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Phytochemical Screening, Cytotoxic Activity, and Proximate Analysis of Split Gill Mushroom *(Schizophyllum commune)*

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ABSTRACT

Mushrooms play an essential role in the ecosystem and have provided humans with numerous benefits in terms of food and medicinal sources. The study was conducted to determine the phytochemical constituents,

cytotoxic activity, and proximate analysis of a widespread mushroom in the region,*Schizophyllum commune*. Qualitative phytochemical analysis and cytotoxic activity of the ethanolic extract were conducted using the test tube method and brine shrimp lethality assay (BSLA).Proximate analysis was carried out using the standards the Association of Analytical Chemists set. Results revealed the presence of saponin, tannins, alkaloids, flavonoids, terpenoids, proteins, and carbohydrates. The linear regression analysis showed that the extracts exhibited a highly cytotoxic activity on brine shrimp with an LC_{50} value of 55.64 ppm. The cytotoxicity of the extract is mainly attributed to its phytochemical content. Proximate analysis revealed that the sample was composed primarily of moisture with high protein and low-fat content levels. Results support that *S. commune* is a good protein source and a healthy diet choice. The study may serve for further ethnobotanical, nutritional, and pharmacological studies.

Keywords **—** Health Science, BSLA, cytotoxic activity, phytochemicals, proximate analysis, *Schizophyllum commune,* Philippines

INTRODUCTION

Human survival, from food, shelter, and other basic needs, has depended on the natural environment. Through time, we have developed ways to use or extract the components of living organisms, primarily plants, for countless ways and advantages. Today, we have continued our exploration to address the emerging concerns on health and well-being as Harvey (2008) emphasized that most of the active ingredients of medicine have been from natural products as the source. In addition, utilizing raw materials from the environment answers the need to develop organically produced and environment-friendly products, given that they are appropriately used(Cooper-Ordoñez et al., 2019; Newman & Cragg, 2020; Veeresham, 2012).

The exploration of natural products has led to the discovery of various important compounds to our benefit, especially in drug discovery. This field has faced challenges; however, improved analytical tools, genome mining, engineering strategies, and microbial culturing advances have opened opportunities for researchers (Atanasov et al., 2021; Khan, 2018).In addition, most of the active compounds have been isolated from plant and microbial sources. However, few studies on the biosynthetic pathways of fungi have been carried out in the past years (Schmidt-Dannert, 2015).

Mushrooms are typically used for food, but they also produce a variety of medicinal substances and carbohydrate-active enzymes (de Mattos-Shipley et al., 2016). *S. commune,* as one of the most widespread fungi in the world, produces a wide range of hydrolytic enzymes such as xylanases, endoglucanases, a large number of protein-coding genes, and expansins-like proteins, which are the potential to be used in a variety of biotechnological processes (Tovar-Herrera et al., 2015). Also, *S. commune* is being studied for gene integration (knock-in) (Vonk & Ohm, 2021); alternative gene splicing (Gehrmann et al., 2016); isolation of the first naturally occurring iminolactones from its fruiting bodies (Liu et al., 2015); sesquiterpenes that inhibit fungal growth and modify bacterial motility (Wirth et al., 2021); and *schizophyllan*, a commercially attractive biopolymer use in various industries (Mohammadi et al., 2018). Studies also showed the potential application of *S. commune* to the biodiesel industry (Singh et al., 2015); the combination of *S. commune* and cellulase resulted in a considerable increase in ethanol production rate (Horisawa et al., 2019).

Commonly known as split gill mushroom, *S. commune* is one of the many fungal species abundant in the Philippines. Field Reyes et al. (2013) cited local terms for the mushroom, including *kurakding* for Bicolanos, *kudopdop* for Visayans, and *kudit* for Ilocanos. This species is described as a tiny, light-brown fungus that clings tomoist rotting tree branches,particularly after a lengthy rain (Ortega, 2012). Tantengco and Ragragio (2018) have underscored that Aeta communities in the Philippines have utilized this mushroom as food and medicine. *S. commune* extracts have been found to have antimicrobial activity (Acanto & Cuaderes, 2021)and anti-inflammatory activity (Du et al., 2016), antioxidant and cytotoxic potential (Romadhonsyah et al., 2022). Kaur et al. (2018) also reported insecticidal potential and genotoxic and cytotoxic effects of the extracts. These properties are due to the polysaccharide *Schizophyllan*, a multipurpose compound applicable in many fields, including the food industry and pharmacy (Zhang et al., 2013).

The abundance of *S. commune* in the local community and its potential have led the researchers to pursue an investigation into this fungus. This study focused on determining the phytochemical constituents and cytotoxic activity of split gill mushroom S. commune ethanolic extracts. Moreover, it sought to determine the values of macronutrients in the sample through proximate analysis; to further enrich the literature and explore the fungus's potential.

The present study aimed to determine the phytochemical constituents and cytotoxic activity of split gill mushroom S. commune ethanolic extracts and conduct a proximate sample analysis.

OBJECTIVES OF THE STUDY

Specifically, the study aimed to (1) determine active compounds present in the extracts through phytochemical analysis, (2) test the cytotoxic activity of the extracts using the Brine Shrimp Lethality Assay, and (3) determine the total protein content, total fat content, crude fiber, total ash content, and free nitrogen extract of the sample through proximate analysis.

Utilizing local raw materials, in this case, *S. commune*, withits potential to benefit humans,would provide opportunities to have a cheaper alternative to producing natural products. Since the compounds are extracted from nature, it would lessen the adverse effects on humans and the environment compared to synthetically-produced ones. Thus, a safer and more environment-friendly option. It may also pave the way for discovering new possibilities for using the species.

In terms of local and cultural significance, this will give value to *S. commune,* which is most often neglected in terms of its importance, especially in the economic aspect. Moreover, people in the community shall be recognized regarding their cultural practices, which involve this fungus. Lastly, this will encourage people to value *S. commune* as part of the natural ecosystem that has a significant role in the balance of nature.

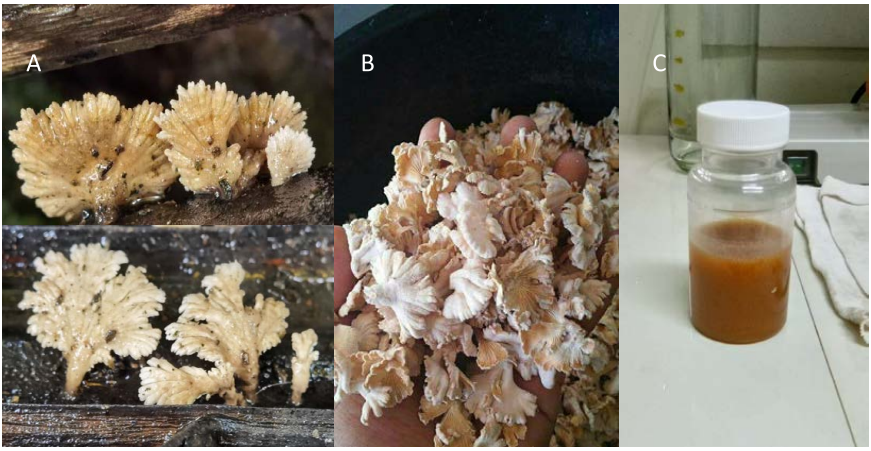
MATERIALS AND METHODS

Preparation of Materials, Chemicals, and Reagents. Laboratory materials needed for the study were obtained from Carlos Hilado Memorial State College (CHMSC) science laboratory. All chemicals and reagents used for phytochemical screening of the samples were of analytical grade. Samples were analyzed at the Negros Prawn Producers Cooperative, Bacolod City, Negros Occidental.

Collection of Schizophyllum commune. The fresh samples were collected in the Minapasuk, Calatrava, Negros Occidental forests and were documented and photographed using a digital camera. *S. commune* growing on the dead logs were scraped using a cutter, put in a resealable bag, and placed in a plastic container to avoid the rapid drying of the sample. The collected samples were brought to the CHMSC Science Laboratory for processing.

The samples were separated and cleaned from unwanted debris, washed with running water, and rinsed with distilled water. After cleaning, the samples were air-dried for a week before analysis. The dried specimens were then covered

with paper, packed in a resealable plastic bag, and put inside the thermo-chest to preserve its freshness and avoid further enzymatic activity during transportation.



A. The *S. commune is* growing in the dead-decaying coconut petioles.
B. The sample after being cleaned from unwanted debris.
C. The ethanolic crude extract of *S. commune.*

Morphological Identification. The samples were identified by comparing the specimen's macroscopic characteristics to published literature and online identification keyssuch as Mushroomobserver.org (n.d.)and MycoKey Morphing Mushrooms Identifier (Petersen & Læssøe, n.d.).

Extraction of the Sample. The sample was thinly chopped and pulverized using a mortar and pestle after being oven-dried for several hours at 50 degrees Celsius. To avoid enzymatic activity, the pulverized material was immediately immersed in 95 percent ethanol and macerated for 48 hours before extraction. After then, the mixture was filtered using filter paper. The collected filtrate was subjected to the rotary evaporator with controlled temperature and revolution to achieve the desired consistency of the extract. A flame test was done to ensure no alcohol mixture was present in the extract.

Phytochemical Screening. Phytochemical screening of *S. commune* ethanolic crude extract was carried out using the test tube method described by Aguinaldo et al. (2005) and (Tiwari et al., 2001). The extract was screened for saponin, tannins, alkaloids, flavonoids, terpenoids, glycosides, proteins, amino acids, and carbohydrates.

Proximate Analysis. Proximate sample analysis for moisture content, carbohydrates, proteins, crude fibers, fats, and ash was carried out using the standards set by the Association of Analytical Chemists (Ensminger, 1976).

Brine Shrimp Lethality Assay (BSLA). Brine Shrimp Lethality Assay was done according to the principles and protocol described by Aguinaldo et al. (2004)with slight modification. Brine shrimp (*Artemia salina)* eggs were placed in a small modified container filled with brine solution, covered with aluminum foil, aerated, and illuminated on the side of the chamber. After forty-eight hours of incubation at room temperature, the active *nauplii* attracted to the brighter side of the hatching chamber were collected using the Pasteur pipette. Samples for testing were prepared using the procedure described by (Peteros & Uy, 2010) by dissolving 50 mg of crude extract in 5 mL of dimethyl sulfoxide (DMSO) and diluted with a brine solution to produce the required concentrations. An appropriate amount of the concentrations was transferred to vials with ten shrimps in each sample vial. Tests were done along with control and different concentrations in triplicate.

Preparation of the Artificial Sea Water.3.8 grams of rock salt was dissolved per 100 mL of distilled water.

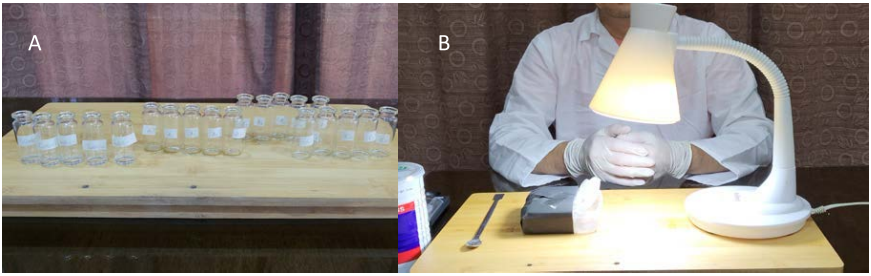
S. commune Extract Preparation. A 50 mg of *S. commune* extract was dissolved in 5 mL methanol to make solution A. Extract 0.5 mL of solution A and added with 10 mL methanol to make solution B. Pipette 100 µL of solution B, 50 µL of solution A and 500 µL of solution A into separate vials, and labeled 1,2, and 3, respectively.A control vial was prepared using one mL of methanol. All bottles were dried under nitrogen gas. Five replicates were made for each dose level.

Hatching the Brine Shrimp. A shallow rectangular dish was filled with artificial seawater. The dish was placed with a plastic divider punched with several 2 cm holes to divide the dish container into two unequal compartments. The larger compartment was covered with black paper to keep away from light and left the smaller chamber uncovered and illuminated with light. After 48 hours, the hatched brownish-orange *nauplii*were pipetted from the illuminated compartment of the dish.

Counting the Nauplii. The nauplii were pipetted and counted macroscopically in the stem of the Pasteur pipette, held against a well-lighted background.

Food for the Brine Shrimp.A 3 mg dry yeast suspension was prepared in 3 mL artificial seawater.

The concentration of S. commune Sample Vials 1, 2, & 3. Each sample vialwas diluted with five mL of artificial seawater to make a final concentration of 10 µg/mL, 100 µg/mL, and 1000 µg/mL. Using the Pasteur pipette, ten (10) nauplii were transferred into each vial labeled 1,2, and 3, and control vials previously prepared. Five mL of artificial seawater was added to each vial, control, and sample. A drop of yeast suspension served as their food was added to each vial and kept under illumination. Survivors were counted after six hours and after 24 hours. Percent of deaths for each dose level and control vials was determined.



A. The sample vials with control and different concentration: 10 ppm, 100 ppm, and 1000 ppm
B. Hatching the nauplii using the rectangular dish and study lamp.

Lethal Concentration Determination. Survivors were counted after 24 hours, and the percentage mortality at each vial and control were determined using the equation:

$$\%\ mortality = \frac{(no.of\ dead\ nauplii)}{(initial\ no.of\ live\ nauplii)}\ x\ 100$$

Statistical Analysis. Percentage composition was used in the sample's proximate analysis and the nauplii's mortality rate. Moreover, Linear regression was used to determine the concentration at which lethality to brine shrimp represents 50% (LC50).

RESULTS AND DISCUSSIONS

Phytochemical Screening. Saponin, tannins, alkaloids, flavonoids, terpenoids, proteins, and carbohydrates were found to be present in the ethanolic extracts of S. commune after the phytochemical screening. However, results revealed

negative results for glycosides and amino acids. These compounds, the secondary metabolites, exhibit physiological activities in humans.

Table 1. Results of phytochemical screening of S. commune ethanolic extract

Secondary Metabolites	Reagents	Positive Results	Experimental Results
Saponin	Distilled water	Continuous frothing	+
Tannins	1% gelatin solution	Green to a black precipitate	+
Alkaloids	Mayer's reagent	Brick-red precipitate	+
Flavonoids	Lead acetate solution	Black cloud/black precipitate	+
Terpenoids	Sulfuric Acid solution	A dark brown/black precipitate	+
Glycosides	Ferric chloride solution	Upper layer: bluish-green color Lower layer: brownish-red color	-
Proteins	4% sodium hydroxide and 1% copper sulfate solution	Violet/pink color formation	+
Amino Acids	5% Ninhydrin solution	Purple color formation	-
Carbohydrates	α-naphthalene solution	Brownish-red precipitate	+

(+) presence (-) absence

Previous literature further confirms results regarding the bioactive compound present in the extract. Flavonoid, phenol, and saponin were present in S. commune mycelial ethanolic extracts (Berfilamen et al., 2013). Also, ethyl acetate extracts of S. commune revealed the presence of phenolics and terpenoids (Sharma et al., 2021).Flavonoids and tannins, as well as steroid and coumarin compounds, were detected in ethanolic extracts (Herawati et al., 2021).

Cytotoxic Activity. The cytotoxic activity of S. commune ethanolic crude extracts using the Brine Shrimp Lethality Assay is shown in Table 2. Mortality of nauplii were 18, 68, and 90 at 10 ppm, 100 ppm, and 1000 ppm, respectively. Utilizing LC50, the ethanolic extracts exhibited cytotoxic activity on brine shrimp with an LC50 value of 55.64 μg/mL or ppm using the linear regression analysis, which is less than 1000 μg/mL or ppm concentration. The extract contains active or potent constituents which are highly toxic to cells (Clarkson et al., 2004; Meyer et al., 1982).This activity of S. commune is due to the different bioactive compounds present in the extract. BSLA is an essential preliminary cytotoxicity assay of plant extract and others based on the ability to kill cultured laboratory larvae (nauplii) (Sarah et al., 2017).However, this method cannot determine the mechanism of action of the bioactive compounds present in the extract.

Table 2. Cytotoxic Activity of schizophyllum commune ethanolic Crude Extracts using the Brine Shrimp Lethality Assay (BSLA)

Extract	Conc. (µg/mL) ppm	No. of Surviving *nauplii* After 24 Hours					No. of Survivors	% Mortality
		Vial 1	Vial 2	Vial 3	Vial 4	Vial 5		
S. commune	10	9	8	7	9	8	41	18
	100	2	3	2	4	5	16	68
	1000	0	0	0	2	3	5	90

The study validated Kaur et al. (2018)results as they reported that S. commune extract had insecticidal activity due to its long-term cytotoxic and genotoxic effects against S. litura. The result on cytotoxicity of the extracts is further confirmed by (Emsen et al., 2017) that acetone and n-hexane extracts of S. commune exerted substantial in vitro cytotoxic effects against the hepatocellular liver carcinoma. Though different solvents and methods were used, it can be resolved that the solvents used were organic, and both were tested in eukaryotic cells. Cytotoxicity of extracts is attributed to the presence of alkaloids (Isah, 2016), flavonoids (Ahmed et al., 2016), and phenolics El-Ansari (2019).In this study, phenolics are present in the form of tannins and flavonoids. Van Dyk et al. (2009) assert that obtaining drugs with different structural features and evaluating the cytotoxicity is a recognition and validation of ethnomedicinal practices. The cytotoxic activity of S. commune recognizes the role of the species in the heritage of the people in the community.

Proximate Analysis. Proximate analysis was developed to provide a broad, top-level classification of food components (Greenfield & Southgate, 2003).Results of proximate analysis of S. commune are presented in Table 3.Data indicates that S. commune is rich in moisture (60.60%), nitrogen-free extract (25.15%), crude protein content (7.63%), and ash content (6.17%).Ash content is the basis of the mineral content of the S. commune. Crude fiber and fat materials are 0.18% and 0.27%, respectively.

Table 3. Proximate Analysis of Schizophyllum commune

Test	Mean
Moisture (Gravimetric-oven drying at 105 ^{0}C)	60.60%
Ash (Oxidation at 550 ^{0}C)	6.17%
Crude Fiber (AOAC method)	0.18%
Fat (Soxhlet Extraction Method)	0.27%
Protein (Kjeldahl Method)	7.63%
Nitrogen Free Extract	25.15%

Edible mushrooms have been part of the human diet in addition to plant and animal sources of food. Chang and Miles (2004) added that besides their high-quality protein, mushrooms are a relatively good source of nutrients such as fat, phosphorus, iron, and vitamins, including thiamine, riboflavin, ascorbic acid, ergosterol, and niacin. Focusing on the protein component of S. commune, the study's results showed a lower protein percentage of the sample of 7.63% than what Longvah and Deosthale (1998) revealed with 16%.The difference in the protein levels is due to the type of substrate the mushrooms are growing (Salami et al., 2016).The same is also true for other components analyzed for the analysis, such as the ash and nitrogen (Hoa et al., 2015).

CONCLUSIONS

The Schizophyllum commune ethanolic crude extract contains phytochemicals or bioactive compounds: saponin, tannins, alkaloids, flavonoids, terpenoids, proteins, and carbohydrates. As the phytochemical analysis has revealed, the cytotoxic activity of the extract against brine shrimp (A. salina) is mainly attributed to the presence of alkaloids and flavonoids. The proximate analysis showed that S. commune can be a source of protein and may be included in the diet. The result of the study supports the traditional medicinal alternative use of S. commune as well as a good source of protein for diet. Based on the possible relationship between brine shrimp lethality and bioactivity, this study could serve for further ethnobotanical, phytochemical, and pharmacological research.

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