IN VITRO STUDY ON SKIN-RELATED BIOACTIVITY OF *PLEUROTUS CYSTIDIOSUS* EXTRACT

PARIYA ATAWONG

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Cosmetic Sciences
2024

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Thesis entitled "*In vitro* study on skin-related bioactivity of *Pleurotus cystidiosus* extract"

By Pariya Atawong

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Cosmetic Sciences of Naresuan University

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ABSTRACT

Pleurotus cystidiosus, distinguished for its nutritional and biomedical attributes due to its rich repository of bioactive compounds, presents promising therapeutic functionalities for cosmetic applications. This study investigated the anti-photoaging and moisturizing potential of *P.cystidiosus* extract (PCE) for cosmetic application, addressing a gap in the scientific knowledge regarding the use of *P.cystidiosus* in cosmetic formulations. Through an extraction process by 60% saturation with ammonium sulfate, PCE1, PCE2, and PCE3 were obtained, exhibiting the method's efficiency with high protein yields and a rich presence of short molecular weight peptides, as confirmed by the Lowry assay and electrophoresis. The extract demonstrated antioxidant activity, assessed via 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,20 -azino-bis-3- ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging assays, alongside anti-tyrosinase activity, primarily attributed to PCH (≤3kDa). Non-anti-bacterial activity of *S. aureus*, *S. epidermidis*, and *C. acnes*. was observed. Particularly, PCE exhibited UV radiation-protective effects against photoaging in Human keratinocytes (HaCaT) cells at 100 µg/mL concentration, suggesting its potential for sunscreen and anti-aging product integration. This study also investigated the skin moisturizing effect of PCE in cosmetic formulations through snake skin moisturizing studies and water contact angle measurements, revealing an

improvement in skin wettability. These results highlighted the critical role of PCE in formulating moisturizing products, integral to skincare routines.

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CHAPTER I

INTRODUCTION

Statement of the Study

Thailand offers an advantageous environment for mushroom cultivation, resulting in the widespread presence of various mushroom species. There are 13 economically significant mushroom types in Thailand, including *Pleurotus pulmonarius*, *Pleurotus osttreatus (Fr.) Kummer*, *Pleurotus ostreatus*, *Auricularia auricula-judae*, *Lentinus polychrous* Lev, *Lentinus squarrosulas*, *Lentinula edodes*, *Coprinus fimetarrius*, *Cyclocybe aegerita*, *Calocybe Indica*, *Schizophyllum commune*, *Ganoderma lucidum*, and *Cordyceps militaris*. As reported by the National Science and Technology Development Agency of Thailand (Chatdanaiphakorn., 2017), mushroom production in the country has exhibited consistent growth. In 2017, 41,322 tons of mushrooms, valued at 2,291 million baht were produced. This increased to approximately 150,000 tons, with a market value of 9,000 million baht in 2020. This rising trend is anticipated in 2024. However, the surplus of locally cultivated mushrooms has introduced challenges, including mushroom spoilage and diminished profitability. Therefore, diversifying the utilization of mushrooms by incorporating them into the cosmetics industry is an interesting and useful use of mushrooms with the potential to augment their value and enhance their efficacy.

Mushrooms have garnered significant attention due to their rich reservoir of diverse bioactive compounds, rendering mushroom extracts a favored choice in skincare and haircare products. These compounds include alkaloids, polysaccharides, flavonoids, glycosides, phenols, polyphenols, proteins, amino acids, saponins, steroids, tannins, and triterpenoids (Y Wu et al., 2016), which collectively demonstrate a wide array of bioactivities, including antibacterial, anti-inflammatory, anti-aging, anti-wrinkle, skin-whitening, and moisturizing effects. The high protein content inherent to mushrooms adds significantly to their value as cosmetic ingredients (Srivastava et al., 2020).

Pleurotus cystidiosus (*P. cystidiosus*) is a newly identified edible mushroom with remarkable nutritional and biomedical properties attributed to its abundant reservoir of bioactive compounds that enable a wide array of therapeutic functions (M. Rahman et al., 2021). Extracts derived from *P. cystidiosus* using ethanol and acetone have been documented to possess antibacterial properties against *Staphylococcus aureus* (Kalaw & Albinto, 2014). To evaluate the potential analgesic effects of *P. cystidiosus*, (Kudahewa et al., 2008) conducted standard hot plate and tail flick tests on male rats during their estrus phase, employing doses of 125, 500, and 1000 mg/kg. Among the male rats, administration of the medium dose of *P. cystidiosus* resulted in an 18% increase in analgesic effects, while the high dose induced a notable 93% increase of 2 hr in the response duration post-administration. Additionally, female rats in estrus exhibited a significant 22% extension in reaction time in the hot plate test. Prior investigations into solvents conducted by (Garcia et al., 2020; Muruke, 2014) have demonstrated that *P. cystidiosus* exhibits antioxidant activity with an efficacy reaching 79.1%, as evidenced by IC_{50} values ranging from 0.035 to 0.150 mg/mL. Additionally, *P. cystidiosus* has displayed substantial iron-chelating capabilities, reaching 98.3% efficacy across diverse experimental settings. In an extensive investigation by (Ching et al., 2011), involving nine different edible mushrooms, *P. cystidiosus* emerged as the most potent antihypertensive agent. This effect was primarily attributed to the presence of a protein with an approximate molecular weight of 8.3 kDa. Another study, by (Lau et al., 2012), scrutinized proteins extracted from *P. cystidiosus* (E1Pc and E5Pc) and *Agaricus bisporus* (E1Ab and E3Ab) in which it was also noted that these proteins exhibited remarkable antihypertensive properties. Proteins extracted from *P. cystidiosus* have a wide range of molecular weights within the range of 3 to 10 kDa, spanning from high to low.

However, *P. cystidiosus* protein extracts have not been reported as being used as a cosmetic ingredient. The objective of this study, therefore, was to investigate the antimicrobial, anti-photoaging, moisturizing activity, and safety of *P. cystidiosus* extract for incorporation into cosmetic formulations to develop effective cosmetic products that leverage the moisturizing effect to enhance skincare.

Objectives of the study

1. To develop a *Pleurotus cystidiosus* extraction method
2. To study the activity of *Pleurotus cystidiosus* extract for cosmetics purposes.

Keywords

Pleurotus cystidiosus, bioactivity, skincare, cosmetics, protein extract

CHAPTER II

LITERATURE REVIEW

Pleurotus cystidiosus

P. cystidiosus, commonly referred to as the oyster mushroom, is an edible fungal species classified within the *Pleurotus* genus. The *Pleurotus* genus includes *P. ostreatus*, *P. sajor- caju*, *P. florida*, *P. flabellatus*, *P. highbing 51*, *P. cystidiosus*, *P. sapidus*, *P. eryngii*, *P. tuberegium*, *P. ulmarium*, *P. pulmonarius*, *P. citrinopileatus*, *P. geesteranus* and others. These mushrooms possess distinctive fan-shaped or oyster-like cap morphology, as shown in Figure 1. The cap exhibits a spectrum of colors, ranging from pale white to various shades of gray, brown, or even pink, depending upon the specific mushroom variety and its developmental stage. Furthermore, oyster mushrooms exhibit white gills that extend down the stem.

Figure 1 *Pleurotus cystidiosus* (Abalone Mushroom).

Source: https://www.out-grow.com/media/catalog/product/cache/

Pleurotus species are notable for being abundant sources of protein, essential minerals (including phosphorus, calcium, iron, potassium, and sodium), and vitamins (such as thiamine, riboflavin, folic acid, and niacin) (Szabová et al., 2013). Beyond their nutritional significance, these mushrooms have gained recognition for their potential medicinal value, particularly in diabetes management and cancer therapy (Sivrikaya et al., 2002). A diverse array of mushroom species harbors a wide range of bioactive metabolites, demonstrating various pharmacological activities, including but not limited to anti-tumor, antigenotoxic, antioxidant, antihypertensive, antiplatelet aggregating, antihyperglycemic, antimicrobial, and antiviral properties (Chang, 2007).

In medicinal mycology, specific oyster mushroom species have garnered particular attention. For example, *P. cystidiosus* has been identified as a potent antioxidant (Li et al., 2007), while *P. ostreatus* exhibits antitumor activity (Chorvathova et al., 1993). *P. ostreatus* is rich in high-quality protein and fiber, primarily consisting of beta-glucans, making it a potentially valuable functional food ingredient, as shown in Table 1-Table 3. The bioactivity and functional properties of polysaccharides depend on factors such as polymerization degree, branching, and the presence of substituents like sulfates. *Pleurotus* species is distinguished by its unique mycochemical composition, as shown in Figure 2. This composition has been substantiated to exhibit antimicrobial, antiviral, anticancer, antioxidant, hypolipidemic, hypocholesterolemic, antihyperglycemic, and immunomodulatory effects (Carrasco-González et al., 2017).

P. cystidiosus is categorized as saprophytic fungi, signifying its reliance on decomposing organic matter as a nutrient source. It is typically found thriving on dead or decaying trees, logs, and similar woody substrates. Notably, it ranks as the third most cultivated mushroom globally due to its prevalence in various regions, including Asia, America, and Europe. This widespread cultivation can be attributed to the straightforward and cost-effective production techniques associated with *Pleurotus* species, coupled with their remarkable biological efficiency (Mane et al., 2007).

In culinary terms, oyster mushrooms are highly esteemed for their gastronomic appeal, boasting a delicate and mild flavor, as well as a tender texture when cooked. They find versatile application in a wide array of dishes, ranging from stir-fries and soups to pasta, and are even employed as a meat substitute in vegetarian and vegan

recipes. Beyond their culinary attributes, oyster mushrooms offer a rich nutritional profile, serving as valuable sources of protein, dietary fiber, vitamin (particularly B vitamin like niacin and riboflavin), and essential minerals such as potassium, phosphorus, and iron. Additionally, these mushrooms are recognized for their potential health-promoting properties.

Table 1 The composition of *Pleurotus* species.

Specie	g/100 g DW															
	Protein				Carbohydrates				Lipids				Ash			
	n	Ẋ	Min	Max	n	Ẋ	Min	Max	n	Ẋ	Min	Max	n	Ẋ	Min	Max
Pa	1	16.1	–	–	–	–	–	–	1	2.3	–	–	1	6.4		
Pci	5	29.4	22.1	36.8	4	38	20.8	44.4	5	2	1.3	2.9	2	7.8	7.7	7.9
Pco	1	17.9	–	–	–	–	–	–	1	2	–	–	1	6.1		
Pcy	7	19.6	15.7	24.5	7	47.1	40.6	52.9	7	2.8	2	3.9	7	6.8	6.3	7.5
Pd	14	23.1	11.3	32.3	7	37.7	35.5	42.4	14	2.4	0.1	4.6	14	7.1	6.2	8.3
Pe	15	17.8	5.8	23.9	2	75.4	69.5	81.4	12	3.5	1.5	6.3	15	6.6	4.9	10.6
Pgi	2	17.3	15.4	19.2	2	65.9	64.7	67.2	2	3.4	3.1	3.7	–	–	–	–
Pge	2	30.3	28.8	31.8	2	32.5	31.6	33.4	2	3.5	3.5	3.6	2	12	11.2	12.8
Ph	1	28	–	–	1	29.2	–	–	1	4.1	–	–	1	11.5	–	–
Pn	1	17.1	–	–	–	–	–	–	1	1.6	–	–	1	5.9		
Po	92	23	7.3	53.3	71	42	13.1	85.8	65	3.4	0.5	7.6	56	7.5	4.1	15.9
Pp	14	22.9	14.4	34	10	9.4	5.9	14.4	11	3.2	1.6	6	4	8.3	6.25	9.8
Psa	8	38.5	20.1	47.2	7	37.3	32	44.5	4	2.4	1.4	3.2	1	6.4		
Psc	24	26	13	44.3	18	38.6	23.5	65.1	19	2.3	0.3	5.3	17	7.5	5.1	8.9
Pt	2	10.8	7.8	13.8	2	43.6	34	53.2	2	0.9	0.7	1.1	2	3.5	2.6	4.9
Pspp	189	22.5	14.7	32.8	133	41.4	33.7	53.2	147	2.7	1.5	4.4	124	7.4	6.0	9.6

n represents the sample size, X_ denotes the average value, Min and Max indicate the minimum and maximum values, respectively. The species abbreviations are as follows: Pa=*P. abalone*; Pci=*P. citrinopileatus*; Pco=*P. cornucopiae*; Pcy=*P. cystidiosus*; Pd=*P. djamor*; Pe=*P. eryngii*; Pgi = *P. giganteum*; Pge = *P. geesteranus*; Ph = *P. high-king*; Pn = *P. nebrodensis*; Po = *P. ostreatus*; Pp = *P. pulmonarius*; Psa = *P. sapidus*; Psc = *P. sajor-caju*; Pt = *P. tuber-regium*; Pspp = *Pleurotus* species.

Source: (Carrasco-González et al., 2017).

Table 2 Dietary fiber and *β*-glucan content in *Pleurotus* species.

Specie	g/100 g DW							
	Crude fiber				β-Glucans*			
	n	X̄	Min	Max	n	X̄	Min	Max
Pci	1	20.7	–	–	–	–	–	–
Pco	1	8.7	–	–	1	37.8	–	–
Pd	5	8.0	7.3	8.9	1	20.5	19.0	22.0
Pe	–	–	–	–	4	34.0	20.4	39.4
Pn	–	–	–	–	3	29.8	23.7	36.6
Po	14	11.8	7.6	15.0	29	29.5	19.3	50.0
Pp	6	7.3	4.8	8.5	–	–	–	–
Psa	1	7.2	–	–	–	–	–	–
Psc	5	8.1	6.7	8.3	6	25.5	23.1	27.5
Ptb	–	–	–	–	3	4.4	3.5	5.9
Pspp	33	10.3	6.6	10.2	47	25.9	18.2	30.2

The *β*-Glucan content was determined using the Megazyme Mushrooms and Yeast Beta-Glucan Assay procedure.

Source: (Carrasco-González et al., 2017).

Table 3 The profile of amino acids and the protein's quality in *P. ostreatus*.

Amino acid	n	Content (g/100 g protein)			AASP		AAS%		PDCAAS%	
		X̄	Min	Max	Child	Adult	Child	Adult	Child	Adult
Lys	33	6.1	1.5	10.7	5.7	4.8	107.5	127.7	95.3	103.1
Leu*	33	6.4	1.9	11.9	6.6	6.1	97.3	105.2		
Val	31	4.8	1.6	8.6	4.3	4.0	112.6	121.0		
Thr	26	4.9	2.3	7.8	3.1	2.5	159.0	197.2		
Ile	33	4.0	1.1	7.1	3.2	3.0	125.0	133.3		
Phe	33	3.9	1.4	6.9	5.2	4.1	155.0	196.6		
Tyr	25	4.1	0.9	11.1						
His	33	3.5	1.1	7.7	2.0	1.6	175.5	219.4		
Trp	14	1.2	0.6	1.6	0.8	0.6	153.8	205.0		
Met	28	1.8	0.5	3.6	2.7	2.3	112.6	132.2		
Cys	24	1.3	0.5	2.3						

n represents the sample size, $\bar{x}$ denotes the average value, Min and Max indicate the minimum and maximum values, respectively. The limiting amino acid is denoted by an asterisk (*). AASP stands for Amino Acid Scoring Pattern, as defined by the Food and Agriculture Organization (FAO) in 2011. AAS refers to Amino Acid Scores, while PDCAAS is the Protein Digestibility Corrected Amino Acid Score, calculated as [(AAS of Limiting Amino acid Protein quality (True Digestibility)]; The true digestibility value used is 98.

Source: (Carrasco-González et al., 2017).

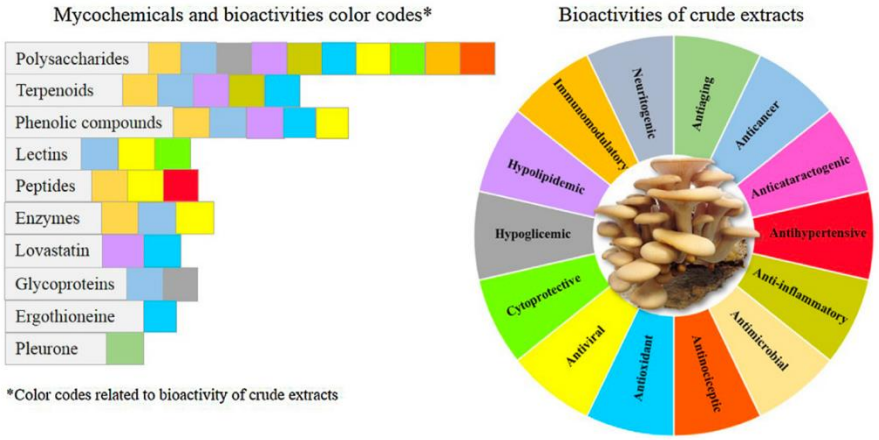


Figure 2 The bioactive properties of extracts from *Pleurotus* species and their associated mycochemicals.

Source: (Carrasco-González et al., 2017).

Bioactive compounds of *Pleurotus* species

The primary constituents of fungal bioactive compounds, referred to as mycochemicals, predominantly include polysaccharides (beta-glucans), peptides, proteins, lectins, lipids, mineral ash, glycosides, alkaloids, volatile oils, tocopherols, phenolic compounds, polyketides, terpenoids, flavonoids, carotenoids, folates, ascorbic acid, enzymes, and organic acids. The bioactive compounds in *Pleurotus* mushrooms include high-molecular-weight polysaccharides, peptides, proteins, low-molecular-weight terpenes, fatty acid esters, and polyphenols. Extracts from different *Pleurotus* species can be used as dietary supplements to boost immunity. Furthermore, they find application in the cosmetics industry and can be incorporated into functional foods as probiotic agents or serve as natural preservatives or constituents in specialized dietary products for individuals with specific medical conditions (Carrasco-González et al., 2017; Golak-Siwulska et al., 2018; Patel & Goyal, 2012).

The therapeutic properties and bioactive compounds identified in select *Pleurotus* species are presented in Table 4.

Table 4 The therapeutic properties and bioactive compounds of *Pleurotus* species.

Activity	Bioactive compounds	Species
Anti-cancer	β-glucans	*P. ostreatus*
	α-glucan	*P. ostreatus*
	proteins	*P. ostreatus*
		P. nebrodensis
Antitumour	polysaccharides	*P. ostreatus*
	proteoglycans	*P. ostreatus*
	lectin	*P. citrinopileatus*
		P. ostreatus
Immunomodulatory	polysaccharides	*P. ostreatus*
		P. cornucopiae
	heteroglycan	*P. ostreatus*
Antihypercholesterolemic	lovastatin	*P. ostreatus*
		P. sajor-caju
		P. florida
	ergosterol	*P. ostreatus*
Antihypertensive	D-mannitol	*P. cornucopiae*
	peptides	*P. cornucopiae*
Anti-atherogenic	angiotensin converting enzyme inhibitor peptide	*P. cornucopiae*
	ergothioneine	*P. eryngii*
	chrysin	*P. ostreatus*
	lovastatin	*P. ostreatus*
Antiviral	proteins	*P. ostreatus*
		P. nebrodensis
	polysaccharides	*P. abalonus*
	lectins	*P. citrinopileatus*
Antibacterial	β-glucans	*P. ostreatus*
	fatty acids esters	*P. eous*
Antifungal	ergosterol	*P. cystidiosus*
	proteins	*P. ostreatus*
		P. eryngii
Anti-oxidative	phenols	*P. citrinopileatus*
	lectins	*P. florida*
	polysaccharides	*P. ostreatus*
		P. tuber-regium
Hypoglycemic	unspecified	*P. ostreatus*
	β-glucans	*P. sajor-caju*
	polysaccharide-peptide	*P. abalonus*
Anti-inflammatory	polysaccharides	*P. ostreatus*
		P. ostreatoroseus
		P. florida
Anti-arthritic	β-glucans	*P. sajor-caju*
		P. ostreatus
Anti-atopic dermatitis	pleuran	*P. ostreatus*
Anticataractogenic	unspecified	*P. ostreatus*
Antinociceptive	β-glucans	*P. eous*
		P. pulmonarius

Source: (Carrasco-González et al., 2017).

1. Mushroom proteins and peptides

Mushrooms are notable reservoirs of natural bioactive proteins and peptides (Erjavec et al., 2012; Xu et al., 2011; Zhou et al., 2019). These emphasize the substantial presence and diversity of bioactive proteins in mushrooms, with a particular focus on enzymes, which have been subject to extensive investigation. While mushrooms offer a wealth of proteins, the identification and characterization of these proteins remain somewhat limited. Mushroom extracts and proteins exhibit antibacterial, antifungal, antiviral, antiparasitic, antitumor, immunomodulatory, insecticidal, and nematotoxic effects. Mushroom proteins are categorized based on their biochemical attributes. Their diversity and characterization highlight mushrooms as a reservoir for novel proteins.

Bioactive proteins with high value can be obtained from higher fungi, either extracted from their fruiting bodies, cultivated mycelium, supernatant, or produced as recombinant proteins. These mushroom proteins offer a unique advantage as they often differ significantly from proteins found in microorganisms, animals, and plants. Many mushroom proteins exhibit exceptional qualities like remarkable thermal and pH stability, as seen in enzymes such as laccases (Hildén et al., 2009), glycosidases (Elvan et al., 2010), protease inhibitors (Sabotič et al., 2009), and lectins (Khan & Khan, 2011), as shown in Table 5. Another benefit associated with bioactive components derived from mushrooms is their perceived safety, which can be substantiated by the careful selection of source materials. This safety is rooted in the extensive historical use of mushrooms and their products, which have demonstrated a favorable track record in terms of human health and well-being. These attributes are highly advantageous for applications in biotechnology, medicine, cosmetics, and agriculture.

Table 5 Bioactive proteins from mushrooms selected according to their biochemical characteristics.

Protein group and biochemical activity	Protein name	Molecular mass (kDa)	sequence[c]	3D structure	mushroom
Lignocellulose-degrading enzymes					
oxidoreductase	laccase	60-80	yes	several	Wood-degrading and litter-decomposing species
	lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP)	35-48 38-62 42-45	yes	several	Wood-degrading and litter-decomposing species
	cellobiose dehydrogenase	90-110	yes	yes (1)	*Phanerochaete chrysosporium*, widespread in basidiomycete and ascomycete species
cellulase	endoglucanase	24-85	yes	no	*Gloeophyllum trabeum, Athelia rolfsii, Serpula inaarssata, Lentinus edodes, Moniliophthora perniciosa* and others
	cellobiohydrolase	36-65	yes	yes (2)	White rot species (rare in brown-rot species), *Agaricus arvencis*
	β-glucosidase	35-66, 90-640	yes	yes (1)	*Ceriporiopsis subvermispora, Phanerochaete chrysosporium, Pleurotus ostreatus, Tricholoma matsutake, Flammulina velutipes* and others
hemicellulase	endoxylanase (endo-1,4-β-xylanase)	21-36	yes	no	*Agaricus bisporus, Gloeophyllum trabeum, Phanerochaete chrysosporium, Schizophyllum commune*
	xylan 1,4-β-xylosidase	35-116	yes	no	*Coprinopsis cinerea, Postia placenta*
	β-mannanase	35-51	yes	no	*Armillariella tabescens, Agaricus bisporus, Coprinopsis cinerea, Lentinula edodes, Phanerochaete chrysosporium*
	β-mannosidase	48, 95-110	yes	no	*Schizophyllum commune, Phanerochaete chrysosporium, Coprinopsis cinerea*
Proteolytic enzymes, peptidases, proteases					
aspartic protease	AmProt1,2	45 and 90	yes	no	*Amanita muscaria*
	CnebAPs	21 to 55	N-term	no	*Clitocybe nebularis*
	LSAP	50	N-term	no	*Laetiporus sulphureus*
	Pleureryn	11.5	N-term	no	*Pleurotus eryngii*
	Polyporopepsin (ILAP)	35	yes	yes	*Irpex lacteus (Polyporus tulipiferae)*
	/	25	no	no	*Phanerochaete chrysosporium*
	/	35	no	no	*Russula decolorans*

Source: (Erjavec et al., 2012).

Table 5 Bioactive proteins from mushrooms selected according to their biochemical characteristics (Continued).

Protein group and biochemical activity	Protein name	Molecular mass (kDa)	sequence[c]	3D structure	mushroom
metalloprotease	AMMP	21	N-term	no	*Armillaria mellea*
	FVP-I	37	N-term	no	*Flammulina velutipes*
	Lysine-specific metalloendopeptidase (MEPs)	18 to 20	yes	yes (1)	*Armillaria mellea, Grifola frondosa, Pleurotus ostreatus*
	Metzincin metalloprotease - PoMTP	/	yes	no	*Pleurotus ostreatus*
	TSMEP1,2	18	N-term	no	*Tricholoma saponaceum*
	/	21	N-term	no	*Lepista nuda*
serine protease	helvellisin	33.5	N-term	no	*Helvella lacunosa*
	PoSI	/	yes	no	*Pleurotus ostreatus*
	cordysobin	31	N-term	no	*Cordyceps sobolifera*
	/	30	N-term	no	*Termitomyces albuminosus*
	/	40	yes	no	*Grifola frondosa*
	/	15	N-term	no	*Amanita farinosa*
	/	19	N-term	no	*Pholiota nameko*
asparaginase	FvNase	85 (13 kDa subunit hexamer)	yes	no	*Flammulina velutipes*
Protease inhibitors					
cysteine protease inhibitor	Clitocypin and macrocypin	16-19	yes	yes (2)	*Clitocybe nebularis, Macrolepiota procera*
serine protease inhibitor	POIA1,2	8	yes	yes	*Pleurotus ostreatus*
	Cnispin, cospin, LeSPI	~ 16	yes	yes (1)	*Clitocybe nebularis, Coprinopsis cinerea, Lentinus edodes*
	proteinase K inhibitors	14-20	no	no	*Trametes versicolor*
	trypsin inhibitors	20, 21.5	no	no	*Abortiporus biennis*
Proteinase A inhibitor	GLPAI	38	no	no	*Ganoderma lucidum*
Lectins					
glycan binding proteins	lectins (over 200 described)	10-100	mostly no	51 structures from 10 species	ubiquitous
Ribosome inactivating proteins (RIPs)					
ribosome inactivation	adustin, calcaelin, flammin, flammulin, hypsin, lyophyllin, pleuturegin, velin, velutin, volvarin	13-40	yes	no	*Polyporus adusta, Calvatia caelata, Flammulina velutipes, Hypsizygus marmoreus, Lyophyllum shimeiji, Pleurotus tuber-regium, Volvariella volvacea*
Ribonucleases (RNAses)					
RNA degradation	RNase	8-18, 28-45	yes/ N-term	no	*Agaricus bisporus, Ganoderma lucidum, Lentinus edodes, Pleurotus ostreatus, Pleurotus, eryngii, Pleurotus sajor-caju* and others
	RNase	14.6	no	no	*Lactarius flavidulus*
	RNase	20	N-term	no	*Schizophyllum commune*
	RNase and DNase	38	N-term	no	*Tricholoma matsutake*
Other proteins					
hydrophobin	hydrophobin-1, 2, 3, B	12-19	yes	no	*Agaricus bisporus*
	hydrophobin-1, 2	12, 14	yes	no	*Pisolithus tinctorius*
	fruiting body protein SC1, SC3, SC6, hydrophobin 6	11-18	yes	no	*Schizophyllum commune*
	other hydrophobins	~ 10-20	yes/ N-term	no	*Coprinopsis cinerea, Lentinus edodes, Pleurotus ostreatus* and others
Biotin-binding protein	tamavidin 1,2	16, 15.5	yes	yes (1)	*Pleurotus cornucopiae*

Source: (Erjavec et al., 2012).

Table 5 Bioactive proteins from mushrooms selected according to their biochemical characteristics (Continued).

Protein group and biochemical activity	Protein name	Molecular mass (kDa)	sequence[c]	3D structure	mushroom
small proteins and peptides	eryngin, cordymin, pleurostrin, agrocybin, ubiquitin-like, ganodermin	9-15	yes	no	*Agrocybe cylindracea, Cordyceps militaris, Ganoderma lucidum, Pleurotus eryngii, Pleurotus ostreatus*
	α-amanitin, β-amanitin, amaninamide, phallacin, phallacidin, phallisacin, desoxoviroidin	~ 1	no	no	*Amanita exitialis*
	antamanide	1.1	yes	no	*Amanita phalloides*
Pore-forming proteins	flammutoxin	31	yes	yes	*Flammulina velutipes*
	ostreolysin	15	Yes	no	*Pleurotus ostreatus, Agrocybe aegerita*
	pleurotolysin A, B	17, 59	yes	yes	*Pleurotus ostreatus*
	Laetiporus sulphureus lectin (LSL)	35	yes	yes	*Laetiporus sulphureus*
Other oxidoreductases	tyrosinase	42-55	yes	yes	*Agaricus bisporus, Lentinus edodes, Pycnoporus sanguineus, Pholiota nameko*
	pyranose 2-oxidase	61-70	yes	yes	*Phanerochaete chrysosporium, Phlebiopsis gigantea, Trametes multicolor, Trametes versicolor, Tricholoma matsutake*
Ether hydrolase	epoxide hydrolase	37	yes	no	*Phanerochaete chrysosporium,* predicted in *Coprinopsis cinerea, Laccaria bicolor*
Phosphoric Monoester Hydrolase	6-phytase	47-50	yes	no	*Agrocybe pediades, Ceriporia sp., Peniophora lycii, Trametes pubescens*

Source: (Erjavec et al., 2012).

There is a growing interest in utilizing high-value functional proteins from edible mushrooms to produce protein hydrolysates. Previous studies have demonstrated the successful generation of enzymatic protein hydrolysates from various mushroom species, such as oyster, abalone, and shiitake (Banjongsinsiri et al., 2016), as well as bunashimeji and enoki mushrooms (Ang & Ismail–Fitry, 2019). Enzymatic hydrolysis of diverse protein sources has led to the production of bioactive peptides with various biological properties, notably antioxidant activity. The characteristics of these bioactive peptides in the hydrolysates can be influenced by multiple factors, including the protein source, pretreatment methods, enzyme type and quantity, substrate concentration, degree of hydrolysis, pH, temperature, and process conditions. Some commonly used proteolytic enzymes in the enzymatic hydrolysis of food proteins include pepsin, trypsin, flavourzyme, alcalase, neutrase, protamex, papain, and bromelain. (Chalamaiah et al., 2019; Laohakunjit et al., 2017). Bioactive compounds of notable

interest include protein hydrolysates and peptides, which demonstrate substantial biological activities encompassing antioxidative, antihypertensive, antimicrobial, and antitumor properties. Additionally, peptides with reduced molecular sizes are known for their facile absorption and minimal toxicity or adverse effects (Chi et al., 2015; García et al., 2013).

Mushroom bioactive peptides (MBPs) are peptides obtained directly or indirectly from edible mushrooms. MBPs are recognized for their diverse range of biological activities, encompassing antioxidative, anti-aging, antibacterial, anti-inflammatory, anti-hypertensive, antitumoral, anti-diabetic, and cholesterol-lowering properties, and memory and cognitive enhancement. MBPs operate by modulating pathways such as MAPK, Keap1-Nrf2-ARE, NF-κB, and TNF (Hseu et al., 2020), thereby exerting antioxidant and anti-inflammatory effects. Furthermore, they enhance antibacterial, anti-tumoral, and anti-inflammatory responses by promoting macrophage proliferation. The bioactive properties of MBPs are closely associated with factors like their molecular weight, charge, amino acid composition, and amino acid sequences (de Castro & Sato, 2015). In comparison to peptides derived from animal sources, MBPs present an advantageous choice as raw materials for the development of health-promoting and functional products. Their advantages include a readily available supply, safety, cost-effectiveness, and the feasibility of large-scale production to deliver valuable nutrients for health preservation and disease prevention (Li et al., 2023).

2. Mushroom protein extracts with antibacterial activity

Within the extensive array of edible mushrooms, the *Pleurotus* species stands out as a prolific source of novel bioactive compounds, often referred to as "mycochemicals" (Deepalakshmi & Sankaran, 2014). In contemporary times, several *Pleurotus* species have gained prominence in commercial cultivation due to their noteworthy mineral content, medicinal attributes, abbreviated life cycle, capacity for reusing specific agricultural and industrial byproducts, and their minimal requirements in terms of resources and technology (Yildiz et al., 2002). The documentation of mushroom proteins, a wide range of biologically active compounds has come to light. These encompass lectins, fungal immunomodulatory proteins, ribosome-inactivating proteins, ribonucleases, laccases, and numerous other proteins. These proteins have garnered significant attention as valuable sources of naturally occurring substances with potential utility in anti-tumor, antiviral, antimicrobial, antioxidant, and immunomodulatory roles (Xu et al., 2011).

Among these *Pleurotus* species (*P.*), *P. ostreatus* displayed the highest antimicrobial and antifungal activities, while *P. eryngii* exhibited comparatively lower effects. Notably, *P. ostreatus* exhibited remarkable inhibition rates, reaching 90.86% against *A. alternata* and 92.67% against *E. coli*. In contrast, *P. eryngii* recorded lower inhibition rates, with values as low as 32.33% for A. flavus and 35.9% for *V. parahaemolyticus* (Figure 3 & Figure 4). These findings underscore the considerable antimicrobial potential of select oyster mushroom strains and suggest their promising role as alternatives to currently used fungicides (Sitara et al., 2023). *Pleurotus* species demonstrate variable activity against Gram-negative pathogenic bacteria and exhibit potent inhibition against Gram-positive pathogenic bacteria, including *Bacillus subtilis* and *Micrococcus luteus* (Khatun et al., 2011).

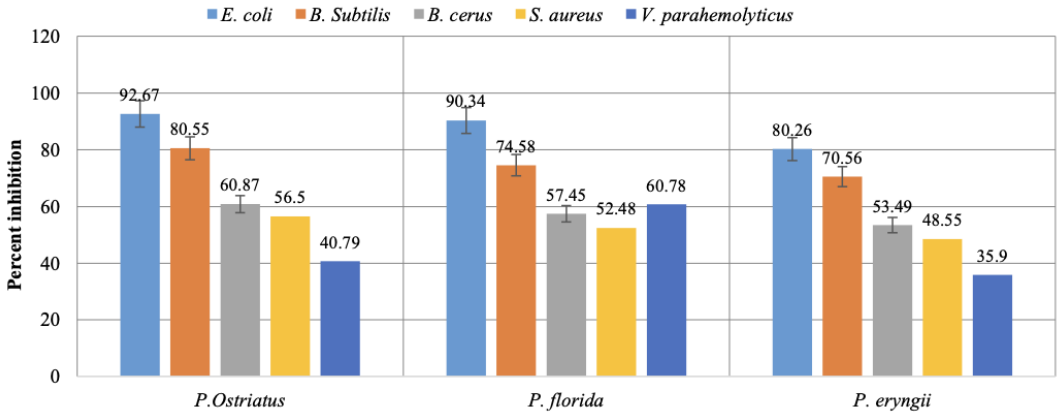
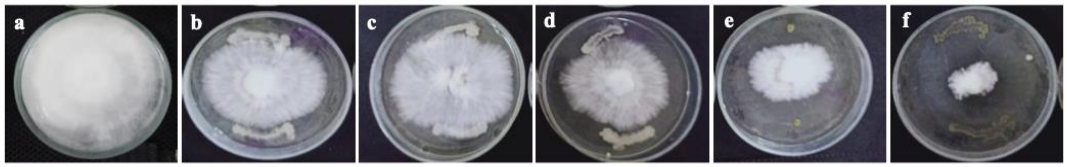


Figure 3 Percent inhibition of *Pleurotus* species (*P.ostreatus*, *P. florida*, and *P.eryngii*) against pathogenic bacteria.

Source: (Sitara et al., 2023).



a = Control (*P. ostreatus*), b = *E.coli*, c = *S. aureus*, d = *B. cereus*, e = *B. subtilis*, f = *V. parahaemolyticus.*

Figure 4 Antibacterial activity of *P. ostreatus* against foodborne bacteria strains *E. coli*, *S. aureus*, *B. subtilis*, *B. cereus*, and *V. parahaemolyticus*.

Source: (Sitara et al., 2023).

The potential antibacterial activity of mushroom extracts has drawn considerable attention. Numerous research investigations have delved into the antimicrobial properties of these extracts against a broad spectrum of human pathogens, encompassing both Gram-positive and Gram-negative bacterial strains. Several mushroom extracts have been documented for their antibacterial properties. Notably,

the acid extract from *P. nebrodensis* and *P. eryngii* exhibited a substantial cationic protein content of approximately 250 µg/mL, with their electrophoretic analysis via SDS-PAGE revealing a predominant band at approximately 14.4 kDa (Figure 5).

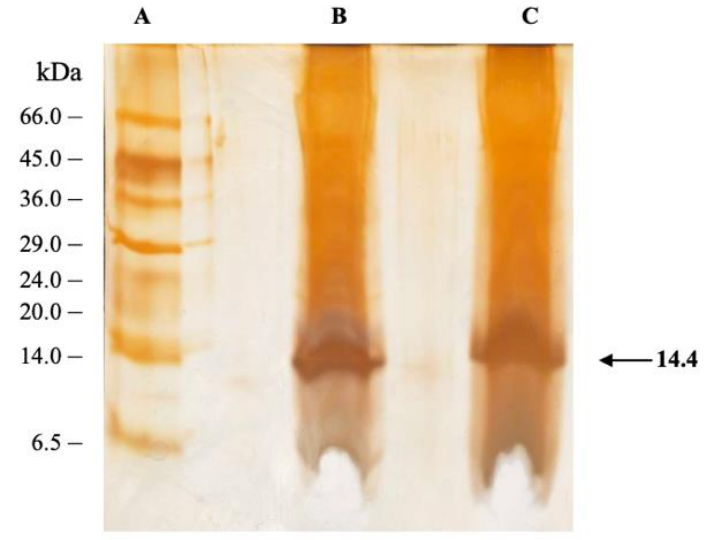


Figure 5 Silver stain of SDS-PAGE (12.5%) of *Pleurotus nebrodensis* (B) and *Pleurotus eryngii* var. *ferulae* (C) cationic protein extract. A = molecular weight markers.

Source: (Schillaci et al., 2013).

The mushroom extract exhibited antibacterial activity against all the bacterial strains tested. Both *P. nebrodensis* and *P. eryngii* var. *ferulae* extracts displayed the capability to inhibit the growth of *S. epidermidis*, with minimum inhibitory concentrations (MICs) of ≤0.025% v/v which is a higher inhibitory effect than *P. eryngii* var. *elaeoselinii* extract which exhibited a MIC of 0.05% v/v. The acidic extract from *P. eryngii* var. *eryngii* demonstrated MIC values of 25% v/v (Table 6), which, while active, was comparatively less effective than extracts from other *Pleurotus* species.

Table 6 Antibacterial activity of examined *Pleurotus* species against Human Pathogenic Microorganisms.

	MIC (% v/v)			
	P. aeruginosa **ATCC 15442**	***E. coli*** **ATCC10536**	***S. aureus*** **ATCC25923**	***S. epidermidis*** **RP62A**
P. nebrodensis	0.05	0.1	0.1	≤0.025
P. eryngii var. *ferulae*	0.05	0.1	0.1	≤0.025
P. eryngii var. *elaoselini*	0.05	0.1	0.1	0.05
P. eryngii var. *eryngii*	25	25	25	25

Activity expressed as MIC (minimum inhibitory concentrations)

Source: (Schillaci et al., 2013).

An assessment of the antibacterial potential of extracts and soluble fractions derived from *P. citrinopileatus*, encompassing solubility in n-hexane, diethyl ether, and ethyl acetate, was conducted against *S. aureus* and *E. coli*. The results conclusively indicated that the n-hexane-soluble fraction (at a concentration of 0.5 mg/mL) displayed substantial antibacterial efficacy, resulting in growth inhibition rates of 79.6% and 75.6% against *S. aureus*, and the ethyl acetate-soluble fraction (at a concentration of 0.3 mg/mL) resulted in growth inhibition rates 87.0% and 60.0% against *E. coli*. These findings suggest the prospective utilization of *P. citrinopileatus* fruiting bodies as a constituent in skincare formulations (Meng et al., 2011).

In a separate investigation, a screening process involved the evaluation of 33 basidiomycetes strains for their antifungal potential (Florianowicz, 2000). Among these strains, five mushroom species, including *P. ostreatus* (commonly known as oyster mushrooms), exhibited notable activity against *Penicillium expansum*. *Lyophyllum shimeji*, a specific mushroom species known to contain a protein that inhibits ribosomes, with this protein having a molecular weight of approximately 20 kDa (Lam & Ng, 2001), displayed antifungal properties against *Physalospora piricola* and *Coprinus comatus*. Researchers isolated a 10 kDa peptide exhibiting antifungal properties from the fruiting bodies of the mushroom species *P. eryngii* (Wang & Ng, 2004). Another antifungal peptide, named Pleurostrin, has been identified in oyster mushrooms (Chu et al., 2005).

These investigations delved into the relationship between chemical structures and fungitoxicity by focusing on specific volatile flavor constituents found in edible mushrooms, with particular attention to *Agaricus bisporus* and *P. florida*. These investigations also showed that 1-octanol exhibits the highest level of fungitoxic activity against *C. gloeosporioides* (Nidiry, 2001). Furthermore, a recent study sheds light on the broad-spectrum antibacterial and antifungal properties of *P. ostreatus* (Iwalokun et al., 2007), along with the antifungal potential of *P. cystidiosus* against *Colletotrichum gloeosporioides* (Menikpurage et al., 2009).

3. Mushroom extracts with anti-photoaging activity

Ultraviolet (UV) radiation is known to contribute to the generation of free radicals, resulting in both immediate pigment-darkening and long-term photoaging effects (Huang et al., 2016). Prolonged exposure of human skin to UV radiation, particularly UVA (315–400 nm) and visible radiation (400–700 nm), can trigger the production of reactive oxygen species (ROS) within the human biological systems (Krutmann et al., 2020). These ROS exhibit cytotoxic and genotoxic effects on cells by oxidizing lipids and proteins and inducing base lesions. It is worth noting that UVA may have more indirect effects on DNA than UVB, primarily through ROS generation, which can also lead to immunosuppression and photocarcinogenesis (de la Coba et al., 2019). Prolonged UVB exposure has the potential to induce skin inflammation and contribute to the process of skin photoaging. Consequently, the utilization of bioactive compounds derived from natural sources for the management and prevention of UV-induced photoaging has gained popularity within the cosmeceutical industry.

The development of wrinkles is a prominent manifestation of skin aging, primarily attributed to the depletion of structural protein, particularly type I collagen, within the dermal layer of the skin (Lupo & Cole, 2007). Elastase, a metalloproteinase, plays a pivotal role in elastin degradation during skin aging, contributing to wrinkle formation, while matrix metalloproteinases (MMPs) are stimulated by the breakdown of collagen and elastin. Notably, extracts derived from the mycelium of *Tricholoma matsutake* (pine mushroom), also known as the pine mushroom, have exhibited a dose-dependent reduction in elastase activity and diminished MMP levels (Kim et al., 2014).

These extracts hold promise as potential biomaterials for anti-wrinkle formulations in cosmetic products. Furthermore, the methanol extract extracted from the fruiting bodies of *Dictyophora indusiate* (veiled lady mushroom), commonly referred to as the veiled lady mushroom, has been investigated for its anti-cholinesterase properties, its ability to mitigate skin wrinkles, and its capacity to inhibit melanogenesis (Nguyen et al., 2013).

Aqueous extracts derived from *P. ostreatus* have demonstrated significant potential as effective agents against photoaging. Additionally, treatment with *P. ostreatus* resulted in the restoration of mRNA levels for PC1, MMP-1, and elastase in UVA-irradiated human dermal fibroblast cells (HDFs), compared to the irradiated control group (Choi et al., 2022). Furthermore, a study investigated the photoprotective potential of exopolysaccharides (ExPFE) and exopolysaccharides (EnPFE) derived from *Pleurotus flabellatus* in the context of UVA irradiation using the human dermal fibroblast cell line (HS-27). The results demonstrate that both ExPFE and EnPFE possess antioxidative properties and confer photoprotective benefits when administered to UVA-exposed HS-27 cells. This research underscores the prospective utility of pink oyster polysaccharides as a natural bioactive compound for shielding the skin against UVA radiation (N. S. A. Rahman et al., 2021).

Previous research by (Choi et al., 2022) identified the antioxidant and antiaging effects of *Pleurotus ostreatus* (PO) and *Hericium erinaceus* (HE) extracts on ultraviolet A (UVA)-induced human dermal fibroblast cells (HDFs). Traditionally utilized for their medicinal properties, PO and HE exhibit significant antioxidant activities, quantified by polyphenol and ergothioneine content as well as through radical-scavenging assays. These extracts demonstrate notable antiaging effects by enhancing the production of procollagen type I (PC1) and simultaneously reducing levels of matrix metalloproteinase-1 (MMP-1) and elastase activity in UVA-damaged HDFs. These findings suggest that extracts of PO and HE hold potential as promising agents for the prevention of photoaging (Figure 6- Figure 8).

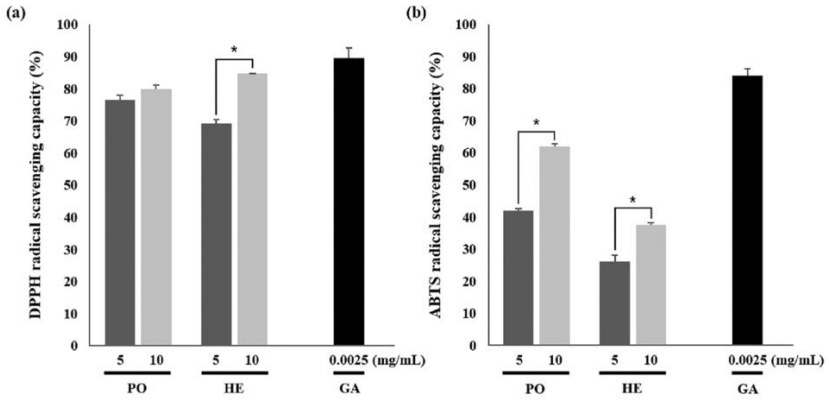


Figure 6 The effect of PO and HE extracts in vitro antioxidant activities. (A) DPPH radical-scavenging activity and (B) ABTS radical-scavenging activity. Each bar shows the mean ± SD (n = 3). *P < 0.05, 5 versus 10 mg/mL of PO and HE in terms of the radical-scavenging ability of ABTS and DPPH.

Source: (Choi et al., 2022)

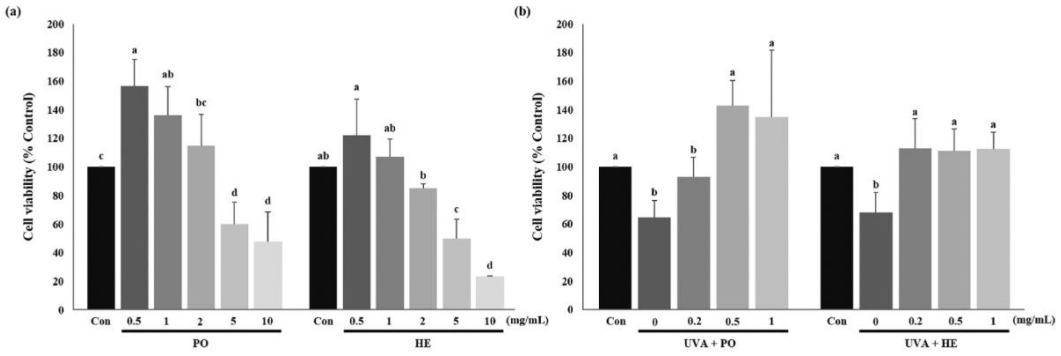


Figure 7 Effect of PO and HE extracts on cell viability in HDFs. (A) HDFs were plated at 4 °C (104 cells/well) and treated with various concentrations of PO and HE for 24 hr. Cell viability was determined by the MTT assay. (B) HDFs were pretreated at different concentrations of PO and HE for 24 hr, followed by exposure to UVA (365 nm, 100 mJ/ cm^2, 38 s), and incubated for another 24 hr. After incubation, cell viability was assessed using the MTT assay. Each bar shows the mean ± SD (n = 3). Different letters indicate significant differences according to ANOVA (P < 0.05).

Source: (Choi et al., 2022)

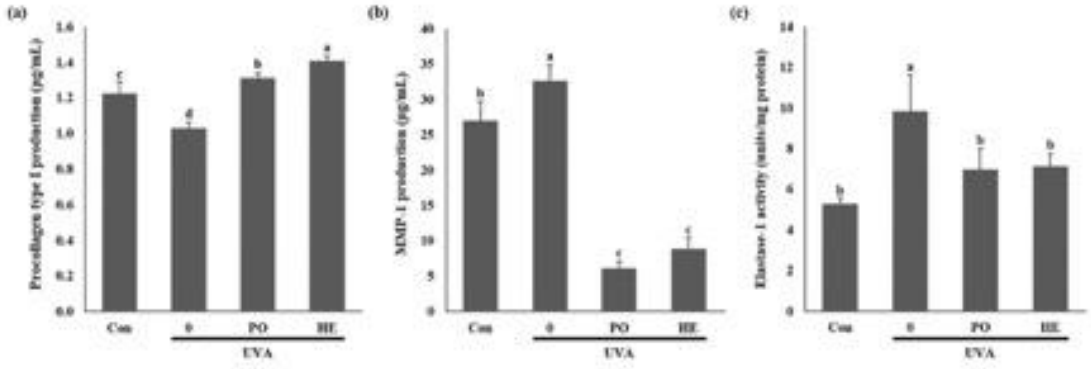


Figure 8 Effect of PO and HE extracts on (A) PC1, (B) MMP-1 production, and (C) elastase-1 activity in UVA-irradiated HDFs. HDFs were incubated with 1 mg/mL of PO and HE for 24 hr, followed by exposure to UVA irradiation (365 nm, 100 mJ/cm^2, 38 s), and incubated for another 24 hr. Each bar shows the mean ± SD (n = 3). Different letters indicate significant differences according to ANOVA (P < 0.05).

Source: (Choi et al., 2022)

Application of *Pleurotus* species in the cosmetics industries

Cosmetics encompass personal care products designed for topical application or oral ingestion, aimed at rejuvenating, safeguarding, and enhancing an individual's physical attributes. These products comprise a blend of chemical compounds, whether synthetic or derived from natural sources, known for their favorable impact on the skin or the targeted areas of application. The cosmetic industry has experienced substantial growth in recent times (Yuanzheng Wu et al., 2016), coinciding with an upsurge in awareness of the potential side effects of chemical-based products and the advantages offered by natural alternatives. Consequently, the younger generation's evolving purchasing preferences have prompted cosmetic brands to explore various botanical extracts (Kapoor et al., 2019).

Among these botanical extracts, mushrooms have gained renewed attention due to their exceptional attributes. Mushrooms contain proteins, lipids, amino acids, glycogens, riboflavin, nicotinic acid, and minerals. Wild mushroom mycelia and fruiting bodies have biologically active compounds like polysaccharides, phenolics,

terpenoids, lectins, ergosterols, and volatile organics. These compounds exhibit antioxidant, antimicrobial, anti-inflammatory, anti-tyrosinase, and anti-hyaluronidase properties (Elsner, 2006). Given their impressive profile of bioactive properties, mushrooms have emerged as pivotal components in diverse cosmetic formulations designed for skin and hair care (Antunes et al., 2020) in Table 7.

The use of *Pleurotus* species in the fields of cosmetology and dermatology is of noteworthy interest. Owing to their rich content of antioxidants and various skin-enhancing components such as anti-aging, anti-wrinkle, skin brightening, and moisturizing agents, extracts from oyster mushrooms hold great potential for the formulation of diverse cosmetics and cosmeceutical products. Specifically, a line of cosmetics featuring β-glucans has been developed, utilizing extracts from several mushroom species, including *P. nebrodensis* (Hyde et al., 2010). Furthermore, the application of extracts from *P. cornucopiae* var. *citrinopileatus* on mice has demonstrated positive effects in managing atopic dermatitis (Tomiyama, 2008). Substantiated research has affirmed the suitability of *P. citrinopileatus* as a valuable source of ingredients for the creation of skincare products (Meng et al., 2011) Notably, extracts from *P. nebrodensis* have exhibited remarkable efficacy as skin brighteners, presenting opportunities for the development of formulations targeting skin discoloration (Dangre et al., 2012). The characteristics exhibited by oyster mushrooms and other mushroom varieties have prompted increased consumer interest in skincare and haircare products that incorporate mushroom-derived ingredients. This trend is primarily driven by consumer awareness and preference for organic and naturally sourced extracts.

Table 7 Various mushroom species utilized in cosmetic formulations, highlighting their bioactive properties.

Mushrooms Scientific name (Common name)	Morphology	Bioactive Properties	Product name	Product image
Lentinula edodes (Shiitake mushroom)		Has moisturizing and tonic effects	One Love Organics Vitamin D Moisture Mist	
		Lift away dirt and make and fight signs of aging	Aveeno Positively Ageless Daily Exfoliating Cleanser	
		Boost collagen, make strides flexible and gives hydration	Kat Burki Form Control Marine Collagen Gel	
Ganoderma lucidum (Reishi mushroom)		Protects the hair from sun damage, also prevents colour fading	Tela Beauty Organics Encore Styling Cream	
		Prevents anti-aging	Menard Embellir Refresh massage	
		Slows down the pre- mature aging and has anti-inflammatory properties	The Face Shop Real Nature Lingzhi Face Mask	
Ganoderma lingzhi (Lingzhi mushroom)		Reduces signs of aging and has skin moisturizing properties	Shiseido Ultimune Power Infusing Concentrate	
Ganoderma lucidum and *Lentinula edodes* (Reishi and Shiitake) mushroom		Has anti-inflammatory and skin reliefing properties	Dr. Andrew Weil for Origins Mega-Mushroom Skin Relief Face Mask	
Ganoderma lucidum and *Pleurotus ostreatus* (Reishi and Oyster mushroom) *Cordyceps sinensis* (Caterpillar mushroom)		Has skin tightening and Anti-hyperlipidemia activity	Hankook Sansim Firming cream	
		Has moisturizing effect, suppress melanin production	Kose Sekkisei Cream	

Source: (Mago et al., 2023).

Table 7 Various mushroom species utilized in cosmetic formulations, highlighting their bioactive properties (Continued).

Mushrooms Scientific name (Common name)	Morphology	Bioactive Properties	Product name	Product image
Agaricus bisporus (Portbello)	 	Helps to prevent sunburn and has anti-microbial properties	Kiss My Face Mineral Sunscreen	 
Trametes versicolor Extract (Cloud mushroom)		Has anti-aging and moisturizing properties Mademoiselle Bio-Cheveux Reduces dandruff and has antibacterial properties Helps in skin tightening	Repairing Cream- High Protection Conditioner- Mademoiselle Bio-Cheveux Intense Protection Mask- Mademoiselle Bio-Cheveux	  
Inonotus obliquus (Chaga)		Has anti-inflammatory properties, also helps to soothes the irritated skin	Root Science RS Reborn Organic Face Mask	
rmis (Snow fungus)		Has moisturizing effect, revitalizes and hydrates the skin	La Prairie Advanced Marine Biology Night Solution	
Tremella Polysaccharide		Moisturizing gel	BeautyDiy Aqua Circulation Hydrating Gel	
Agaricus subrufescens (Almond mushroom)		Antioxidant, keeps the skin hydrated	Surkran Grape Seed Lift Eye Mask	
Phellinus linteus (Sang Hwang mushroom)		Has skin revitalizing properties Has anti-inflammatory and anti-oxidant properties. Also beneficial for eczema	Vitamega Facial Moisturizing Mask Grow Hyal B5 Toner	

Source: (Mago et al., 2023).

Moisturizing Effect

The water content of the stratum corneum and skin surface lipids impact skin appearance and function (Sator et al., 2003). Moisturizers slow moisture loss from the skin, reducing the appearance of fine lines. They increase stratum corneum hydration, improving the skin's physical and chemical properties, and making it moisturized, smooth, and soft (Baumann, 2007). Traditional moisturizing ingredients like pantothenic acid, 6-palmitoyl-L-ascorbic acid, and hyaluronic acid are believed to have potent moisturizing effects in cosmetic formulations.

Moisturizers can be broadly divided into two main categories: hydrophilic (water-loving) and lipophilic (oil-loving). Hydrophilic moisturizers are often called humectants, while lipophilic ones can be further classified as occludents or emollients (Rawlings et al., 2004; Sethi et al., 2016). Table 8 provides examples of each of these moisturizer types, while Figure 9 illustrates their effects on the skin. These ingredients are either identical or similar to natural substances in the stratum corneum (Spada et al., 2018). They are typically used in combination, with certain ingredients providing overlapping properties (Rawlings et al., 2004).

Table 8 The most common moisturizers and their effect on the skin.

Moisturizer Type	Examples	Effects on the Skin
Humectants	Glycerin, gelatin, propylene glycol, butylene glycol, panthenol, sorbitol, urea, hyaluronic acid, glycolic acid, lactic acid, sodium pyrrolidine carboxylic acid	Mostly low molecular weight substances which attract and hold water in the skin May cause water to evaporate into the environment and therefore need to be used with an occludent
Emollients	Cholesterol, squalene, linoleic acid, stearic acid, oleic acid, fatty alcohols	Saturated and unsaturated variable length hydrocarbons which improve the 'feel' of the skin by filling the spaces in between corneocytes Provide what has been termed 'skin slip' or lubricity, imparting a sense of softness and plasticity to the skin Improve the overall appearance and texture of the skin Often used in combination with emulsifiers
Occludents	White soft paraffin/petrolatum, beeswax, mineral oil, dimethicone, lanolin, carnauba wax, cetyl alcohol, caprylic/capric triglyceride	Oils and waxes which form an inert layer on the skin and physically block transepidermal water loss (TEWL) Some occludents enter the intercellular space and interact with the stratum corneum lipids, reinforcing the skin barrier

Note that some examples have multiple properties and effects on the skin.

Source: (Barnes et al., 2021).

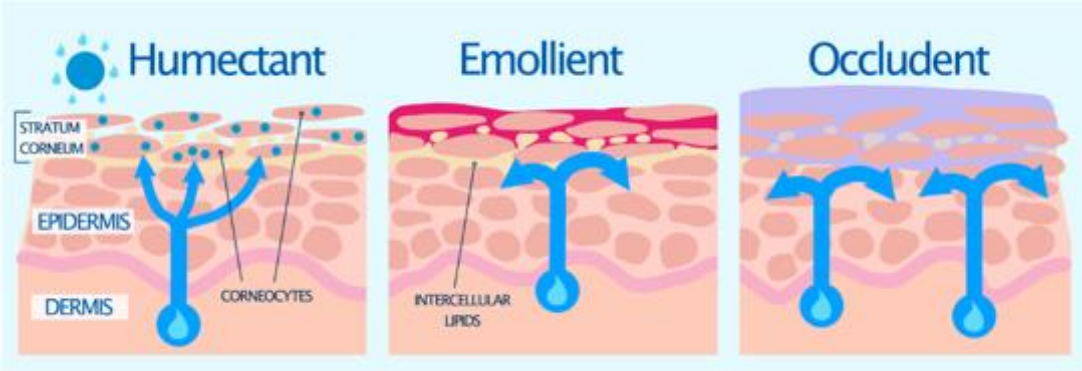


Figure 9 Mechanism of action of moisturizer.

Source: (Barnes et al., 2021).

1. Humectants

Humectants, characterized by their hygroscopic nature, share similarities with the natural moisturizing factor (NMF) found in the skin. These compounds possess the ability to permeate the stratum corneum (SC) and function akin to biological sponges, attracting and retaining water within the skin. This process involves the extraction of water from the dermis to the epidermis or from the surrounding environment when atmospheric humidity levels exceed 80%. However, the sole use of humectants can lead to water loss into the environment, necessitating their combination with occlusive agents to mitigate or prevent trans-epidermal water loss (TEWL). This combined approach aids in fortifying the epidermal barrier function and enhancing hydration levels. Moreover, certain humectants also exhibit emollient properties. Many humectants share molecular similarities with components of the NMF, such as lactic acid, pyrrolidone carboxylic acid (PCA), and amino acids. Humectants like glycerol, triacetin, and polyols are often incorporated into aqueous-based formulations, such as gels, to bolster their moisturizing and occlusive attributes, which typically exhibit lesser efficacy when compared to creams and ointments (Barnes et al., 2021).

2. Emollients

Emollients mimic the structure of intracellular bilayers within the stratum corneum (SC), enhancing the tactile sensation of the skin by filling the intercellular spaces between corneocytes and imparting a sensation of softness and suppleness, commonly referred to as "skin slip" or lubricity. This contributes to an overall enhancement in skin texture and appearance. Essential fatty acids like linoleic, stearic, and oleic acids, found in natural oils such as lanolin and coconut oil, can oxidize into eicosanoids. These molecules are key in signaling inflammation and immune responses, suggesting a significant impact on skin physiology (Barnes et al., 2021).

3. Occludents

Occlusive agents function by acting upon the natural sebum and lipid components present on the surface of the skin, thereby diminishing TEWL. They achieve this by forming a hydrophobic barrier film over the skin, effectively preventing water from evaporating from the stratum corneum (SC) and entrapping moisture within the upper skin layers. The effectiveness of moisturizers like soft white paraffin, lanolin, and various waxes increases when applied to damp skin. Lipophilic agents like petrolatum can infiltrate the stratum corneum's intercellular space, enhancing the lipid barrier to reduce water loss. Petrolatum, known for reducing transepidermal water loss by over 98%, is considered the most effective ingredient for dry skin. Other oily occlusive agents, such as mineral oil, silicone, and lanolin, typically reduce TEWL by approximately 20% to 30%. Nonetheless, these agents are often associated with drawbacks such as greasiness, potential allergenicity, and unpleasant odor (Barnes et al., 2021).

CHAPTER III

RESEARCH METHODS

Materials

1. *Pleurotus cystidiosus* (Phitsanulok Mushroom Cultivation Group, Phitsanulok, Thailand)
2. Acrylamide/Bis-acrylamide solution 30% (29:1) (HiMedia Laboratories GmbH, Einhausen, Germany)
3. Ammonium sulfate (Elago Enterprises Pty Ltd., N.S.W., Australia)
4. BLUltra Prestained Protein Ladder（6.5 to 270 kDa) (Bio-Helix Co. Ltd., New Taipei City, Taiwan)
5. Bovine serum albumin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
6. Butan-1-ol, AR. (RCI Labscan Limited., Bangkok, Thailand)
7. Copper (II) Sulfate (Antibioticos S.P.A., Milano, Italy)
8. Folin's reagent or Sodium 1,2-naphthoquinone-4-sulfonate (Loba Chemie PVT. Ltd., Mumbai, India)
9. Formaldehyde 35 - 40%, AR. (RCI Labscan Limited., Bangkok, Thailand)
10. N,N,N',N'-Tetramethyl ethylenediamine (Temed) (Merck KGaA, Darmstadt, Germany)
11. Potassium Sodium tartrate (Ajax Finechem Pty. Ltd., NSW, Australia)
12. Silver nitrate (RCI Labscan Limited., Bangkok, Thailand)
13. Sodium carbonate (Elago Enterprises Pty. Ltd., NSW, Australia)
14. Sodium chloride (Elago Enterprises Pty Ltd., N.S.W., Australia)
15. Sodium hydroxide (Ajax Finechem Pty. Ltd., NSW, Australia)
16. DMEM medium (Gibco, Thermo Fisher Scientific Inc., Massachusetts, U.S.)
17. Fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific Inc., Massachusetts, U.S.)
18. Antibiotic/Antimycotic solution (Gibco, Thermo Fisher Scientific Inc., Massachusetts, U.S.)
19. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Singapore)

20. Thiazolyl blue tetrazolium bromide (MTT) (Bio Basic Inc., Markham, Canada)
21. Phosphate buffer saline (PBS) (Gibco, Thermo Fisher Scientific Inc., Massachusetts, U.S.)
22. Mueller Hinton (MHA) agar (HiMedia Laboratories LLC, Kennett Square, U.S.)
23. Brain heart infusion (BHI) agar (HiMedia Laboratories LLC, Kennett Square, U.S.)
24. Butylene Glycol (P0622O37ACN, Krungthepchemi Co.,Ltd., Bangkok, Thailand)
25. Polyethylene Glycol 400 (0024239303, Krungthepchemi Co., Ltd., Bangkok, Thailand)
26. Glycerin 99.7% (CMCT0017-20220510, Cheme Cosmetics, Bangkok, Thailand)
27. Phenoxyethanol (PHG-S2220719-26, Krungthepchemi Co.,Ltd., Bangkok, Thailand)

Apparatus

1. Digital analytical balance with 0.01 mg readability (AB204, Mettler-Toledo, Greifensee, Switzerland)
2. Digital analytical balance (TM-02, Tiger, Taiwan)
3. Cell culture ware (Corning Incorporated, New York, U.S.)
4. Centrifuges (Model 5922, KUBOTA, Tokyo, Japan)
5. UVGL-25 Compact UV Lamp (Analytik Jena GmbH+Co. KG., Cambridge, UK)
6. Beckman Coulter J2-MC High-Speed Centrifuge (Beckman Coulter, Inc., California, USA)
7. Refrigerator (4 oC) (Sanden International (USA), Inc., Texas, USA)
8. Hotplate and Magnetic Stirrer (Model RCT basic, BEC Thai Bangkok Equipment & Chemical Co., LTD., Bangkok, Thailand)
9. 1 RC Dialysis Membrane Tubing 6000 to 8000 Dalton MWCO (Spectrum Labs, Inc., California, USA)
10. Microplate reader (Biotek, Synergy HT, Biotek Instruments, Winooski, USA)
11. Olympus CKX41 microscope (Thermo Fisher Scientific Inc., Massachusetts, U.S.)
12. Vortex mixer (G-560E, Scientific Industries, INC., New York, USA)
13. Incubator shaker (GFL 1086 shaking water bath, Burgwedel, Germany)
14. Hot air oven (Model 600, Memmert, Schwabach, Germany)
15. MSC-Advantage™ (Thermo Fisher Scientific Inc., Massachusetts, U.S.)

16. Forma™ Series 3 Water Jacketed CO2 Incubator, 184L (Thermo Fisher Scientific Inc., Massachusetts, U.S.)
17. Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific Inc., Massachusetts, U.S.)
18. Carl Zeiss™ Axio Vert.A1 inverted Microscope (Thermo Fisher Scientific Inc., Massachusetts, U.S.)
19. DSA25 Drop Shape Analyzer (A.KRÜSS Optronic GmbH, Hamburg, Germany)
20. Snake skin™ Dialysis Tubing 10,000 MWCO (Thermo Fisher Scientific Inc., Rockford, U.S.A.)
21. Analytical Balance (AB204-S, Mettler Toledo, Mettler Toledo Co., Ltd., Bangkok, Thailand)

Methodology

1. Preparation of *P. cystidiosus* extract (PCE)

The *P. cystidiosus* extraction was executed in adherence with the protocol outlined by (Deng et al., 2020). Initially, 3 kg of fresh fruiting body of *P. cystidiosus* was shredded and blended with 0.15 M NaCl in a 1:2 (w/v) ratio. The resulting mixture was centrifuged at 15,300 × g at 4 °C for 30 min, resulting in 7 L of supernatant, which was subsequently collected for the protein precipitation. Precipitation was achieved by 60% saturation with ammonium sulfate. The precipitate was collected and resuspended in distilled water at a volume ratio of 3:50 (v/v), resulting in 700–750 mL of protein solution, which underwent repeated centrifugation. The 650–700 mL protein suspension was dialyzed at intervals of 6 hr over 1-3 days to eliminate salt, followed by freeze-drying of PCE, which was stored at -80 °C until further analysis.

The PCE was hydrolyzed with a 10:1 ratio of pepsin to 5 mM potassium phosphate. Hydrolysis was performed at 37 °C for 24 hr at pH 6.8 by using pepsin (5% enzyme/substrate), and the protein sample was then heated at 95 °C for 20 min to stop the reaction. The protein hydrolysate was then prepared using the following steps (i) centrifugation to separate the hydrolysate from any remaining solids and collect the supernatant, (ii) purification of the supernatant by filtering through ultrafiltration membranes with molecular cut-off of ≤ 3- kDa giving hydrolyzed PCH (≤3 kDa), and (iii) freeze-drying the filtered supernatant to yield the protein hydrolysates. The PCH (≤3kDa) was stored at -20 °C until required for further analysis.

2. Specification of PCE

2.1 Protein content

PCE samples were placed in 96-well plates and the protein levels were measured using the Lowry assay adapted from (Bollag & Edelstein, 1991). Solutions were prepared as follows: Solution A with 5 g of $CuSO_4$ $5H_2O$ in 100 mL of distilled water; and Solution B with 21.2 g of Na_2CO_3 and 4 g of NaOH in 1 L of water. Solution C combined 1 mL of Solution A with 50 mL of Solution B, and Solution D mixed 10 mL of Folin-Ciocalteu reagent with 10 mL of distilled water. The sample preparation procedure entailed the dissolution of PCE and PCH (≤3kDa) in deionized water,

resulting in the formation of a protein solution derived from PCE and PCH (≤3kDa). A 20 µL sample of both PCE and PCH (≤3kDa) was mixed with 200 µL of Solution C and allowed to stand at room temperature for 15 min. Subsequently, 20 µL of Solution D was added, and the mixture was left for another 30 min at room temperature. Absorbance was measured at 750 nm using a microplate reader, and protein concentration was calculated against a BSA standard curve from the same plate.

2.2 Electrophoresis

The electrophoretic profile of the PCE was determined following the protocol used by (Jovanovic et al., 2007). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-Page) analysis was used to distinguish between proteins based on their molecular weight, ranging from 6.5 to 270 kDa and 3 to 260 kDa. Each PCE protein concentration was 10 µg/30 µL. A sample buffer was mixed with the samples to achieve a weight ratio of 1:5. The buffer sample contained 3 mL of 20% SDS; 3.75 mL of 1M Tris-HCl pH 6.8; 9 mg of bromophenol-blue; 2.4 mL of 0.0001% (w/v) β-mercaptoethanol; and 4.5 mL of 20.2% (v/v) glycerol in a beaker. The volume was then adjusted to 15 mL with distilled water. The supernatant of each sample was boiled at 95 °C for 15 min before performing gel electrophoresis. A 10% de-staining solution was prepared. The resolving gel (15%) contained 30% Acrylamide and bis-acrylamide solution; 4.5 M Tris-HCl (pH 8.8); 10% SDS; 10% ammonium persulphate; and N,N,N',N'-tetramethyl ethylenediamine (TEMED). The stacking gel (4%) contained 30% acrylamide and bis-acrylamide solution; 0.5 M Tris-HCl (pH 6.8); 10% SDS; 10% ammonium persulphate; and TEMED. The electrophoresis was run at 120V for 2 hr using a General Electric Healthcare Bioscience diagnostic equipment, EPS301. The running buffer consisted of 25 mM Tris base; 192 mM glycine; and 0.1% SDS. The BLUltra pre-stained protein marker was used to identify and compare the molecular weight of the proteins. The gel was tinted with a silver and a coomassie brilliant blue (R250). The gel was washed with a de-staining solution, (40% (v/v) methanol and 10% (v/v) acetic acid), until the background was clear upon inspection. The gel was then viewed and photographed with a gel documentation (GelDoc) system.

3. The bioactivity of PCE

3.1 DPPH radical scavenging activity

The radical scavenging ability (RSA) of PCE was assessed using a DPPH assay, adapted from (Chen et al., 2021). The assay involved dissolving 0.1 mM DPPH in methanol to prepare the reagent. The test sample included 20 µL of PCE at three different protein concentrations: 50, 100, and 250 µg/mL. Then, 100 µL of DPPH reagent was added to a microplate and the mixture was incubated for 30 min in the dark at room temperature. Absorbance was measured at 517 nm. The results were benchmarked against 0.5 mg/mL of Trolox, used as a positive control. Lower absorbance indicated higher free-radical-scavenging activity. The capability to scavenge radicals in the DPPH was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{OD517}_{\text{blank}} - \text{OD517}_{\text{sample}})}{\text{OD517}_{\text{blank}}} \times 100 \quad (1)$$

3.2 ABTS radical scavenging activity

The $ABTS^{\cdot+}$ scavenging activity of PCE was assessed following the method described by (Chen et al., 2021). The ABTS reagent was created by dissolving 7 mM ABTS in distilled water. Samples of 20 µL each at concentrations of 50, 100, and 250 µg/mL were prepared. A 2.45 mM potassium persulfate and 7 mM ABTS were added to a microplate. The mixture was incubated for 30 min in the dark at room temperature, and absorbance was measured at 734 nm. Results were compared against 0.5 mg/mL Trolox as a positive control. Lower absorbance indicated higher radical-scavenging activity. The capability to scavenge the radical of the ABTS was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{OD734}_{\text{blank}} - \text{OD734}_{\text{sample}})}{\text{OD734}_{\text{blank}}} \times 100 \quad (2)$$

3.3 Anti-tyrosinase activity

The anti-tyrosinase activity of PCE was assessed with modifications to the method by (Song et al., 2020). A mixture was prepared by combining 120 µL of 5 mM L-dopa with 70 µL of 5 mM potassium phosphate buffer (pH 6.8). Then, 15 µL of tyrosinase (EC 1.14.18.1) and PCE were added. Kojic acid served as the positive control. The mixture was incubated at 37 °C for 20 min, and the formation of DOPA-chrome was measured at 475

nm, indicating tyrosinase inhibition. The anti-tyrosinase activity was calculated using the following equation:

$$\text{Tyrosinase inhibition (\%)} = \frac{(\text{OD475}_{\text{control}} - \text{OD475}_{\text{sample}})}{\text{OD475}_{\text{control}}} \times 100 \quad (3)$$

3.4 Determination of total phenolic content of the PCE

The total phenolic content (TPC) within the PCE was assessed using the Folin–Ciocalteu assay, following the method introduced by (Singleton & Rossi, 1965), with adaptations for 96-well microplates as outlined by (Granato et al., 2015). The sample was dissolved in deionized water for a concentration of 1 mg/mL. Following preparation, 25 µL of the solution was mixed with 25 µL of threefold diluted Folin–Ciocalteu reagent and 200 µL of ultrapure water. After a 5-min incubation, 25 µL of saturated sodium carbonate solution (10.6 g/100 mL) was added. The microplate was shaken for 20 sec and left to stand for 60 min in the dark at 25 °C. Absorbance was measured at 725 nm using a Biotek Synergy HT microplate reader (Biotek, Synergy HT, Biotek Instruments, Winooski, USA). The total phenolic content was quantified and reported in milligrams of gallic acid equivalents per milliliter.

3.5 Anti-bacterial Activity

The antibacterial activity of the PCE was evaluated with slight modifications to the protocol described by (Viyoch et al., 2006). Fresh bacterial cultures were cultivated on Mueller Hinton (MHA) Agar for *S. aureus* and *S. epidermidis* and Brain Heart Infusion (BHI) Agar for *C. acnes*. The direct colony suspension method involved inoculating bacterial cultures (*S. aureus*, *S. epidermidis*, and *C. acnes*) in 0.85% normal saline to achieve concentrations of 1-1.5×10^8 CFU/mL. A 2 mg/disc test disc was prepared by dissolving the sample in distilled water to a concentration of 100 mg/mL, followed by filtration through a sterile 0.22 µm syringe filter. Subsequently, 20 µL of the test sample was pipetted onto a 6 mm sterile filter and dried at 37 °C for 30 min. The inhibition zones were assessed by placing the test sample strips on the agar surface, with tetracycline (30 µg) serving as the positive control for *S. aureus* and *S. epidermidis*, clindamycin (2 µg) for *C. acnes*, and distilled water as the negative control. Cultures of *C. acnes* were maintained at 37 °C in an anaerobic

environment for 4 days, while *S. aureus* and *S. epidermidis* were incubated at 37 °C for 18–24 hr. Inhibition zones were determined by measuring the diameter of clear zones in millimeters. Each test was conducted in triplicate to ensure the reliability of the results.

4. *In vitro* cytotoxicity determination

The cytotoxicity of PCE on human keratinocytes (HaCaT) was evaluated with minor adjustments to the protocol by (Gaweł-Bęben et al., 2020). HaCaT cells were cultured in DMEM supplemented with 10% FBS and 1% Anti-Anti (100U/mL) in a 5% CO_2 environment at 37 °C. Cell cytotoxicity and viability were assessed using the MTT assay. Cells were seeded at 2,000 cells/well in a 96-well plate and incubated for 24 hr before treatment with various concentrations of PCE for another 24 hr. Post-treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. After 3 hr, purple formazan crystals formed which were washed away, and the precipitates were dissolved in 100 µL DMSO per well. Absorbance was measured at 540 nm using a microplate reader after 10 min of dissolution. Cell viability was assessed using the following equation:

$$\text{Cell viability (\%)} = \frac{(\text{OD540}_{\text{control}} - \text{O540}_{\text{treated}})}{\text{OD540}_{\text{control}}} \times 100 \quad (4)$$

5. The assessment of UV light source and its effect on cell viability through UV exposure test

UV lamps emitting UVA (320-400 nm) were employed for irradiation, utilizing an *in vitro* UVGL-25 Compact UV lamp (L118304) equipped with a sensor calibrated at 365 nm to measure consistent power output in units of J/cm². Exposure duration was calculated using the equation:

$$\text{Dose (J/cm}^2\text{)} = \text{exposure time (s)} \times \text{output intensity (W/cm}^2\text{)}. \quad (5)$$

Rigorous quality control procedures were implemented to assess lamp performance and exposure time accuracy before each use to account for potential fluctuations in power output.

The assessment of the protective effect of PCE on cell viability was conducted using the MTT assay. Initially, cells were seeded into a 96-well culture plate at a density of 2,000 cells/well and cultured for 24 hr. The cells were then exposed to UV radiation at intensities of 0, 1.59, and 3.18 J/cm². Following the exposure, the cells were treated with candidate protein hydrolysates at both low and high concentrations for durations of 24 hr and 48 hr. Similarly, the evaluation of the therapeutic effect of PCE on cell viability involved seeding the cells into a 96-well culture plate at a concentration of 2,000 cells/well, followed by incubation for 24 hr. The cells were then subjected to UV radiation at intensities of 0, 1.59, and 3.18 J/cm². Post-exposure, the cells were treated with the candidate protein hydrolysates at low and high concentrations for 24 hr and 48 hr.

6. Preparation of hydrating water formulations

Hydrating water formulations were prepared by combining hydrophilic substances in water. Water was mixed with butylene glycol, polyethylene glycol 400, and glycerin. PCE and phenoxyethanol were added, and the solution was then homogenized. All three formulations included varying percentages of components (see Table 9).

Table 9 Ingredients and functions of substances in the hydrating water formulations.

Ingredients	Formulation (%w/w)			Function
	F1	F2	F3	
Water	90.00	79.00	89.96	Solvent
Butylene glycol	4.00	8.89	4.00	Humectant
Polyethylene glycol 400	4.00	8.89	4.00	Humectant
Glycerin	1.00	2.22	1.00	Humectant
Phenoxyethanol	1.00	1.00	1.00	Preservative
PCE	-	-	0.04	Active ingredient

7. Snake skin moisturizing study

7.1 Water Loss in Snake Skin

The efficacy of hydrating water formulations was conducted by incorporating slight modifications to the protocol as outlined by (Arezki et al., 2017) to determine weight loss in snake skin. Snake skins sourced from (Thermo Fisher Scientific; 10,000 MWCO) were cut into 2x2 cm^2 segments, which were immersed in water at 4 °C for 24 hr. The snake skin segments were then dried on filter paper to eliminate residual water. Next, each sample received 10 g of deionized (DI) water, with 25 µL of hydrating water formulations added to ensure even dispersion. Following an air-drying period of 20–30 min, the samples were weighed using a Mettler Toledo AB204 scale with a precision of 0.1 mg. Percentage weight loss was assessed at predetermination times (0, 1, 2, 4, 6, and 8 hr) under ambient conditions. This experimental procedure was replicated six times to ensure experimental reliability.

7.2 Contact angle measurement

The drop methodology was employed to determine contact angles on other snake skins that were treated with hydrating water, following procedures modified from (Badami & Bui, 2021). Contact angle measurements were carried out using a DSA25 Drop Shape Analyzer (A.KRÜSS Optronic GmbH, Hamburg, Germany). The substrates were fixed on microscope slides measuring 5x2 cm using tape, and 80 µL of the sample was applied and the slides were left to dry overnight at room temperature before measurement. Deionized water was utilized as the liquid phase in all experiments for wettability assessments, with each sample allocated an experimental duration of 10 sec. Contact angle measurements were conducted using a liquid-phase droplet volume of 10 µL. Real-time monitoring of liquid volume was facilitated through image analysis techniques employing ADVANCE Software (KRÜSS Scientific). Automated contact angle analysis by ADVANCE software (see Figure 10) was conducted over time, tracking the droplet circumference to ascertain the angle formed at the interface between the solid surface, the liquid drop, and the surrounding vapor phase. Reported values represent the average of three distinct samples, with three measurements taken on each sample at six discrete locations on the given substrate.

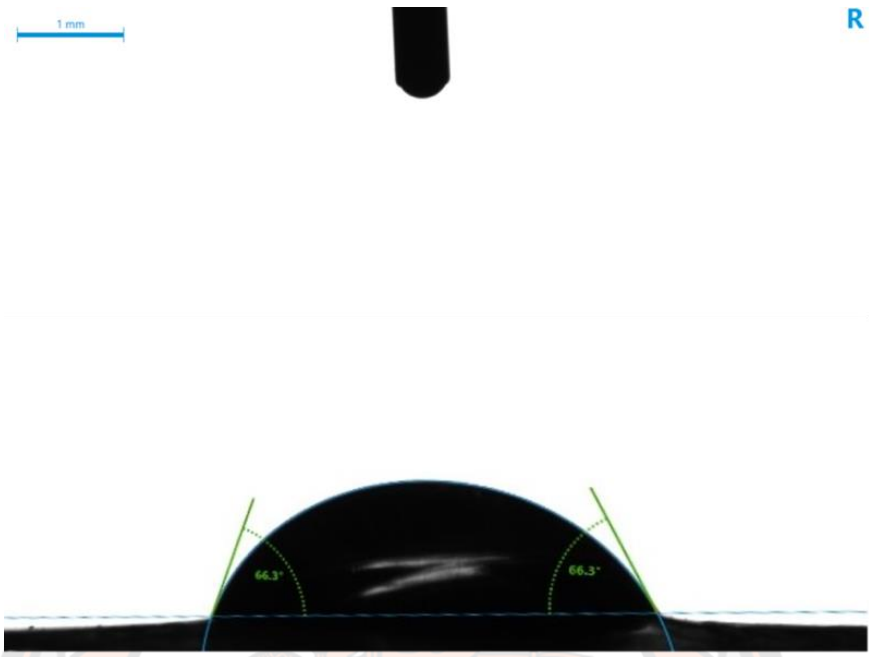


Figure 10 The contact angle of water droplets on hydrating water application substrates was assessed, with automated analysis conducted by the measuring instrument.

8. Accelerated stability

PCE powder and hydrating water were stored in sealed glass bottles for accelerated stability testing. Initially assessed at 24 hr (t_0) and again at 90 days, the samples were exposed to temperatures of 4 ± 2 °C, ambient temperature, and 45 ± 2 °C. Stability was evaluated based on organoleptic tests, including pH, appearance, and sensory characteristics.

9. Statistical analysis

Data are reported as the mean ± SD from independent experiments. Statistical analysis was conducted using Student's t-test and One-way ANOVA, with a significance threshold set at $\rho<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

1. *P. cystidiosus* extract

1.1 Specification of PCE

PCE extraction was performed to facilitate industrial upscaling for cosmetic ingredient production. The characteristics of PCE were assessed for each batch. *P. cystidiosus* was subjected to extraction in three batches to ensure consistency in characteristics.

The percentage yields of PCE were 82 mg/100 g for PCE1, 64 mg/100 g for PCE2, and 41 mg/100 g for PCE3 from the fresh fruiting body of *P. cystidiosus*. Total protein content percentages were 72.00% for PCE1, 85.00% for PCE2, and 100.00% for PCE3, as shown in Table 10. The total protein content observed in PCE1 and PCE2, lower than in PCE3 indicated the presence of residual salts from the extraction procedure. This result is explained by the conductivity of the protein solution before the freeze-drying process, where PCE1 and PCE2 displayed approximately 2500 μS/cm. Conversely, the longer dialysis process (PCE3) clearly showed lower conductivity, measuring at 549.37 ± 0.25 μS/cm. This approach holds the potential for improving protein yield. Previous research suggested that pre-extraction salt removal before protein extraction explained the decline in conductivity of the alkali-soluble protein fraction from seaweed pulp (Juul et al., 2022).

Table 10 The percentage of yields and protein of PCE.

Protein from	Conductivity (μS/cm)	Yield (mg/100g)		Total protein content in PCE (%)
		PCE	Protein in PCE	
PCE1	2505.22 ± 1.67	82.00	59.00	72.00
PCE2	2513.58 ± 1.73	64.00	55.00	85.00
PCE 3	549.37 ± 0.25	41.00	41.00	100.00

The powder of PCE1-PCE3 exhibited as a fine brown powder, as shown in Figure 11. The powders varied in hue, with PCE1 being the darkest brown, followed by the progressively lighter shades of PCE2 and PCE3. This color variation resulted from prolonged dialysis, which yielded a lighter brown color influenced by the original color of the mushrooms blended before extraction. The extraction process for peptides with a molecular weight of ≤3kDa (PCH) produced a relatively low yield, suggesting the need for an improved extraction method, potentially through the addition of pepsin enzymes to enhance digestion efficiency.

Previous studies indicated that the selection of appropriate enzymes or chemical reagents should consider the target proteins' characteristics, such as amino acid composition and hydrophobicity, as well as their compatibility with the digestion technique's pH or solvent conditions. Various methods existed to accelerate digestion to mere seconds, potentially benefiting specific applications like membrane protein digestion. However, protocol optimization was necessary, particularly for protein or biomarker quantification, to avoid less reproducible results (Switzar et al., 2013).

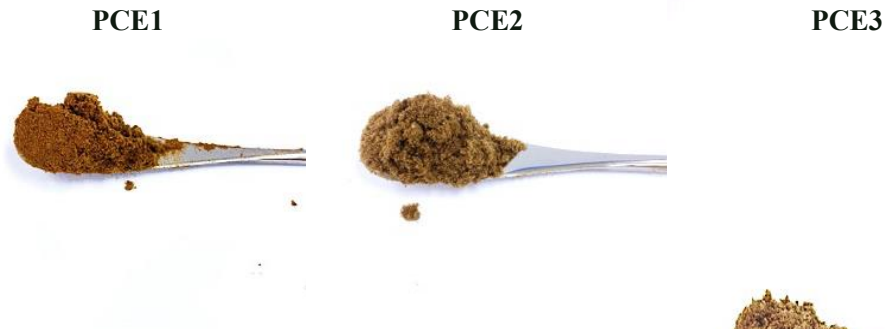


Figure 11 Physical characteristics of PCE.

1.2 Profile of protein from PCE

Ensuring the consistency and quality of protein extraction in each batch requires analyzing the molecular weight pattern. The protein profile of PCE was assessed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), revealing bands spanning from 3 to 270 kDa. Predominantly, protein bands below 20 kDa were observed in PCE1, PCE2, and PCE3, as depicted in Figure 12.

Notably, PCE demonstrated uniform protein profiles across all batches, suggesting its viability as a standard for prospective industrial-scale manufacturing.

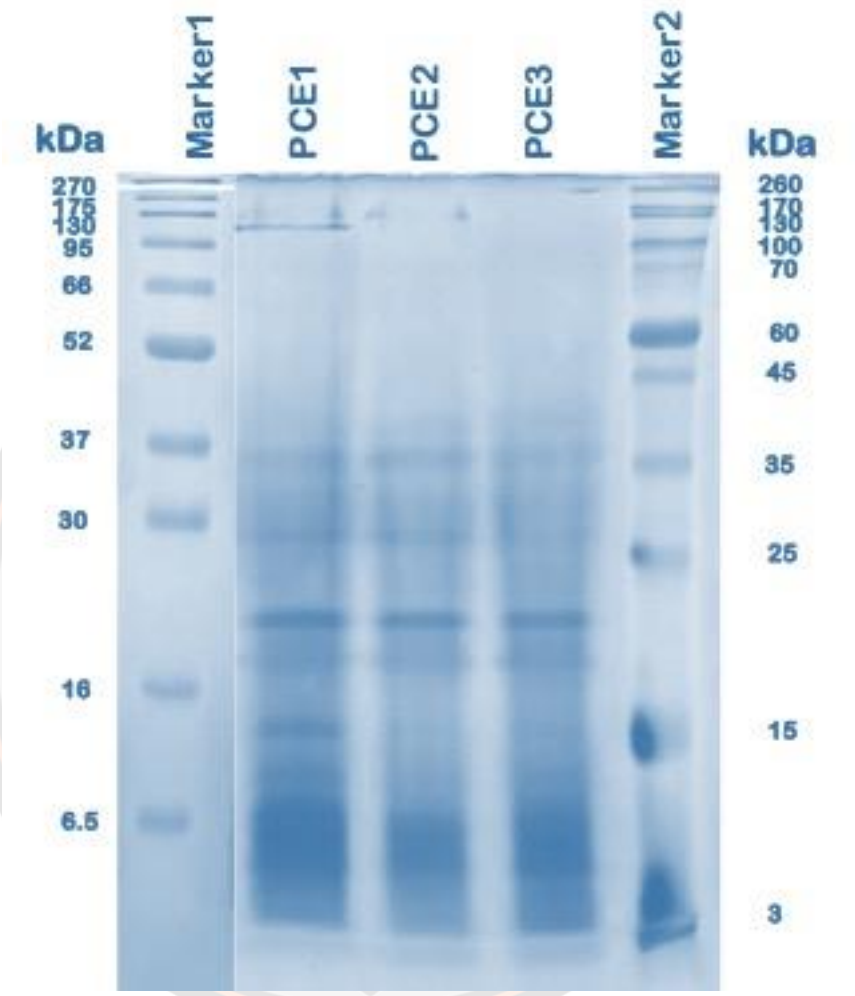


Figure 12 Protein profile of PCE (PCE1, PCE2, and PCE3). Maker1: Standard marker (6.5-270 kDa) and Maker2: Standard marker (3-260 kDa).

2. The bioactivity of PCE

2.1 Antioxidant activity

There is rising interest in adding antioxidants to cosmetics due to market demand and ingredient advancements. Bioactive peptides from animal and plant protein hydrolysates are recognized for effectively combating oxidative stress (Yang et al., 2021). In this study, the antioxidant activity of the protein extracted from *P. cystidiosus* was determined by DPPH and ABTS assays. The percentage of DPPH and ABTS radical scavenging activity, or % antioxidant index, was determined for PCE1, PCE2, PCE3, and PCH at concentrations of 50, 100, and 250 µg/mL, with Trolox serving as the reference standard at 500 µg/mL. The DPPH radical scavenging activity of PCE indicated low activity across the three batches initially, but an increase was observed with higher concentrations of PCE. At a concentration of 250 µg/mL, PCE1 exhibited an antioxidant activity of 13.43 ± 0.57% (376 µg/mL), PCE2 showed 10.12 ± 0.96% (283 µg/mL), and PCH demonstrated 24.24 ± 1.17% (700 µg/mL), outperforming protein concentrates from *P. ostreatus* (González et al., 2021). Additionally, the IC_{50} values for DPPH (1.4 ± 0.2 mg/mL) confirmed the superior antioxidant activity of PCE1, PCE2, and PCH. A similar pattern was observed in the ABTS assay, at a concentration of 250 µg/mL, PCE1 exhibited an antioxidant activity of 8.06 ± 0.75% (2.47 mg/mL), PCE2 showed 7.03 ± 0.37% (2.15 mg/mL), PCE3 showed 16.99 ± 0.57% (5.20 mg/mL), and PCH demonstrated 32.14 ± 2.13% (9.83 mg/mL), outperforming protein concentrates from *P. ostreatus* (González et al., 2021). Additionally, the IC_{50} values for ABTS (15.3 ± 0.3 mg/mL) confirmed the superior antioxidant activity of PCE1, PCE2, PCE3, and PCH, as shown in Table 11. However, notable antioxidant activity was observed for the PCH. This suggests that proteins with a molecular weight below 3 kDa exhibit superior antioxidant activity compared to those with higher molecular weights, consistent with previous studies showing that low molecular weight protein hydrolysates (below 3 kDa) have greater DPPH• and •OH scavenging activities (You et al., 2010). Additionally, peptides with molecular weights ranging from 12 to 35 kDa demonstrate significant bioactivity (González et al., 2021).

Table 11 The DPPH, and ABTS radical scavenging activity of PCE.

Protein concentration (µg/mL)	DPPH radical scavenging activity (%)					ABTS radical scavenging activity (%)				
	PCE1	PCE2	PCE3	PCH (≤3 kDa)	Trolox 500 µg/mL	PCE1	PCE2	PCE3	PCH (≤3 kDa)	Trolox 500 µg/mL
50	3.12 ± 0.42	3.76 ± 0.42	0.00±0.00	16.12±1.11	-	5.63±0.89	6.49±0.32	9.31±0.17	24.24±0.50	-
100	7.99 ± 0.81	9.64 ±0.83	2.57±0.73	18.39±0.61	-	6.98±0.34	6.88±0.15	11.26±0.53	26.41±0.68	-
250	13.43 ± 0.57	10.12 ± 0.96	6.70±0.61	24.24±1.17	-	8.06±0.75	7.03±0.37	16.99±0.57	32.14±2.13	-
	-	-	-		81.98±0.28	-	-	-		89.21±0.17

Results are given as mean ±standard deviation.

2.2 Anti-tyrosinase activity

Skin pigmentation is induced by melanin production from melanocytes due to UV radiation exposure. Tyrosinase plays an important role in converting tyrosine into melanin. Inhibiting tyrosinase activity is a property of a substance that can disrupt melanin production, reducing skin pigmentation (Hearing & Tsukamoto, 1991; Meng et al., 2012). In this study, the tyrosinase inhibitory percentages for PCE1, PCE2, PCE3, and PCH were evaluated at concentrations of 50, 100, and 250 µg/mL, with kojic acid at 100 µg/mL as the reference standard (Table 12). The inhibitory activity was determined using a standard tyrosinase inhibition assay. The results indicated that PCE did not significantly inhibit tyrosinase, likely due to its low concentration of low molecular weight proteins. In contrast, PCH at 50-250 µg/mL demonstrated higher tyrosinase inhibitory activity (49.89% to 63.41%). Compared to the tyrosinase inhibitory activities of hot water extracts from *P. ostreatus* fruiting bodies (0.125-1.0 mg/mL) reported by (Alam et al., 2010) which ranged from 9.60% to 49.60%, PCH showed superior inhibition, suggesting the efficacy of small proteins. This aligns with findings from previous research indicating that low molecular weight proteins exhibit stronger tyrosinase inhibitory activity compared to larger counterparts (Feng et al., 2021). Additionally, prior studies have highlighted a positive correlation between peptide antioxidant activity and their inhibitory effects against LOX, tyrosinase, and XOD (Addar et al., 2019; Grancieri et al., 2019; Zhong et al., 2021).

Table 12 Inhibitory tyrosinase activity of PCE.

Protein concentration (µg/mL)	Inhibitory tyrosinase activity (%)				
	PCE1	PCE2	PCE3	PCH (≤3 kDa)	Kojic acid 100 µg/mL
50	nd	nd	nd	49.89 ± 1.69	-
100	nd	nd	nd	58.21 ± 1.28	-
250	nd	nd	nd	63.41 ± 0.25	-
	-	-	-		82.66 ± 1.03

Results are given as mean ±standard deviation, and nd: not detected.

2.3 Total phenolic content of the PCE

In this study, total phenolic content for PCE1, PCE2, PCE3, and PCH were evaluated at concentrations of 50, 100, and 250 µg/mL, with Gallic acid as the reference standard. The results indicated undetectable total phenolic contents in PCE (Table 13). Compared to the higher total phenolic content reported by (González et al., 2021) for protein concentrate from *P. ostreatus* mushrooms, the freezing and freeze-drying processes after extraction may partially account for the decreased phenolic content and radical scavenging ability. This indicates that the antioxidant and anti-tyrosinase activities described in Sections 2.1-2.3 do not correlate with the phenolic content in these extracts, consistent with findings that total phenolic content reflects the concentration of phenolic compounds associated with enhanced antioxidant (Duan et al., 2007; Pan et al., 2008; Zhao et al., 2006) and tyrosinase inhibitory effects (Baek et al., 2008; Kim et al., 2008). PCH was not tested for total phenolic content due to insufficient quantity and will not be included in this or subsequent experiments.

Table 13 Total phenolic content of PCE.

Protein concentration (µg/mL)	TPC (mg gallic acid equivalent (GAE)/mL)		
	PCE1	PCE2	PCE3
50	nd	nd	nd
100	nd	nd	nd
250	nd	nd	nd

Results are given as mean ±standard deviation, and nd: not detected.

2.4 Anti-bacterial activity

The anti-bacterial activities of the PCE samples were evaluated against *S. aureus, S. epidermidis,* and *C. acnes,* all three of which are the cause of acne. The results showed the PCE exhibited no discernible activity against *S. aureus, S. epidermidis,* and *C. acnes,* as shown in Table 14. This was consistent with previous research findings, as documented by (Hapsari et al., 2012), who identified alkaloids, saponins, phenolics, and tannins as prominent components within oyster mushrooms. Tannins are

recognized for their significant involvement in microbial cell wall degradation. Additionally, (Egra et al., 2019) highlighted the antibacterial properties of tannins, attributing their efficacy to protein precipitation. The antibacterial mechanism of tannins involves interaction with the cell membrane, enzyme inactivation, and disruption of genetic material functionality.

Table 14 The PCE's inhibitory zone diameter.

Bacterial	Inhibition zone (mm)		
	PCE (100 mg/mL)	Negative control	Positive control*
S. aureus	NA	NA	35
S. epidermidis	NA	NA	13
C. acnes	NA	NA	65

*Antibiotics: Tetracycline 30 µg for positive control of *S. aureus* and *S. epidermidis*.
: Clindamycin 2 µg for positive control of *C. acnes.*

NA: No activity

3. *In vitro* cytotoxicity assessment of PCE

In this cytotoxicity assessment, HaCaT cells were exposed to varying concentrations (100 - 5000 µg/mL) of protein from PCE for 24, 48, and 72 hr. As shown in Figure 13, increasing PCE concentration resulted in decreased cell viability of HaCaT cells. Concentrations exceeding 500 µg/mL exhibited clear cytotoxic effects at all time points. However, the concentration of 100 µg/mL appeared safe for HaCaT cells, with cell viability percentages at 24 hr (86.71±5.33%), 48 hr (85.32±4.54%), and 72 hr (105.73±6.86%). Consequently, the concentration of 100 µg/mL of PCE was deemed suitable for further investigation as a cosmetic ingredient in this study. After inoculation at 104 cells/mL, HaCaT cells exhibited a lag phase of 24 to 48 hr, a population doubling time of 26.4 hr, and a cell density at a confluence of 105 cells/cm². These results were used to select optimal experimental conditions for assessing cytotoxicity (at 24 hr) and antiproliferative activity (over 7 days) of the drugs. A 72 hr treatment with PCE may not be suitable for cytotoxicity testing, as the multiwell

colorimetric assay (MTT) can distinguish cell densities from 2,500 to 40,000 cells/well (Pessina et al., 2001), but after 72 hr, cell numbers had increased significantly.

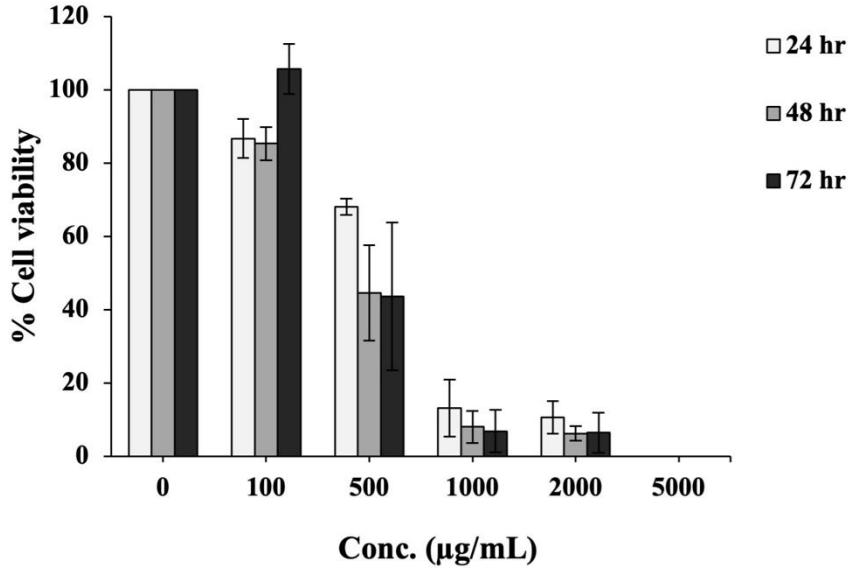


Figure 13 The percentage of cytotoxicity attributed to the PCE.

4. Effect of PCE on cell viability before and after UV exposure

The protective and therapeutic effects of PCE were evaluated on HaCaT cells exposed to UV radiation at doses of 0, 1.59, and 3.18 J/cm² for 24 and 48 hr (Figure 14). The PCE concentration selected from the *in vitro* cytotoxicity assessment was evaluated in this experiment. Treatment with PCE at a concentration of 100 µg/mL before UVA exposure at doses of 1.59 and 3.18 J/cm² resulted in increased cell viability compared to control cells at 24 hr post-UVA radiation (Figure 14A), suggesting its potential as an anti-photoaging adjuvant. This aligns with the (N. S. A. Rahman et al., 2021) study, which evaluated the photoprotective properties of exopolysaccharides (ExPFE) and EnPFE from *P. flabellatus* (pink oyster mushroom) extracts on UVA-irradiated human dermal fibroblast (HS-27) cells using the Cell Titer-Blue® assay. The cells were exposed to UVA doses ranging from 0 to 76 J/cm² at 24 hr, and both 0.001-10 mg/ml of ExPFE and EnPFE demonstrated photoprotective effects. The superior results shown in the PCE treatment group from prolonged UVA exposure (Figure 14B) were, however, not significantly different from the results

shown from prolonged UVA exposure in the untreated group (See Figure 14C and D). These results may indicate that PCE at a concentration of 100 µg/mL may offer some protection against UVA-induced epidermal damage in keratinocytes. These findings align with a previous study (Choi et al., 2022) where aqueous extracts of *P. ostreatus* and *Hericium erinaceus* demonstrated positive DPPH and ABTS radical-scavenging activity. The extracts promoted procollagen type I production and reduced matrix metalloproteinase-1 and elastase activity in human dermal fibroblast cells exposed to UVA damage.

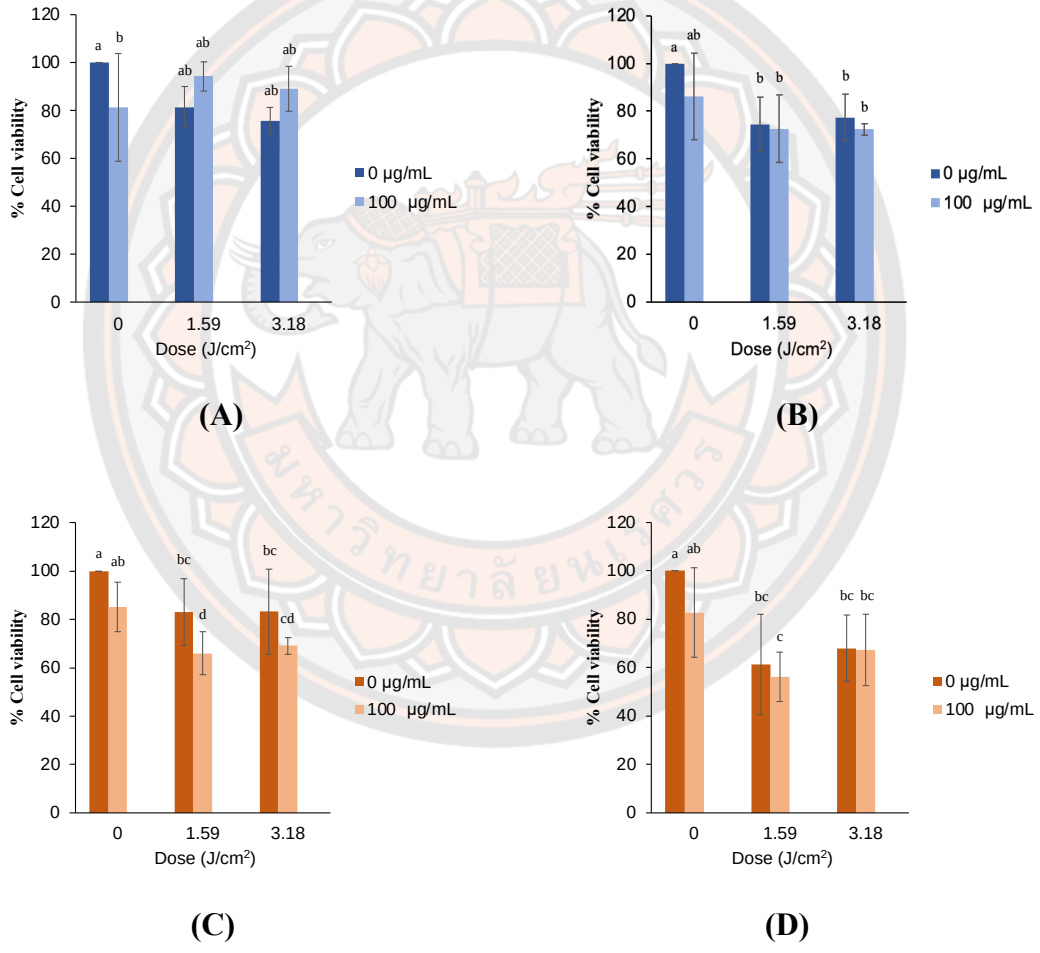


Figure 14 Effects of PCE on HaCaT cell viability before and after UV exposure: A) pre-UV (protective) at 24 hr, B) pre-UV (protective) at 48 hr, C) post-UV (therapeutic) at 24 hr, D) post-UV (therapeutic) at 48 hr.

5. Hydrating water containing PCE

A hydrating water product is a water-based skincare product that is aimed at replenishing moisture levels in the skin for a hydrated, plump, and refreshed complexion. In this study, the hydrating water containing PCE was prepared to assess its *in vitro* moisturizing effect in a cosmetic product and the final product's stability. From Table 9, formulations F1, containing 9% w/w humectant, and F2 (without PCE) containing 20% w/w humectant, both exhibited clear liquid solutions. The addition of PCE led to a transparent yellow liquid appearance. Stability tests of the three formulations were conducted under accelerated conditions at 4 ± 2 °C, ambient temperature, and 45 ± 2 °C for 90 days, as shown in Figure 15. None of the formulated hydrating waters exhibited changes in pH, color, or odor. The hydrating water containing PCE was further evaluated for its moisturizing properties in a snake skin moisturizing study and water contact angle test.

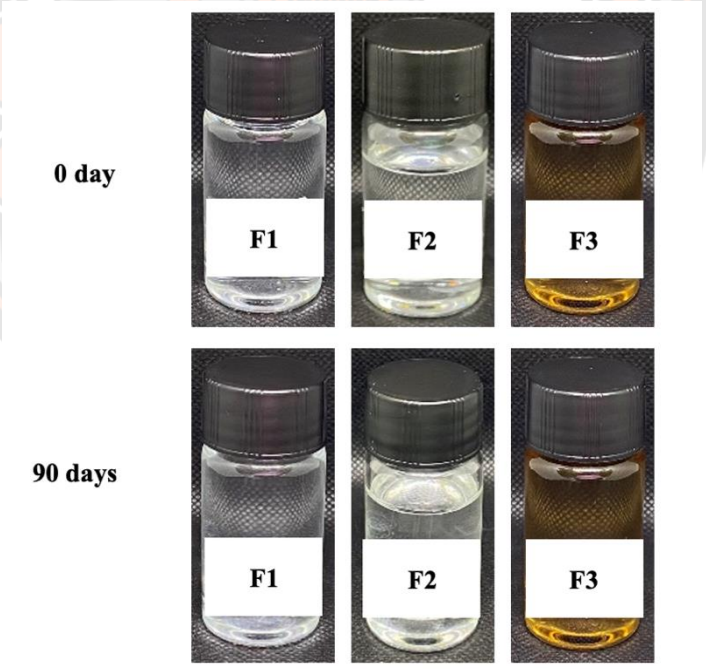


Figure 15 The appearance of a hydrating water formula with different ratios of components in 0 days and 90 days.

6. The water loss of snake skin treated with hydrating water

Hydrating water is frequently employed to enhance the moisturizing properties of the skin by attracting moisture into the skin. The assessment of weight loss from the snake skin can explain the efficacy of hydrating water in the moisturizing property (Arezki et al., 2017). Figure 16 illustrates the rate of water loss from the snake skin surfaces treated with hydrating waters. The untreated snake skin exhibited the highest percentage of water loss. However, the application of hydrating waters (F1, F2, and F3) reduced the rate of water loss. At 8 hr, treatments with F1, F2, and F3 showed a lower percentage of water loss compared to untreated skin, indicating the positive moisturizing effect of hydrating water containing PCE. F3, containing 0.04% w/w PCE, was particularly effective in reducing water loss. This aligns with (Ścibisz et al., 2008)., who reported that high molecular weight proteins from animal and plant sources exhibit excellent moisturizing properties. Additionally, proteins with molecular weights below 3000 Da can penetrate deeper into the skin (dermis) and hair (cortex). Hydrolyzed proteins are often combined with other moisturizing agents such as glycerin, sodium lactate, or free amino acids. Polar peptides, being hydrophilic, can stabilize o/w emulsions by increasing the viscosity of the external phase, while hydrophobic proteins with low solubility might stabilize emulsions through their affinity for the water-oil interface.

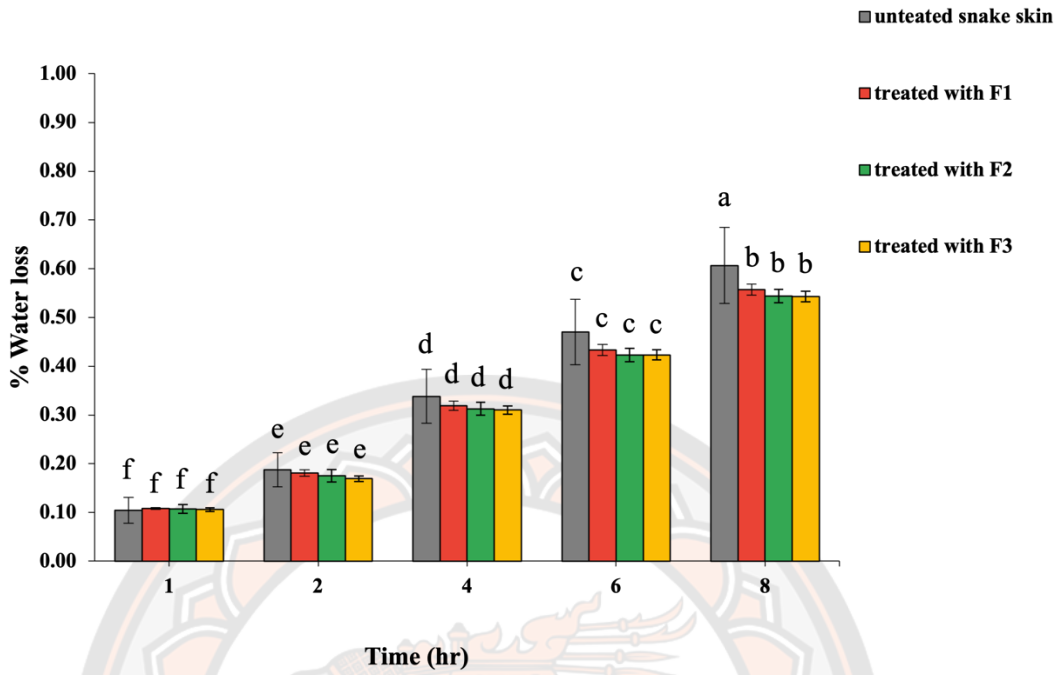


Figure 16 The percentage of water loss from hydrating water on snake skin surfaces.

7. Water contact angle of hydrating water on snake skin surfaces

The water contact angle is an important parameter for assessing skin wettability after treatment with cosmetic products. A lower water contact angle indicates better wetting properties (Wang et al., 2024), potentially improving spreadability and absorption on the skin. The snake skin treated with hydrating water exhibited a significant reduction in water contact angle (Figure 17). Notably, the inclusion of PCE in the F3 formulation resulted in the lowest water contact angle value, indicating enhanced skin wettability conferred by PCE. The protein's low molecular weight could serve two functions: first, as a humectant; it binds water from the lower layers of the epidermis to the stratum corneum, and also as an occlusive as it reduces trans-epidermal water loss (Mokrejs et al., 2017). This aligns with findings that low molecular weight hydrolysates can form strongly adsorbed monomolecular layers that are difficult to remove. Conversely, larger molecules tend to form weakly anchored films with a greater capacity to bind water molecules, making high molecular weight polypeptides more effective as moisturizing film formers. Low molecular weight

peptides, on the other hand, are used in products requiring high substantivity, such as hair regeneration treatments, where they integrate into hair keratin to restore its proper structure (Ścibisz et al., 2008).

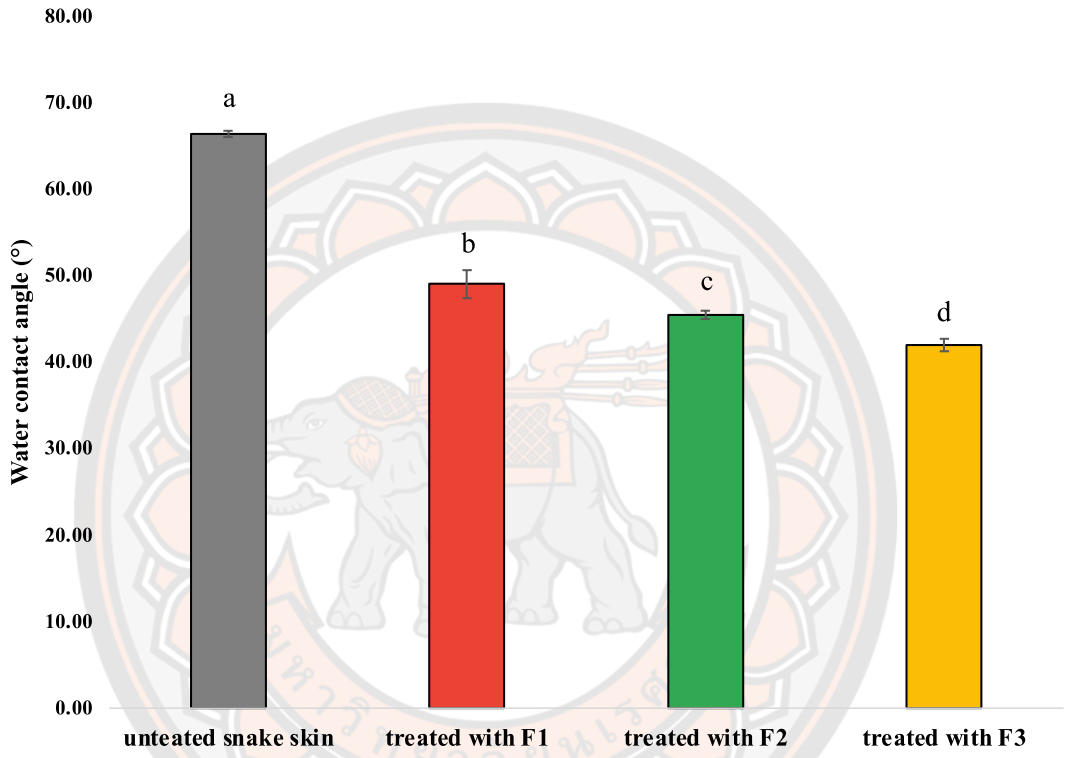


Figure 17 The water contact angle of snake skin surface treated with hydrating water.

CHAPTER V

CONCLUSION

In conclusion, *P. cystidiosus* extracts (PCE) exhibit potential for cosmetic applications, particularly in anti-photoaging and moisturizing formulations. The extraction process yielded varying results across different batches, with PCE1, PCE2, and PCE3 showing yields of 82 mg/100 g, 64 mg/100 g, and 41 mg/100 g, respectively, and total protein contents of 72.00%, 85.00%, and 100.00%. The lower conductivity observed in PCE3 suggests improved protein yield due to a longer dialysis process.

PCE displayed promising characteristics in its protein profile, with a high protein content. PCE also demonstrated antioxidant and anti-tyrosinase activities, attributed to its low molecular weight (≤3 kDa). Antibacterial activity was not shown by the extract of *S. aureus*, *S. epidermidis*, and *C. acnes*.

Cytotoxicity assessments indicated that PCE is safe at a concentration of 100 µg/mL, supporting its suitability for cosmetic use. In UV radiation tests, PCE exhibited protective effects against photoaging in keratinocytes, suggesting its potential as a sunscreen and anti-aging adjuvant. The incorporation of PCE into hydrating water tends to enhance its moisturizing properties, as evidenced by the water loss identified in snake skin and water contact angle results. The hydrating formulations containing PCE also demonstrated good physical stability over three months, indicating a favorable shelf-life for cosmetic products. However, further testing for chemical stability is necessary. The promising bioactivities of PCE highlight its potential for incorporation into cosmetic products aimed at anti-photoaging and moisturizing applications, pending further optimization and validation of extraction methods and long-term stability assessments.

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APPENDIXS

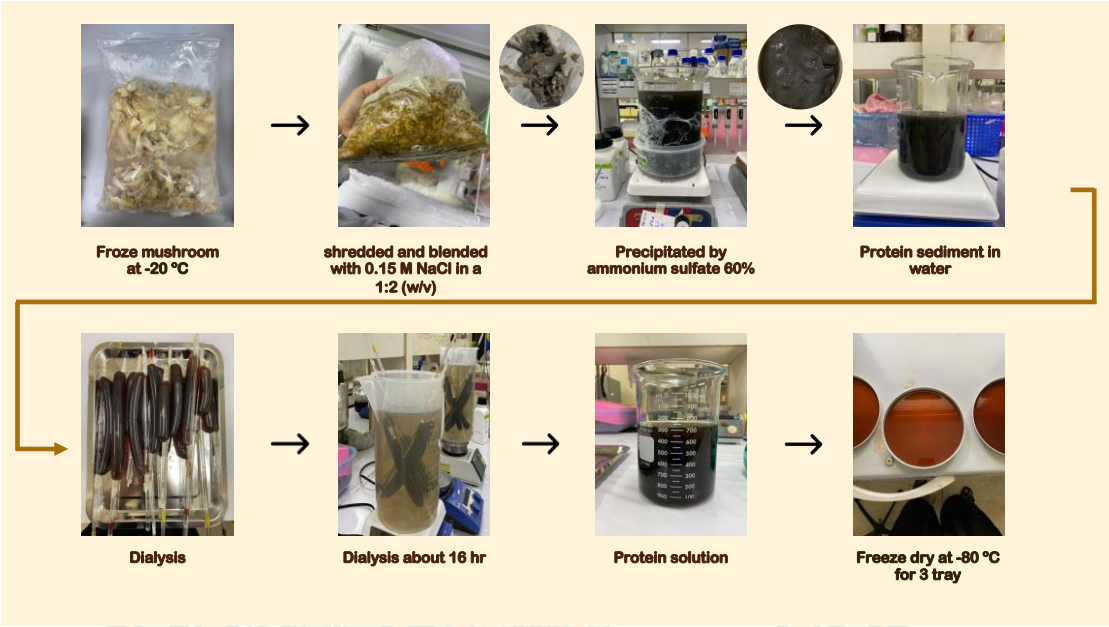

Figure 18 Preparation of PCE process.

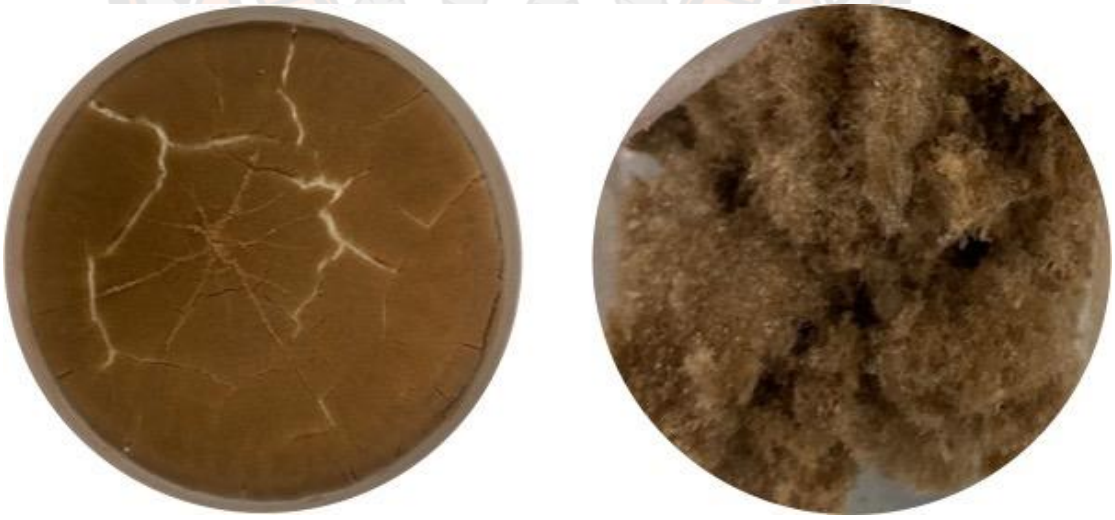

Figure 19 Appearance of PCE after freeze-drying.

Figure 20 Appearance of PCE at 1 mg/mL in water.

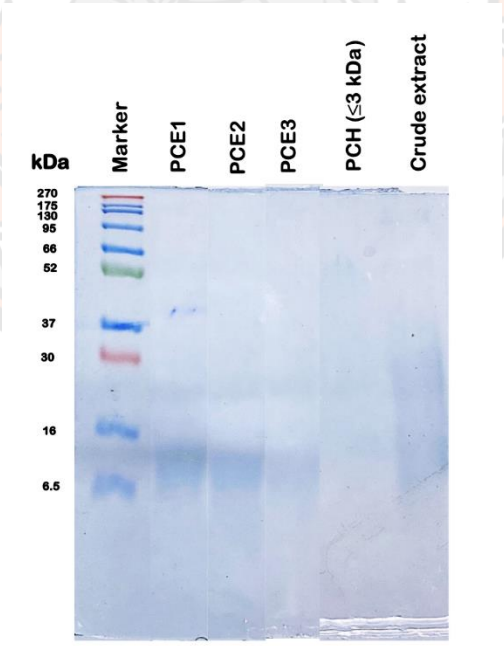

Figure 21 Profile of 20 µg of protein from PCE, PCH (≤3 kDa), and crude extract.

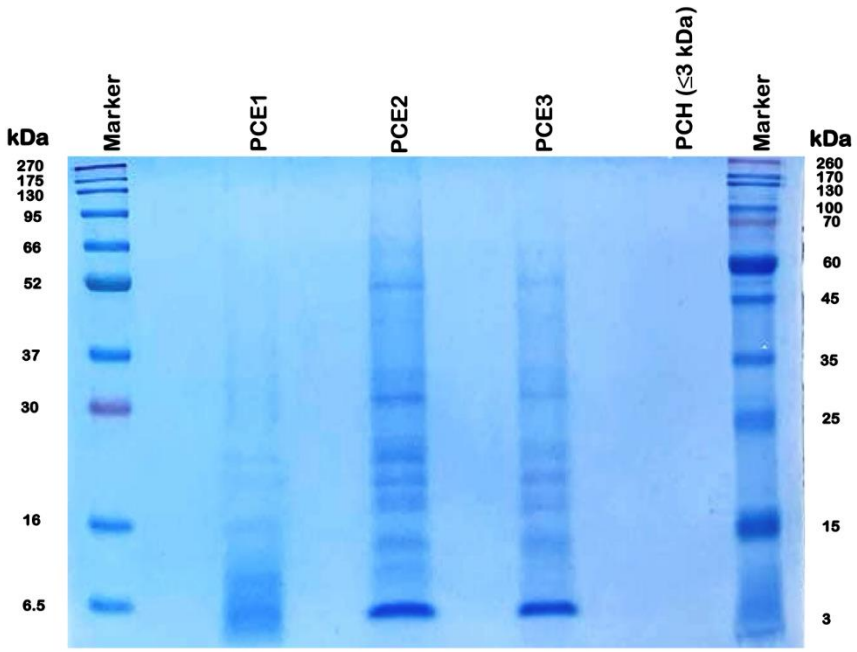

Figure 22 Profile of 25 µg of protein from PCE and PCH (≤3 kDa).

Table 15 Data of *P. cystidiosus* protein extraction.

	Extract by NaCl (L)	Protein sedimentation (mL)	Supernatant (mL)
PCE1	7.3	725	700
PCE2	7.7	717	690
PCE3	7.5	700	650

Table 16 The yield percentages of PCE and PCH (≤3 kDa).

Protein from	Weight of fresh fruiting body of *P. cystidiosus* (g)	Weight of crude extract from *P. cystidiosus* (g)	Yield (mg/100g)
PCE1	3000	2.46	82
PCE2	3000	1.93	64
PCE3	3000	1.22	41
PCH (≤3 kDa)	2500	0.30	12

Table 17 The protein content of PCE, PCH (≤3 kDa), and crude extract

Protein from	Protein content (μg/μL)
PCE1 (1mg/mL)	0.72±0.17
PCE2 (1mg/mL)	0.85±0.10
PCE3 (1mg/mL)	1.10±0.10
Crude extract	2.11±0.07
PCH (≤3 kDa)	4.15±0.62

Table 18 The pH levels of hydrating water formulations at various times and temperatures.

Formulations	pH (Mean ± S.D.)					
	0 Day		45 Days		90 Days	
F1	4°C:	5.05 ± 0.01	4°C:	5.07 ± 0.01	4°C:	5.02 ± 0.02
	Ab:	5.04 ± 0.02	Ab:	4.93 ± 0.01	Ab:	4.74 ± 0.01
	45°C:	5.04 ± 0.01	45°C:	4.77 ± 0.02	45°C:	4.40 ± 0.02
F2	4°C:	6.55 ± 0.01	4°C:	6.38 ± 0.02	4°C:	6.18 ± 0.01
	Ab:	6.53 ± 0.01	Ab:	6.50 ± 0.01	Ab:	6.53 ± 0.01
	45°C:	6.53 ± 0.02	45°C:	6.51 ± 0.00	45°C:	6.48 ± 0.03
F3	4°C:	6.97 ± 0.02	4°C:	6.61 ± 0.01	4°C:	6.22 ± 0.01
	Ab:	6.96 ± 0.01	Ab:	6.45 ± 0.01	Ab:	6.46 ± 0.02
	45°C:	6.97 ± 0.01	45°C:	6.09 ± 0.02	45°C:	6.05 ± 0.02

Table 19 The percentage of water loss at various times when using hydrating water formulations on snake skin.

Times	% Water loss (Mean ± S.D.)			
	Untreated	Treated with F1	Treated with F2	Treated with F3
1 hr	0.1046 ± 0.0263	0.1081 ± 0.0022	0.1059 ± 0.0036	0.1068 ± 0.0089
2 hr	0.1876 ± 0.0349	0.1810 ± 0.0066	0.1692 ± 0.0059	0.1753 ± 0.0124
4 hr	0.3381 ± 0.0551	0.3190 ± 0.0095	0.3101 ± 0.0085	0.3127 ± 0.0135
6 hr	0.4701 ± 0.0673	0.4334 ± 0.0115	0.4234 ± 0.0108	0.4228 ± 0.0139
8 hr	0.6067 ± 0.0778	0.5572 ± 0.0114	0.5433 ± 0.0108	0.5439 ± 0.0139

Table 20 The contact angle of hydrating water formulations on snake skin.

	Contact angle (°)
Untreated	66.37 ± 0.35
Treated with F1	49.07 ± 1.62
Treated with F2	45.47 ± 0.47
Treated with F3	42.00 ± 0.76

BIOGRAPHY

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Publication	Atawong P., Sriprom N., Sitti S., Attarat J., Kaewkong W. and Kriangkrai W. Formulation and stability testing of hydrating water containing protein hydrolysate from *Pleurotus ostreatus* (FR.) Guel. 2022; 44. (The Proceeding of Cosmetic & Beauty International Conference 2022). Atawong P., Sagasae W., Attarat J., Kaewkong W. and Kriangkrai W. Extraction and Evaluation of Hydrolysate Protein from *Pleurotus* spp. for Cosmeceutical Applications. 2023; 143-44. (The Proceeding of Consortium for Globalization of Chinese Medicine 2023).