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Screening of antimicrobial and antioxidant properties of ethyl acetate extracts from wild edible mushrooms

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ABSTRACT

Objective: The current work was undertaken to evaluate *in vitro* antibacterial, antifungal and antioxidant properties, together with total phenolic contents of ethyl acetate extracts from wild edible mushrooms collected in September 2017 from the rehabilitated forest area in the northeast of Thailand. **Results:** All the wild edible mushrooms collected could be classified into 6 families and 13 species namely *Macrolepiota rhacodes*, *Heimiella japonica, Boletus persoonii, Mycoamaranthus cambodgensis, Cantharellus cibarius, Amanita princeps, Amanita hemibapha, Russula cyanoxantha, Russula eburneureolata, Russula virescens, Russula emetica, Russula violeipes,* and *Termitomyces clypeatus*. To the best of our knowledge, there have been no previous reports on the antimicrobial and antioxidant properties of the two mushrooms, *B. persoonii* and *H. japonica*. For antibacterial analysis, the minimum inhibitory concentration (MIC) values of the ethyl acetate extracts toward *Escherichia coli* and *Staphylococcus aureus* were in the ranges of 1.25-10 and 0.63-10 mg/ml, respectively. The best MIC values for *E. coli* and *S. aureus* (1.25 and 0.63 mg/ml) were obtained in the extract of *B. persoonii* followed by *M. cambodgensis* (2.5 and 1.25 mg/ml). Determination of antifungal activity indicated that among all the 13 ethyl acetate extracts, 5 extracts (38.46%) displayed antifungal effect at the concentration of 50 μg/ml on *Candida albicans*. In antioxidant activity study, the ethyl acetate extracts exhibited the percentage of 1,1-diphenyl-2-picrylhydrazyl radical inhibition ranging from 9.95 ± 0.64 to 87.62 ± 0.15 and their total phenolic contents were between 3.83 ± 0.09 and 192.33 ± 0.23 mg gallic acid equivalent (GAE)/g extract. The extract of *B. persoonii* showed the highest antioxidant capacity with average percent inhibition of 87.62% followed by *M. cambodgensis* (79.11%) whose activities were higher than the standard ascorbic acid (75.16%). In addition, *M. cambodgensis* contained the highest average content of phenolic compounds at 192.33 mg GAE/g extract. **Conclusion:** The results of this study clearly suggested that the wild edible mushrooms, *B. persoonii* and *M. cambodgensis* could be potent sources of natural antimicrobials and antioxidants that might be used for medicinal applications in the prevention or treatment of various diseases.

Keywords: Antibacterial activity, antifungal activity, antioxidant activity, total phenolic content, wild edible mushroom

INTRODUCTION

The trouble of drug resistance in human pathogenic bacteria including *Mycobacterium tuberculosis*, *Staphylococcus* sp., *Streptococcus* sp., and others leading to infectious bacterial diseases is still a major threat to public health. In addition, the human population is encountering more fungal infections as a result of the AIDS epidemic and increased immune-compromised patients. Hence, there is an increasing need for more and better antibacterials and antimycotics.[1,2] Besides the drug resistance in bacteria and the fungal infection, free radicals are also one of the global serious health topics. Free radicals are found to be a major cause of oxidative stress preceding chronic health problems

such as aging, cancers, cardiovascular diseases, inflammatory diseases, and Parkinson's disease.[3,4] Increased efforts are, therefore, urgently required to search for newer and more effective antioxidants to prevent pathological conditions and develop drugs to deal with these diseases.[5]

Microorganisms, in particular fungi, have been known to be natural sources of pharmaceutical interests and medicines such as penicillin, cephalosporin, griseofulvin, and lovastatin.[6] The estimated numbers of fungi on the Earth are over 1.5 million species. To date, however, few of the world's fungi, approximately 5% of all fungal species have been studied.[1,6] Despite the fact that fungi are one of the most diverse groups of organisms worldwide, they are the most poorly investigated. Some relatively unexplored fungal groups derived from ecosystems, for example, endophytic fungi from medicinal plants, entomopathogenic fungi from insects, fungi from marine origins, and macrofungi from tropical forests are probably potential sources for the production of a diverse array of bioactive metabolites.[7,8] Mushrooms are an enormous variety of macrofungi belonging to basidiomycetes and ascomycetes.[9] The number of mushrooms around the world is estimated at 140,000, yet maybe only 10% (approximately 14,000 named species) are known.[10] Cumulatively from the previous reports, more than 100 medicinal functions are produced by mushrooms and fungi, and some key applications are antiallergic, antibacterial, anticancer, antidiabetic, antifungal, antioxidant, antitumor, antiviral, immunomodulating, cardiovascular, detoxification, and hepatoprotective effects. The medicinal values of mushrooms can be attributed to a variety of bioactive compounds including alkaloids, carotenoids, nucleotides, polyketides, polysaccharides, saponins, steroids, terpenes, and tocopherols.[11,12] In addition to the biologically active substances, mushrooms contain various phenolic compounds such as polyphenolics such as flavonoids and phenolic acids such as gallic, *p*-coumaric, and *p*-hydroxybenzoic acids which are shown to act as antioxidants. Phenolics are important secondary metabolites commonly found in fungi and reported to exert multiple biological effects including the capacity to reduce oxidative cellular damage caused by free radicals.[13,14] Consequently, the search for medicinal agents from mushrooms has become a matter of great interest in the past decade.[15,16]

Thailand is located in a tropical area, furnishing a great biodiversity of wild edible mushrooms which are considered as functional food and traditional medicine. They are becoming more important for their nutritional, sensory, and especially pharmacological characteristics. However, very little is known about the biological activity of naturally wild edible mushrooms.[17,18] These background data led us to speculate that Thai wild edible mushrooms might be a potential source of medicinal and therapeutic properties. In this research, screenings of antimicrobial and antioxidant activities of 13 wild edible mushrooms in Thailand were carried out. The total phenolic contents of their ethyl acetate extracts were also evaluated. To the best of our knowledge, we herein disclose the first report on *in vitro* antimicrobial and antioxidant activities of two Thai wild edible mushroom species including *Boletus persoonii* and *Heimiella japonica*.

MATERIALS AND METHODS

Collection and Identification of Wild Edible Mushrooms

Thirteen morphologically different types of wild edible mushrooms at the mature stage (cap opened) collected from the rehabilitated forest area of Yasothon Province located in the northeast of Thailand were procured in September 2017 as shown in Table 1. The wild edible mushrooms were identified not only by comparing their morphological characteristics with descriptions and photographs given in the references and keys[19,20] but also by discussion and direct interview with local people. The different mushroom samples were placed in ziplock plastic bags and stored at cool condition. The time interval between sample collection and extraction is limited at the maximum period of 3 h to maintain freshness of samples.

Preparation of Ethyl Acetate Extracts

Fresh fruiting bodies of wild edible mushrooms were cleaned from soil debris and contaminants under running tap water and air-dried at room temperature. Each air-dried mushroom sample (200 g) was cut into small pieces using a sterile blade and transferred into a 1000 ml conical flask for extraction. The mushroom material was macerated with methanol at room temperature for 2 days. The methanol extract was evaporated under reduced pressure and then added with 200 ml of distilled water and partitioned twice with an equal volume of ethyl acetate. The ethyl acetate was evaporated to dryness and the crude ethyl acetate extract was obtained.

Determination of Antibacterial Activity

Antibacterial activity of crude ethyl acetate extracts toward two pathogenic bacteria, *Escherichia coli* ATCC 25922 (Gram-negative bacteria) and *Staphylococcus aureus* ATCC 25923 (Gram-positive bacteria), was determined using broth microdilution assay according to the method described by Chomcheon *et al*.[21] The tested bacteria were provided from Faculty of Agriculture and Life Sciences, Chandrakasem

Table 1: List of wild edible mushrooms used in this investigation

Sample	Family	Scientific name
1	Agaricaceae	*Macrolepiota rhacodes*
2	Boletaceae	*Heimiella japonica*
3		*Boletus persoonii*
4		*Mycoamaranthus cambodgensis*
5	Cantharellaceae	*Cantharellus cibarius*
6	Pluteaceae	*Amanita princeps*
7		*Amanita hemibapha*
8	Russulaceae	*Russula cyanoxantha*
9		*Russula eburneureolata*
10		*Russula virescens*
11		*Russula emetica*
12		*Russula violeipes*
13	Termitophilae	*Termitomyces clypeatus*

Rajabhat University and cultured on Mueller-Hinton agar for 24 h at 37°C. Selected fresh single colonies were inoculated into 5 ml of tryptic soy broth and incubated in shaking incubator for 2–3 h at 37°C. The turbidity of the bacterial suspension was adjusted with sterile normal saline solution to match the turbidity of 0.5 McFarland standard (optical density 0.1 at 625 nm). Then, the suspension was diluted with Mueller-Hinton broth (MHB) to contain 1×10^6 colony-forming units/ml. Solution of mushroom crude extract in dimethyl sulfoxide (DMSO) (25.6 mg/ml) was diluted with MHB for the assay of antibacterial activity. The crude extracts were determined over the final concentration range of 10–0.5 mg/ml and oxacillin was used as positive control. The minimum inhibitory concentration (MIC) is defined as the lowest concentration that inhibits the growth of test microorganism.

A 50-μl volume of MHB containing crude extract was dispensed into each well of sterile microtiter plates (96-flat-bottom wells). Sterile extract-free medium containing the corresponding amount of DMSO was dispensed in the growth control well. The final adjusted bacterial suspensions were inoculated into each well in a volume of 50 μl. Crude extract-free MHB in a volume of 100 μl was used as the sterility control. After incubation at 37°C for 24 h, a 20-μl of *p*-iodonitrotetrazolium solution (1 mg/ml) was added into each well. The antibacterial assay plates were further incubated for 1 h. Growth in each well was estimated by a color change from colorless to violet. Crude extract inhibiting bacterial growth would prevent the development of a violet color. The well presenting no change in color indicated the antibacterial activity of the crude extract. All the MIC measurements were carried out in duplicate.

Determination of Antifungal Activity

Antifungal activity of mushroom ethyl acetate extracts was performed using colorimetric assay according to Espinel-Ingroff and Pfaller[22] with some modifications. The extracts were determined at a final concentration of 50 μg/ml and *Candida albicans* was employed as a test organism. 2,3-Bis-(2-methoxy-4-nitro-5-phynylamine carbonyl-2H-tetrazolium hydroxide was used as an oxidation-reduction indicator measuring metabolic activity of the yeast fungus. Amphotericin B was used as positive control. The half-maximal inhibitory concentration (IC_{50}) is defined as the concentration which results in 50% inhibition of fungal growth compared with untreated control. The antifungal activity was analyzed at the bioassay service of the National Center for Genetic Engineering and Biotechnology, Bangkok, Thailand.

Determination of Antioxidant Activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of mushroom ethyl acetate extracts was measured photometrically according to the method of Lourith *et al*.[23] with some modifications. Each extract with a concentration of 0.5 mg/ml was mixed with equal volume of DPPH ethanol solution freshly prepared at a concentration of 0.06 mM. The mixture was shaken well and left to stand for 30 min in the dark at room temperature. The absorbance of the mixture was measured at 517 nm and ascorbic acid (0.5 mg/ml) was employed as standard. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ Inhibition} = (A_{blank} - A_{sample})/(A_{blank}) \times 100$$

Where, A_{blank} and A_{sample} are the absorbance of the control (without extract) and the absorbance of the test extract, respectively. The experiment was done in triplicate.

Determination of Total Phenolic Content

Total phenolic contents of mushroom ethyl acetate extracts were estimated colorimetrically according to the method of Lourith *et al*.[23] with some modifications. Briefly, each extract (800 μl) with a concentration of 5 mg/ml was mixed with Folin–Ciocalteu reagent (80 μl). Then, 1.6 ml of 7.5% sodium carbonate solution were added and the mixture was made up to a final volume of 4 ml. The mixture was shaken well and allowed to stand for 60 min at room temperature. The absorbance was measured at 765 nm and gallic acid was taken as standard. The standard curve of gallic acid in a concentration range between 0 and 40 μg/ml was generated [Figure 1]. The total phenolic contents of the extracts are expressed as mg of gallic acid equivalent (GAE) per g of extract. The test was done in triplicate.

RESULTS AND DISCUSSION

The wild edible mushrooms could be classified into phylum *Basidiomycota*, 6 families, 8 genera, and 13 species as presented in Table 1. All the species of mushroom fruiting bodies were collected from the rehabilitated forest area of Yasothon Province located in the northeast of Thailand. This region has been known to have a great variety of wild mushroom species. The mushrooms collected are practically important for their edibility, availability, and medicinal utility. Various chemical constituents of certain mushrooms studied in this research have been reported previously and some of them are efficient bioactive metabolites exerting a wide range of beneficial effects

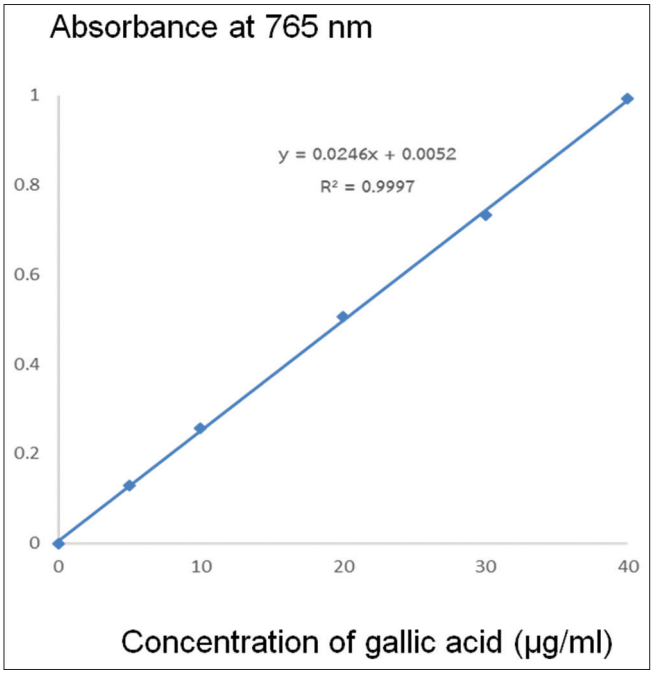

Figure 1: Standard curve of gallic acid

on human health. Alkaloids, anthraquinones, flavonoids, phenols, saponins as well as terpenoids were reported from the methanolic and ethanolic extracts of the fruiting body of *Cantharellus cibarius* which had antibacterial, antifungal, and antioxidant activities.[24] For *Russula virescens*, the presence of polysaccharides, sterols, and triterpenes was detected in its aerial parts. Some of the polysaccharides including 1,3-beta-d-glucan were found to exhibit antioxidant properties.[25] In addition, the nutraceutical phenolic compounds, quercetin, myricetin, and naringenin were found to be present in the fruiting bodies of *Amanita princeps*, *C. cibarius*, *Russula emetica*, *Russula violeipes*, and *Termitomyces clypeatus*.[17] As revealed by the result of mushroom extraction, the obtained yields varied ranging from 125 to 550 mg shown in Table 2. The small or large yield variation was commonly observed with respect to the ability of the mushrooms to produce metabolites. The difference in the yields could be attributed to the difference in the amount or content of the compounds present in the extracts.

From the *in vitro* antibacterial screening, the MIC of ethyl acetate extracts of wild edible mushrooms ranged between 1.25 and 10.0 mg/ml for *E. coli* and between 0.63 and 10.0 mg/ml for *S. aureus* as presented in Table 2. The difference in the result of antibacterial activity could be due to the production of either broad-spectrum antibacterial substances or several compounds with different activities. In addition, the results revealed that the ethyl acetate extracts inhibited the Gram-positive than Gram-negative pathogenic bacteria in accordance with many researches.[26-28] Hydrophobic molecules can pass through the cell wall of the Gram-positive bacteria which are composed predominantly of peptidoglycan more easily than that of the Gram-negative bacteria of which the lipopolysaccharide layer in outer membrane acts as a strong permeability barrier toward hydrophobic molecules.[29,30] The least MIC value (1.25 mg/ml) was obtained in *B. persoonii* against *E. coli* followed by *Mycoamaranthus cambodgensis* (2.5 mg/ml). The highest antibacterial activity against *S. aureus* with the best MIC value (0.63 mg/ml) was produced by *B. persoonii* followed by *M. cambodgensis* (1.25 mg/ml) and *C. cibarius* (2.5 mg/ml). In similar researches, a number of macrofungi in the genera *Boletus* and *Cantharellus* showed good inhibition rates against some pathogenic bacteria.[31] Based on the obtained results, the wild edible mushrooms, *B. persoonii* and *M. cambodgensis,* are very interesting due to their remarkable effects on *E. coli* and *S. aureus* at low concentrations and high yields of crude extracts, suggesting that both mushrooms are promising good sources of natural antibacterial agents.

For the antifungal evaluation, all the ethyl acetate extracts were tested against *C. albicans* by colorimetric method. *C. albicans* has been the most extensively investigated pathogen in studies of antifungal resistance due to the morbidity associated with infections in immunocompromised patients.[32] Of 13 extracts, 5 extracts (38.46%) of *B. persoonii*, *M. cambodgensis*, *C. cibarius*, *A. princeps*, and *Amanita hemibapha* were found to have antifungal activity against *C. albicans* with IC_{50} ≤50 μg/ml while the eight remaining extracts (61.54%) with IC_{50} >50 μg/ml were considered to be inactive as shown in Table 3. The five active extracts exhibited a significant antifungal effect on the tested strain at low concentration, indicating that the wild edible mushrooms could be potential sources of antifungal agents. It should be noted that the results expressed in this study are the first information on the antimicrobial activities of the wild edible mushrooms, *B. persoonii* and *H. japonica*.

The antioxidant activity of all the ethyl acetate extracts was determined using the DPPH radical scavenging assay. The DPPH assay is most widely used among antioxidant assays and considered to be the most efficient method that is not affected by metals and enzyme inhibition.[33] The obtained results disclosed that the ethyl acetate extracts exhibited the percentage of inhibition in the range of 9.95 ± 0.64–87.62 ± 0.15 as displayed in Table 4. The highest percentage scavenging

Table 2: Yield and MIC of wild edible mushroom extracts

Sample	Mushroom species	Yield of extract (mg/200 g wet weight)	MIC (mg/ml)	
			Escherichia coli	***Staphylococcus aureus***
1	*Macrolepiota rhacodes*	250	I	10.0
2	*Heimiella japonica*	210	10.0	5.0
3	*Boletus persoonii*	350	1.25	0.63
4	*Mycoamaranthus cambodgensis*	475	2.50	1.25
5	*Cantharellus cibarius*	160	10.0	2.50
6	*Amanita princeps*	130	10.0	5.0
7	*Amanita hemibapha*	460	10.0	5.0
8	*Russula cyanoxantha*	125	10.0	10.0
9	*Russula eburneureolata*	550	I	10.0
10	*Russula virescens*	540	I	10.0
11	*Russula emetica*	490	10.0	5.0
12	*Russula violeipes*	195	10.0	5.0
13	*Termitomyces clypeatus*	440	I	I
	Oxacillin		0.5 mg/ml	1.0 μg/ml

I: Inactive, MIC: Minimum inhibitory concentration

Table 3: Half-maximal inhibitory concentration of wild edible mushroom extracts

Sample	Mushroom extract	IC_{50} (μg/ml) of antifungal activity (*Candida albicans)*
1	*Macrolepiota rhacodes*	I
2	*Heimiella japonica*	I
3	*Boletus persoonii*	≤50
4	*Mycoamaranthus cambodgensis*	≤50
5	*Cantharellus cibarius*	≤50
6	*Amanita princeps*	≤50
7	*Amanita hemibapha*	≤50
8	*Russula cyanoxantha*	I
9	*Russula eburneureolata*	I
10	*Russula virescens*	I
11	*Russula emetica*	I
12	*Russula violeipes*	I
13	*Termitomyces clypeatus*	I

I: Inactive (IC_{50}>*50* µg/ml)

Table 4: DPPH free radical scavenging activity and total phenolic content of wild mushroom extracts

Mushroom extract	% Inhibition of DPPH±SD*	Total phenolic content (mg GAE/g extract)±SD*
Macrolepiota rhacodes	28.26±0.45	14.64±0.29
Heimiella japonica	12.59±0.25	14.54±0.29
Boletus persoonii	87.62±0.15	80.07±0.22
Mycoamaranthus cambodgensis	79.11±0.33	192.33±0.23
Cantharellus cibarius	38.63±0.57	19.47±0.20
Amanita princeps	16.54±0.41	8.44±0.08
Amanita hemibapha	16.01±0.52	3.83±0.09
Russula cyanoxantha	19.45±0.15	6.08±0.16
Russula eburneureolata	23.78±0.30	10.55±0.16
Russula virescens	11.37±0.64	10.66±0.09
Russula emetica	9.95±0.64	6.70±0.26
Russula violeipes	18.39±0.55	13.78±0.33
Termitomyces clypeatus	12.95±0.12	8.36±0.24
Ascorbic acid (0.5 mg/ml)	75.16±0.31	

*n=3. DPPH: 1,1-Diphenyl-2-picrylhydrazyl, GAE: Gallic acid equivalent, SD: Standard deviation

power was found in species *B. persoonii* (87.62%) followed by *M. cambodgensis* (79.11%) whose activities were higher than the standard ascorbic acid (75.16%) while species *R. emetic* gave the lowest value (9.95%). As the results indicated, the wild edible mushrooms, *B. persoonii* and *M. cambodgensis,* could be regarded as rich sources of natural antioxidant metabolites. To the best of our knowledge, there is no previous report on the antioxidant activity of *B. persoonii* and *H. japonica* species. There are only few data of the antioxidant property of some mushrooms studied in this work. For instance, Kosanic *et al*.[34] reported that the IC_{50} values for the antioxidant activity of acetone extracts of *Russula cyanoxantha* and *C. cibarius* were 86.30 and 156.40 μg/ml, respectively. It was also reported that *A. princeps*, *A. hemibapha*, *R. violeipes*, and *T. clypeatus* showed antioxidant capacities with percent inhibition range of 59.4–83.07.[17] In addition, the total antioxidant capacity of *M. cambodgensis,* 0.44 mmol Trolox equivalent/100 g was reported by Srikram and Supapvanich.[18] The differences in biological activities of wild edible mushrooms are probably a consequence of the presence of different components with various bioactivities. This generally depends on species and life cycle stage of mushrooms, together with environmental and other factors such as the effect of climate, soil composition, extractant, and method of testing.[8,35,36]

The total phenolic content of mushroom ethyl acetate extracts was estimated by Folin–Ciocalteu colorimetric method. Ethyl acetate was used as extraction solvent because it has a significant selectivity in the extraction of low-molecular-weight phenolic substances and high-molecular-weight polyphenols.[37] Similarly, Conde *et al*.[38] reported that ethyl acetate allowed the highest phenolic content and the selective removal of non-phenolic compounds. According to the result of total phenolic content determination in Table 4, the ethyl acetate extracts showed a large variation between 3.83 ± 0.09 and 192.33 ± 0.23 mg GAE/g extract. The extract of *M. cambodgensis* gave the highest average total phenolic content with the value of 192.33 mg GAE/g extract, followed by the extract of *B. persoonii* (80.07 mg GAE/g extract) while the extract of *A. hemibapha* exhibited the lowest average value (3.83 mg GAE/g extract). Compared to the previous findings, most ethyl acetate extracts of the wild edible mushrooms in Thailand studied in the present work contained more total phenolic content than the extracts of selected edible mushrooms in Malaysia (0.90–6.03 mg GAE/g extract).[39] Moreover, the total phenolic contents of the ethyl acetate extracts from *M. cambodgensis* and *B. persoonii* were found to be significantly higher than those of the extracts from some Portuguese and Serbian wild edible mushrooms which reported in the ranges of 4.58–58.14 and 9.36–173.13 mg GAE/g extract, respectively.[40,41] Based on the outcomes in Table 4, there was the parallelism found between the antioxidant potential and total phenolic content of most extracts. The ethyl acetate extracts with high antioxidant activities had high total phenolic contents implying that phenolic compounds were a major contributor to the antioxidant capacities of the extracts and could be used as an index of antioxidant metabolites. The antioxidant activity of phenolics may be related to their ability to chelate metals, inhibited lipoxygenase and scavenge free radicals.[42] Phenolic compounds are known to be the main source of antioxidants inhibiting arise of many diseases such as cancers and heart and lung diseases by preventing free radical reactions.[43] They have also been reported for other biomedical activities including antihyperglycemic, anti-inflammatory, and antimicrobial efficiency.[44]

CONCLUSION

The present study focused on the antimicrobial and antioxidant screening of the wild edible mushrooms in Thailand that are the important step to provide valuable information sources of bioactive metabolites for further development of drugs. To the best of our knowledge, this is the first report on *in vitro* antimicrobial and antioxidant activities of the wild edible mushroom species including *B. persoonii* and *H. japonica*. The results of this work strongly supported that wild edible mushrooms could be alternative potential supply sources of natural bioactive compounds with antimicrobial and antioxidant properties. Particularly, the wild edible mushrooms, *B. persoonii* and *M. cambodgensis,* exhibited notable antibacterial and antifungal activities and also demonstrated high levels of phenolic compounds that possessed strong antioxidant capacities. Further studies of the bioactive agents of these two wild edible mushrooms would be interesting and should be pursued that might yield novel lead compounds with potent antimicrobial or antioxidant effects for improving human health and promoting quality of life.

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