides information insight from *C. insigne* characteristics.

Table 1: Secondary metabolites, antioxidant activities, and protein in spore extraction of *C. insigne*

Secondary metabolites/Antioxidant Activities	Content (mean ± SD)
Total phenolic content (mg GAE/g of CE)	9.28 ± 0.51
Total flavonoid content (mg QE/g of CE)	242.41 ± 23.82
Ferric reducing antioxidant power (mM FeSO4/g of CE)	25.81 ± 2.06
Inhibition concentration at 50% (IC_{50}) of DPPH radical (µg/ml)	483 ± 47.07
Protein content (mg/g of CE)	4.38 ± 0.00

Antioxidant Activities

Crude extraction of *C. insigne* showed ferric reducing antioxidant power assay value was described as millimoles of $FeSO_4$ per gram of crude extract presented as FRAP power 25.81 ± 2.06 mM $FeSO_4$/g of CE (Table 1). The result of DPPH radical scavenging activities showed the concentration-dependent manner ($P < 0.05$) (figure 3) and was exhibited 483 ± 47.07 µg/ml as concentrations of inhibitory at

50% (IC_{50}) as shown in Table 1. A lower IC_{50} values indicated higher DPPH scavenging activity.

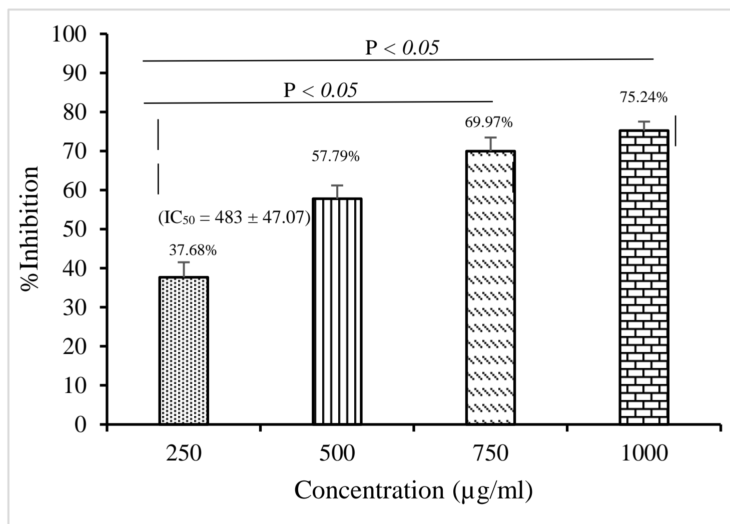

Figure 3: ANOVA post-hoc analysis with Tukey's multiple comparisons of the percent of inhibition of DPPH radical of *C. insigne* extraction. Data was expressed as mean ± SD from five replicates. The significant difference was set at P-value < 0.05

High-Performance liquid chromatography

Phenolic and flavonoid contents were assessed with three different wavelengths (280 nm, 380 nm, and 450 nm) to identify the compound by using HPLC analysis through the comparison of the retention times of peak sample with the various standards including gallic acid, chlorogenic acid, and luteolin as shown in Table 2 and Figure 4. The concentrations of these two contents were analyzed based on the peak area of the compound, while the linear regression equation showed $y = 77020x + 7436.1$ with a good correlation coefficient (R^2) was 0.9993. The compound luteolin was identified in *C. insigne* at the retention time of 44.86 min with the concentrate 15.83 ± 0.01 mg/g of crude extraction. The results presented are shown in Table 2.

Table 2: The HPLC analysis of the phenolics and flavonoid standard display retention time and detection with three different wavelengths

Standard Compounds		Retention Time (min)			Detected amount (mg/g of crude extract)
		280 nm	380 nm	450 nm	
Hydroxybenzoic Acids	Gallic acid (1)	6.82 ± 0.02	n/a	n/a	n/a

Standard Compounds		**Retention Time (min)**			**Detected amount (mg/g of crude extract)**
		280 nm	**380 nm**	**450 nm**	
Hydroxycinnamic Acids	Chlorogenic acid (2)	17.06 ± 0.02	17.06 ± 0.02	n/a	n/a
Flavone	Luteolin (3)	44.86 ± 0.00	44.86 ± 0.00	n/a	15.83 0.01

*n/a= not applicable

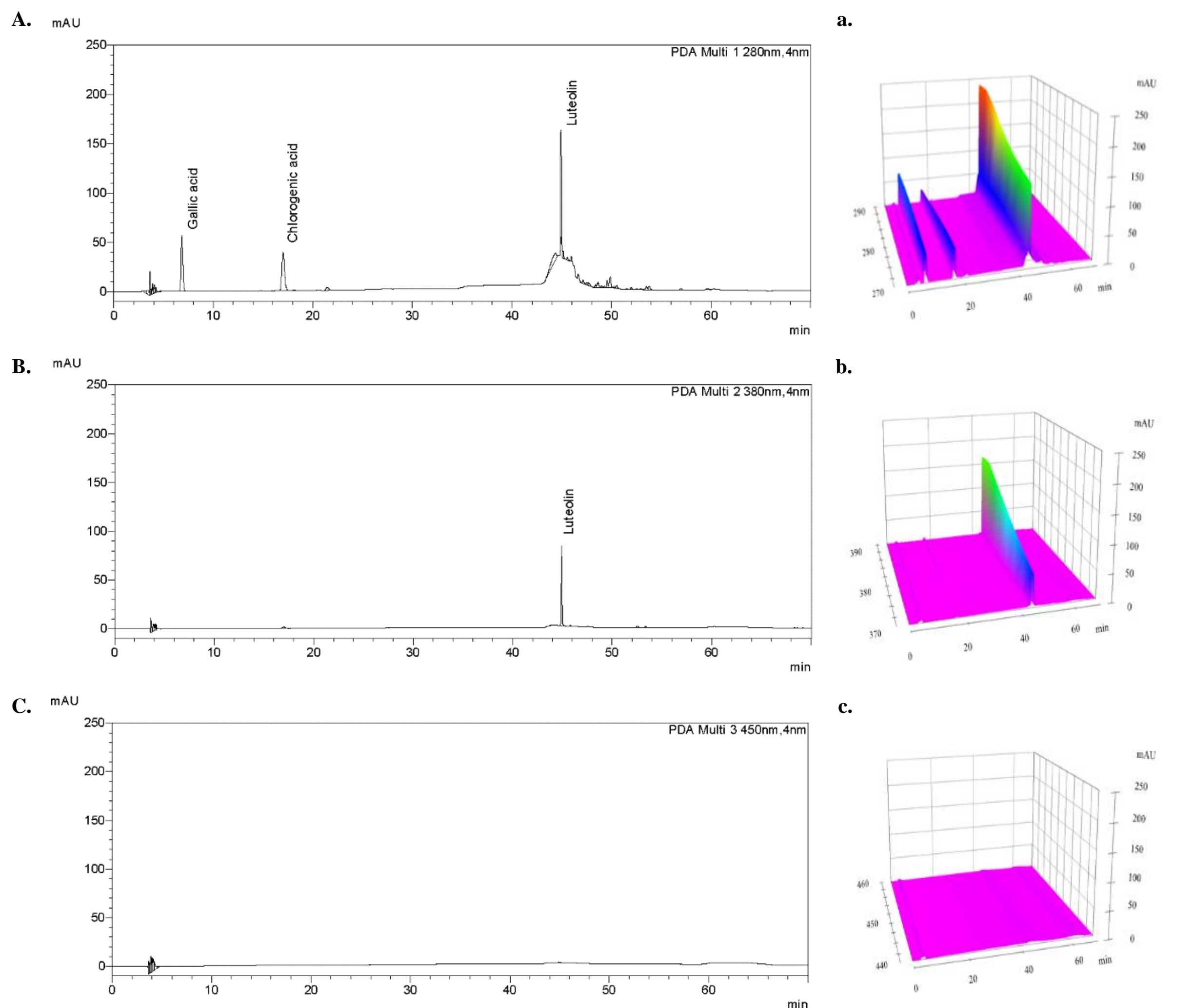

Figure 4: The mixture of three standards of phenolics and flavonoid in each wavelength A.280 nm, B. 380 nm and C. 450 nm. Big capital letter showed the 2D chromatogram while small letter showed 3D chromatogram

Correlational characteristics of *C. insigne* were shown in Table 3, which examined antioxidant capacities (FRAP and DPPH) and their relationship with secondary metabolites from chemical screening (TPC, TFC, and luteolin). The results revealed a significant negative correlation of FRAP with TFC ($r = -0.879$, $P < 0.05$), suggesting that TFC was

presented in extraction play a crucial role with FRAP value. TFC could be the biomarkers in extraction with high-reducing antioxidant power. However, luteolin was not with any antioxidant capacity of crude extraction.

Table 3: Illustration of correlation coefficient (r) between phytochemicals in *C. insigne* and antioxidant capacity (DPPH and FRAP). If the correlation coefficient r value is close to +1, it suggests the strong positive relationship or an inverse relationship. If r is near 0, it suggests a weak or no relationship

Test	TPC	TFC	LUT	FRAP	DPPH
TPC	**1.00**	-0.031 (P = 0.961)	-0.328 (P = 0.590)	-0.401 (P = 0.504)	0.459 (P = 0.437)
TFC	-0.031 (P = 0.961)	**1.00**	0.280 (P < 0.648)	-0.879* (P < 0.05)	0.087 (P = 0.999)
LUT	-0.328 (P = 0.590)	0.280 (P < 0.648)	**1.00**	-0.071 (P = 0.909)	-0.036 (P = 0.955)
FRAP	-0.401 (P = 0.504)	-0.879* (P < 0.05)	-0.071 (P = 0.909)	**1.00**	-0.857 (P = 0.064)
DPPH	0.459 (P = 0.437)	0.807 (P = 0.099)	-0.036 (P = 0.955)	-0.857 (P = 0.064)	**1.00**

*Statistically significant is noted as P < 0.05. Abbreviations used are TPC = total phenolic content, TFC = total flavonoid Content, LUT = luteolin, DPPH = DPPH radical scavenging activity, FRAP = ferric-reducing antioxidant power

4. Discussion

Our study may serve as the fundamental scientific evidence of *C. insigne* in Cambodia with its secondary metabolites including antioxidant activities and protein content properties. Among extraction solvent, methanol mostly is significantly chosen based its chemical properties to obtain the phenolics and flavonoid compounds from plants and mushroom, i.e., *Pleurotus floraida, Macrolepiota procera, Psilocybe Natalensis, Cantharellus cibarius, Hydnum repandum, Lepista sorduda, Lycoperdon pyriforme, Neolentinus lepideus,* and *Russula virescens* mushrooms were extracted with methanol to obtain crude extraction to study phytochemical contents and antioxidant activities in their research [26-28]. Following to the author *Worachot et.al* [13], studied on gelatinous body of *C. insigne* found TPC (1.00 ± 0.05 mg GAE/g extract), TFC (3.51 ± 0.18 mg RE/g extract), FRAP (1.73 ± 0.01 mg FeSO4/g extract), which were lower than our study in powdery spore which was found TPC (9.28 ± 0.51 mg GAE/g of CE), TFC (242.41 ± 23.82 mg QE/g of CE), and FRAP (25.81 ± 2.06 mM $FeSO_4$/g of CE), respectively. Those could explain why the whole mushroom contains benefit pharmacology for people's health, and they can eat both gelatinous bodies and powdery spores of *C. insignis*. As mentioned above, *C. insigne* is a wild mushroom. Research on various edible wild mushrooms from

different regions found secondary metabolites and antioxidant activities to offer health benefits compared to other mushrooms. For instant, *Pleurotus eous* had TPC equal to 13.03 ± 1.05 µg GAE/mg of extract, and Pleurotus ostreatus found TFC equal to 3.71 ± 0.73 mg GAE/ g of extract were considered as value phytochemical contents and beneficial therapeutic properties for human health [29]. Additional studies have reported *Paraleptista flaccida* had TPC (32.86 ± 0.52 mg GAE/mg of extract) and TFC (10.34 ± 0.60 mg catechin equivalent /g of extract); meanwhile, *Leptista nuda* had TPC (25.52 ± 0.56 mg GAE/mg of extract) and TFC (19.02 ± 0.80 mg catechin equivalent/g of extract) [30]. DPPH IC50 value also was found in *Armillaria mellea* (1.32 ± 0.09 mg/ml of extract) and *Marcrolepopta procera* (1.31 ± 0.05 mg/ml of extract) [31]. Phenolic and flavonoid compounds were reported to explain pharmacological activities against disease including antioxidant, anticancer, antidiabetic, or anti-inflammatory activities [32]. In our study, TFC revealed a significant positive correlation of FRAP ($r = -0.879$, $P < 0.05$), suggesting that TFC was presented in extraction play a crucial role with FRAP value via hydrogen atom transferred mechanism [33]. HPLC is particularly employed to identify and quantify the chemical contents in mushrooms [34]. The result showed a luteolin peak that was presented phenolic and flavonoid in *C. insigne* mushroom. Luteolin is known as a flavonoid that applies therapeutic from natural polyphenols to provide health benefits such as hypertension, anti-inflammatory, antioxidant, and cancer [35, 36]. However, TFC showed a strong statistically significantly positive correlation with FRAP value explained as $r = -0.879$, $P < 0.05$, indicating the strong reducing antioxidant power via a single electron transfer mechanism [37]. Beside the therapeutic properties of wide edible mushroom, there are many reports on its nutritional status typically, rich in protein and their low fat or fiber content [38]. In our result presented about *C. insigne* that had a relatively low protein content (0.44%). However, *Agaricus bisporus* from USA, found a high level of complete protein about 20% and could potentially replace traditional protein source [39]. According to the article from China [40], different mushroom species exhibited significant variation in protein content such as *Volvariella volvacea* stood out with the highest protein around 28 to 35% and *Pleurotus ostreatus* provide protein around 21.1 to 34.5%, while *Flammulina*

velutipes also offered a notable protein 18.6 to 28% with *Agaricus bisorus* around 14.1 to 27.10%. Furthermore, a study conducted in Malaysia found protein content in *Lentinula edodes* 16%, *Pleurotus ostreatus* 18.20%, *Hypxizygus marmoreus* 14.0%, and *Flammulina velutipes* 15.80% [41]. Also, in Indonesia that found *Volvariella volvacea* had protein content 2.70% [42]. The results of these detained the protein content of various mushroom species that expressed both in grams and as percentages to provide information and categorize the protein levels as high or low depending on specific species of mushrooms.

These findings support the use of mushroom powder as a base material for producing mushroom protein isolates and concentrates by demonstrating taste-related components and nutritional value. However, wild mushrooms can also be toxic and threaten health risk. Further toxic compounds screening should be explored. Wild mushrooms generally displayed various secondary metabolites and antioxidant activities depending on specific species.

5. Conclusion

The finding of this research provided the broad range of biological activities about secondary metabolites that exhibited antioxidant-included nutrition for human health benefits. The results from this research are crucial for realizing the potential benefits of developing *C. insigne* into pharmaceutical supplements, exploring its traditional uses, and apply it in food processing. This included innovations in natural products related to nutrition and cosmetic. Additionally, sharing knowledge about potential properties can help raise awareness and interest in fugal biodiversity. Moreover, future studies on this mushroom will be concentrated on different potential applications deep the understanding.

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