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A Comparative Study on Chemical Composition and Pharmacological Effects of *Paecilomyces hepiali* and Wild *Ophiocordyceps sinensis*

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Abstract

This study looked at comparison of chemical components and pharmacological activity between wild *Ophiocordyceps sinensis* and *Paecilomyces hepiali*. The chemical components investigated included amino acids, vitamins, dietary elements, protein, lipid, ash, carbohydrates, crude fibre, ergosterol and mannitol. Studies on pharmacological activity included anti-platelet aggregation, inhibitory effect on IL-8 gene expression, anti-mutagenic activity, skin whitening effect and improvement activity on human skin texture. The results show that *P. hepiali* has a larger total content of seven essential amino acids (leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine and valine) than *O. sinensis*, 8580 mg/100g and 6180 mg/100g respectively. The total content of dietary elements analysed (potassium, magnesium, zinc, copper, manganese and selenium) was also higher in *P. hepiali* (3135 mg/100g) than that in *O. sinensis* (2445 mg/100g). The total content of four vitamins (B1, B2, B6 and E) was almost equal for both fungi. *Paecilomyces hepiali* had more content of protein, lipid, ash, carbohydrate, ergosterol and mannitol than *O. sinensis*. However, the contents of lipid and ash were not significantly different between the two fungi. The hot water extract of *P. hepiali* has been shown to have a number of pharmacological effects which include 1) inhibition of aggregation on human platelet, 2) inhibition of IL-8 gene expression, 3) anti-mutagenic activity, 4) inhibition effect on production of melanin, and 5) improvement on human skin texture. Anti-platelet aggregation effects on human platelet, inhibition of IL-8 gene expression, anti-mutagenic activity and improvement effect on human skin texture were greater in *P. hepiali* treatments than that in wild *O. sinensis*.

Keywords

Chemical Composition, Pharmacological Effects, *Paecilomyces hepiali*, *Ophiocordyceps sinensis*

1. Introduction

Ophiocordyceps sinensis has been used as a traditional Chinese medicine (TCM) since time immemorial. Winkler [1] reported that the earliest known documentation of *yartsa gunbu* (*O. sinensis*) was by Nyamnyi Dorje, a Tibetan physician and lama who lived from 1439 to 1475. The range of therapeuctic uses claimed for *O. sinensis* is far reaching, although many of them have yet to be adequately investigated. In TCM, this fungus has been used to treat conditions including respiratory and pulmonary diseases; renal, liver, and cardiovascular diseases; hyposexuality; and hyperlipidemia. It is also used in treatment of immune disorders and as an adjunct to modern cancer therapies (chemotherapy, radiation treatment, and surgery). *Ophiocordyceps sinensis* is believed, particularly in and around Tibet, to be a remedy for weakness and fatigue, and is often used as an overall rejuvenator for increased energy while recovering from serious illness [2]-[4].

Due to rarity and exorbitant prices of the wild fruit bodies of *O. sinensis*, a number of isolates have been used in large-scale mycelia production as substitutes. One of such isolates is *Paecilomyces hepiali*. This isolate has been popularly used as an anamorph of *O. sinensis*. Most of the reported medicinal benefits of artificially cultivated mycelia of *O. sinensis* are based on clinical trials which had been conducted on *P. hepiali* Chen (strain Cs-4). The aseptically fermented mycelia of *P. hepiali* Chen (strain Cs-4) underwent extensive human testing and clinical trials during the 1980's and resulted in a popular commercial product in China, known as Jin-Shui-Bao capsule [5]. However, current reports are discarding this fungus as a correct anamorph of *O. sinensis*. *Hirsutella sinensis* is currently receiving general acceptance as the true anamorph of this mushroom [6]-[8]. Using spontaneously hypertensive rats, Chioza and Ohga [9] demonstrates that hot water extract of *P. hepiali* has antihypertensive effects.

Chemical profiles and pharmacological effects of fungi vary in terms of species as well as strain. This study was aimed at investigating pharmacological effects of the *P. hepiali* strain used in this study and comparisons were made with wild *O. sinensis* on the same. In addition to that, the chemical compositions of *P. hepiali* and *O. sinensis* were also compared.

2. Materials and Methods

2.1. Fungal Material and Preparation of Samples

The fungal strain used in this study was acquired from the mushroom culture bank at the Laboratory of Forest Resources Management, Kyushu University. It is assigned to accession number KUMB108 in the culture bank. The fruit bodies of *P. hepiali* were produced using a substrate composed of 40 g brown rice, 0.325 g glucose, 0.65 g sucrose, 2 g peptone and 65 ml corn steep liquor as described by Chioza and Ohga [10]. The fruit bodies were as shown in **Figure 1(b)**. The wild fruit bodies of *O. sinensis* (**Figure 1(a)**) were obtained from Tibet, China. The dried samples of these fungi were ground into fine powder (16-mesh size) using a Waring blender. These powders were used in chemical analyses and making hot water extracts. The powders were extracted in ten volumes of hot water (80°C, 1 hour). For comparison, hot water extract of *Lentinula edodes* fruit bodies was also prepared in the same manner.
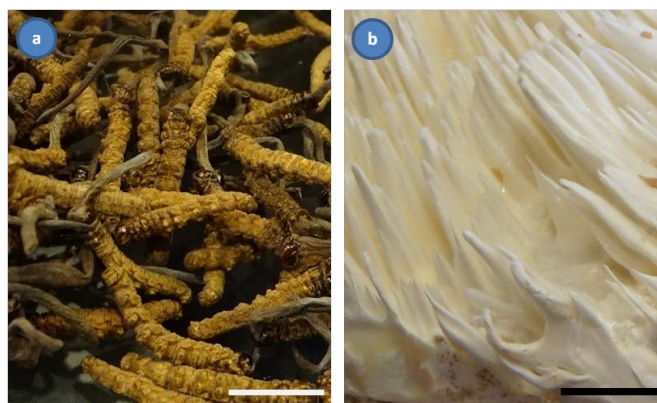

Figure 1. Wild *Ophiocordyceps sinensis* (a) and cultured *Paecilomyces hepiali* (b). Each scale bar represents 2 cm.

2.2. Analysis of Chemical Components

Powders of wild *O. sinensis* and *P. hepiali* were subjected to various chemical analyses. Amino acids were determined using an automatic amino acid analyser (L-8900 amino acid analyzer, Hitachi Co. Ltd., Japan). Vitamins, ergosterol and mannitol were analysed by HPLC. Protein was analyzed according to Micro-Kjeldahl method. Magnesium, zinc, copper and manganese were determined by ICP spectrometry. Fluorescence spectrometry was used to analyse selenium. Potassium was determined using Atomic Absorption Spectrophotometry (AAS).

2.3. Inhibition of Platelet Aggregation

Blood obtained from healthy adults who had not taken any drug known to affect platelet function for two weeks was centrifuged (1000 rpm, 20 minutes, room temperature) to separate and collect Platelet-rich Plasma (PRP). After collecting PRP from the upper layer, the lower layer was further centrifuged (3000 rpm, 5 minutes, room temperature) to separate and collect Platelet-poor Plasma (PPP). Each extract, dissolved in 2 μl of 2% of Dimethyl sulfoxide (DMSO) solution, was added to the preheated (37˚C) PRP and PPP (223 μl). After incubation for three minutes at 37˚C, 25 μl of PAF (500 nM) or an aqueous solution of arachidonic acid (500 nM) was added to induce platelet aggregation. As a control, ion-exchanged water was used. An aggregometer (MCM Hema Tracer 313 M, MC Medical Co. Ltd., Japan) was used to measure aggregation.

2.4. Inhibition of IL-8 Gene Expression

Normal human skin fibroblasts were cultured on Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in 6 cm diameter petri dishes. Six factors were used in this experiment, namely *O. sinensis* extract, *P. hepiali* extract, *L. edodes* extract, hydrocortisone, tumor necrosis factor (TNF-*α*) and control. The fungal extracts and hydrocortisone were added to the medium at concentrations of 0.01% (dry weight) and 10^{-7} M respectively. Hydrocortisone (Hcon) was used as a positive control. TNF-*α* was added at a concentration of 1 ng/ml to promote chemokine (IL-8) gene expression. The medium in which only TNF-*α* was added (Tcon) and another without fungal extracts as well as TNF-*α* (Control) served as other controls. The cells were incubated for 6 hours at 37˚C. Using conventional methods, RNA was isolated from the cells and then synthesized cDNA. Real-time quantitative PCR (TaqMan PCR method) analyses were performed to quantify the expression of IL-8 genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control in the PCR analyses. The percentage of inhibition was calculated using the comparative C_T method described by Leutenegger *et al*. [11] and in this study TNF-*α* control (Tcon) was used as a reference sample (100%).

2.5. Assessment on Anti-Mutagenic Effect

The anti-mutagenic effect of wild *O. sinensis* and *P. hepiali* extracts was evaluated using *Salmonella typhimurium* strain TA98 and a mutagen 2-Amino-3-Methyl-3H-Imidazo[4,5-F]Quinoline (IQ). A 2% solution of extract dissolved in ion exchange water (1.9 ml) was added to a 0.2 mM mutagen solution (0.1 ml) and the mixture was allowed to stand for 15 minutes at room temperature. Then 2 ml of molten top agar, 0.1 ml of cell suspension culture of *Salmonella typhimurium* (TA98) and 0.5 ml of S9 mix (Wako Pure Chemical Industries, Ltd.) were added to 1 ml of the mixture. The entire mixture was poured into 96-well microtiter plates and incubated at 37˚C for 48 hours. Five plates were used for each treatment. The method used by Yen and Chen [12] was applied in calculating percent inhibition: Inhibition (%) = [1 − (number of revertants in the presence of fungal extract/number of revertants in the absence of fungal extracts)] × 100%.

2.6. Effect on Production of Melanin

B16 melanoma cells, at a concentration of 1.0×10^5 cells/ml, were seeded in 24-well plate (1 ml/well). After incubation (37˚C, 5% CO_2) for 24 hours, the cells were "passaged" to fresh medium containing 998 μl of EMEM and 2 μl solution of fungal extract dissolved in Dimethyl sulfoxide (DMSO) at various concentrations. After 48 hours, the medium was changed again in the same way. After an additional 24 hours, the medium was removed and thereafter cells were lysed with 1 ml of 1 N NaOH. Melanin content was measured by absorbance at 405 nm by using a plate reader. Kojic acid and arbutin, blended whitening cosmetics on the market, were used instead of fungal extract as positive controls.

2.7. Effect on Skin Roughness/Texture

Effect of the fungal extracts on texture of the human skin was determined by using the replica method. The skin replicas were taken from the faces of healthy women with normal skin and observed by an optical microscope (×20). Each extract was diluted 10 times with ion exchange water. At a fixed position on the face, 10×10 mm gauze soaked with the extract solution was coated twice per day for 2 weeks. As a control, ion exchange water without the fungal extract was used.

The rate of improvement was evaluated based on points obtained in a scoring procedure. The scores were awarded as follows: One point to the panel which had disappearance of sulcus cutis and crista cutis, and peeling of the stratum corneum in a wide range, two points to the panel which had unclear sulcus cutis and crista cutis, and peeling of the stratum, three points to the panel which had flat sulcus cutis and crista cutis, four points to the panel with clear sulcus cutis and crista cutis, five points to the panel with clear and neat sulcus cutis and crista cutis.

2.8. Statistical Analysis

All statistical analyses were performed by analysis of variance (ANOVA) followed by Tukey's post hoc test. All the analyses were done at 0.05 level using Minitab 17 Statistical Software (Minitab Inc.). Apart from data on amino acid content and chemical components, all data are presented as means of five replicates. The error bars in all graphs represent standard deviation.

3. Results and Discussion

3.1. Comparison of Chemical Components

The chemical components analysed included amino acids, vitamins, dietary elements, protein, lipid, ash, carbo-hydrates, crude fibre, ergosterol and mannitol. The results (**Table 1** and **Table 2**) show that *P. hepiali* has a larger total content of seven essential amino acids (leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine and valine) than *O. sinensis*, 8580 mg/100g and 6180 mg/100g respectively. The total content of dietary elements analysed (potassium, magnesium, zinc, copper, manganese and selenium) was also higher in *P. hepiali* (3135 mg/100g) than *O. sinensis* (2445 mg/100g). The total content for four vitamins (B1, B2, B6 and E) was

Table 1. Amino acids content in wild *Ophiocordyceps sinensis* and *Paecilomyces hepiali*.

Amino acid	Wild *O. sinensis* (mg/100g)	*P. hepiali* (mg/100g)	Analysis method
Alanine (Ala)	1400	1890	Amino acid analyzer
Arginine (Arg)	1210	2310	Amino acid analyzer
Aspartic acid (Asp)	2090	3310	Amino acid analyzer
Cysteine (Cys)	390	670	Amino acid analyzer
Glutamic acid (Glu)	3230	4890	Amino acid analyzer
Glycine (Gly)	1350	1520	Amino acid analyzer
Histidine (His)	460	480	Amino acid analyzer
Isoleucine (lle)	860	940	Amino acid analyzer
Leucine (Leu)	870	1320	Amino acid analyzer
Lysine (Lys)	1380	1970	Amino acid analyzer
Methionine (Met)	260	410	Amino acid analyzer
Phenylalanine (Phe)	850	1350	Amino acid analyzer
Proline (Pro)	2570	2630	Amino acid analyzer
Serine (Ser)	1780	1810	Amino acid analyzer
Threonine (Thr)	1340	1890	Amino acid analyzer
Tryptophan (Trp)	240	490	HPLC
Tyrosine (Tyr)	890	1120	Amino acid analyzer
Valine (Val)	1690	1920	Amino acid analyzer

Table 2. Comparison of various chemical components in wild *Ophiocordyceps sinensis* and *Paecilomyces hepiali*.

Component	Wild *O. sinensis*	*P. hepiali*	Analysis method
(mg/100g)			
Protein	24,500	36,700	Micro Kjeldahl method
Lipid	2050	2300	Soxhlet extraction method
Ash	6300	6900	Complete ashing method
Carbohydrates	4300	6800	Beltran method
Crude fiber	24,600	61,000	Balance method
β-Glucan	10,600	18,800	Enzyme method
Ergosterol	327	884	HPLC
Mannitol	9860	12,980	HPLC
Potassium (K)	2350	3010	AAS
Magnesium (Mg)	89	119	ICP spectrometry
Zinc (Zn)	4.21	4.08	ICP spectrometry
Copper (Cu)	0.42	0.78	ICP spectrometry
Manganese (Mn)	1.13	1.32	ICP spectrometry
Selenium (Se)	0.017	0.018	Fluorescence spectrometry
(mg/g)			
Vitamin B1	0.83	0.48	HPLC
Vitamin B2	1.13	1.28	HPLC
Vitamin B6	0.33	0.42	HPLC
Vitamin E	0.25	0.31	HPLC

almost equal for both fungi. *Paecilomyces hepiali* had more content of protein, lipid, ash, carbohydrate, ergosterol and mannitol than *O. sinensis*. However, the contents of lipid and ash were not significantly different between the two fungi.

Mycelia are commonly used as substitutes for fruit bodies of most medicinal mushrooms in production of various medicinal products. Therefore, a comparison of chemical composition of fruit bodies and cultivated mycelia of culinary mushrooms is very important as indicted by Chang and Wasser [13]. On a dry-weight basis, mushrooms normally contain 19% to 35% protein, as compared to 7.3% rice, 12.7% in wheat, 38.1% in soybean and 9.4% in corn [13]. As **Table 2** shows, in the present study the protein content obtained for wild *O. sinensis* was 24,500 mg/100g (24.5%) which falls within the generalised range of protein content for mushrooms mentioned earlier. The protein content for *P. hepiali* (36,700 mg/100g) was significantly higher than that of wild *O. sinensis* and this value is also slightly higher than the generalised range of protein content in mushrooms. The mycelial protein content of *P. hepiali* (36.7%) obtained in this study is more than the mycelial protein contents reported by Ulziijard and Mau [14] for *Sparassis crispa* (32.61%), *Inonotus obliquus* (25.59%), *Grifola frondosa* (26.40%) and *Anthrodia camphorata* (9.49%). Leung *et al.* [15] found slightly higher protein content in mycelium of a *Tolypocladium* sp. (11.7 mg·g^{-1}) than in wild *C. sinensis* (9.54 mg·g^{-1}). They isolated *Tolypocladium* sp. from wild *C. sinensis*.

3.2. Restriction on Platelet Aggregation

In this study, hot water extracts of both *P. hepiali* and *O. sinensis* have been shown to have inhibition effect on human platelet aggregation. *Paecilomyces hepiali* exhibited a higher value than wild *O. sinensis* as regard to restriction percentage (**Figure 2**). Both *P. hepiali* and *O. sinensis* showed significantly greater anti-platelet aggregation effect than *L. edodes* (**Figure 2**). The levels of restriction for *O. sinensis*, *P. hepiali* and *L. edodes* on platelet aggregation were slightly higher in arachidonic acid-induced aggregation than PAF-induced aggregation.
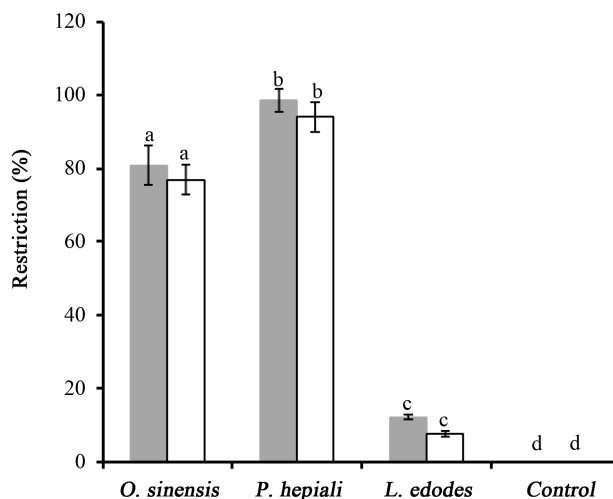
Figure 2. Anti-platelet aggregation test on human platelet. Solid columns are arachidonic acid，open columns are PAF. The error bars are representing standard deviation (n = 5). Bars not sharing the same letter represent means that are significantly different (p < 0.05).

Platelet aggregation is important in the formation of a hemostatic plug when normal blood vessels are injured. However, the interaction between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction [16]. As indicted by Davi & Patrono [17], precise control of platelet function must occur to prevent thrombotic event. This study has demonstrated that both *P. hepiali* and *O. sinensis* have inhibiting activity on human platelet aggregation. Other mushrooms have also been reported to have similar effect and these include *Schizophyllum commune* Fr., *Lentinus tigrinus* (Bull.) Fr., *Lentinus sajor caju* Fr., *Ganoderma lucidum* (Curtis: Fr.) P. Karst. and *Pleurotus florida* (Mont.) Singer [18]. Jose *et al*. [19] also demonstrated that methanol extract of *P. florida* has marked inhibitory effect on aggregation of washed human platelet induced by Adenosine 5'-diphosphate (ADP). An ethanol extract of *Inonotus obliquus* ASI 74003 mycelium has also been shown to have high platelet aggregation inhibitory activity. A tripeptide with sequences Trp-Gly-Cys is reported to be the platelet aggregation inhibitor in *I. obliquus* [20].

3.3. Effect on IL-8 Gene Expression

The hot water extract of *P. hepiali* significantly inhibited the expression of interleukin-8 (IL-8) gene stimulated by tumor necrosis factor-α (TNF-α) on human fibroblasts. The inhibition activity by hot water extract of *P. hepiali* was significantly higher than hot water extracts of wild *O. sinensis* and *L. edodes* and was almost equal in effect to hydrocortisone which was used as a positive control (Hcon) in this investigation (**Figure 3**).

Interleukin-8 is a member of the CXC chemokine subfamily. Baggiolini *et al.* [21] indicated that chemokines are distinguished depending on the arrangement of the first two of four conserved cysteines, which are either separated by one amino acid (CXC chemokines) or adjacent (CC chemokines). Chemokines are a type of cytokines that mainly act on neutrophils, monocytes, lymphocytes and eosinophils [22]. Cytokines are important mediators of inflammation and are associated with pathogenesis of many inflammatory diseases [23]. This study has shown that *P. hepiali*, wild *O. sinensis* and *L. edodes* have inhibitory effect on IL-8 gene expression induced by TNF-α on human fibroblasts (**Figure 3**). Results of this study are suggesting that these fungi have a potential role in treating inflammatory conditions.

3.4. Anti-Mutagenic Effect

The anti-mutagenic effects of wild *O. sinensis* and *P. hepiali* extracts were evaluated using *Salmonella typhimurium* strain TA98 and a mutagen 2-Amino-3-Methyl-3H-Imidazo[4,5-F]Quinoline (IQ). **Figure 4** shows results of the test. Both *P. hepiali* and wild *O. sinensis* exhibited significant anti-mutagenic activity and the re-
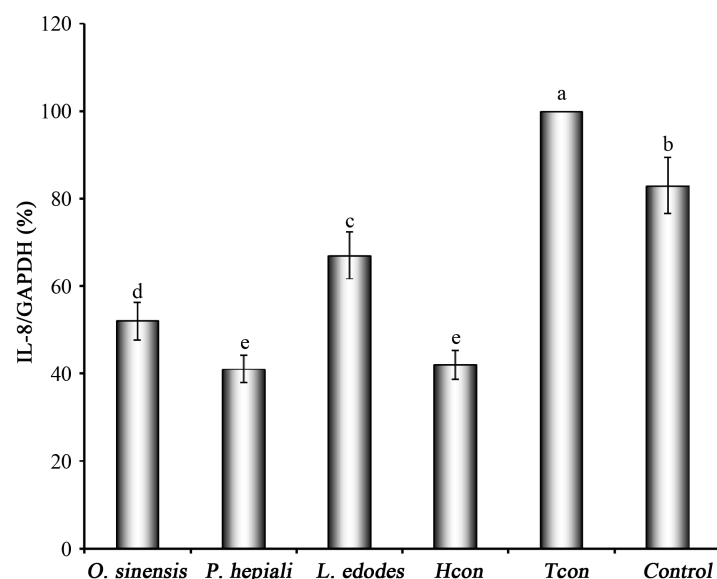
Figure 3. Inhibition test of IL-8 gene expression stimulated by TNF-α on the human fibro blasts. Hcon: hydrocortisone as a positive control. The error bars are representing standard deviation (n = 5). Bars not sharing the same letter represent means that are significantly different (p < 0.05).
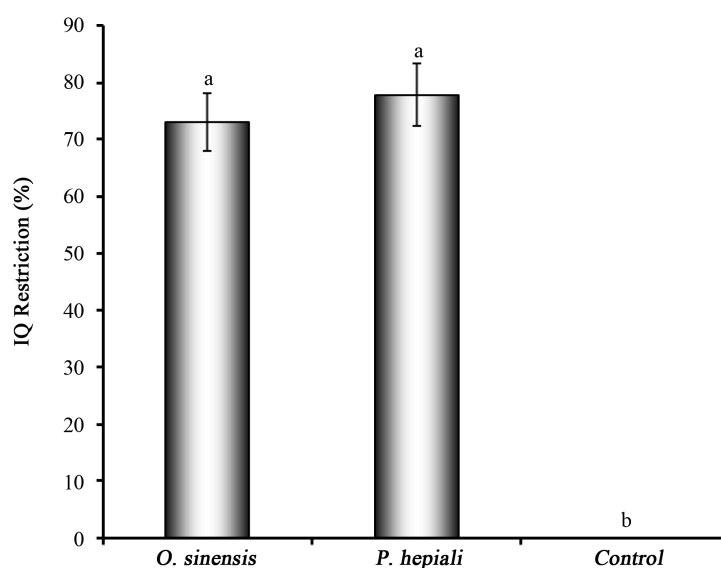

Figure 4. Inhibition effect of hot water extracts of wild *O. sinensis* and *P. hepiali* to the mutagen, IQ. The error bars are representing standard deviation (n = 5). Bars not sharing the same letter represent means that are significantly different (p < 0.05).

striction percentages to IQ between the two fungi were not significantly different.

The mutagen, IQ was first isolated from broiled Sardines [24]. IQ is highly mutagenic and concern thus exist that is could be a factor in the etiology of human cancer [25]. The results in this study are demonstrating the potential role these fungi would play in cancer treatment.

3.5. Skin Whitening Effect

The skin whitening effect of *P. hepiali* was conducted by evaluating its inhibition activity on melanin produced

by B16 melanoma cells. Comparisons were made with arbutin and kojic acid, compounds which are popularly used as skin whitening agents. **Figure 5** shows results of this investigation. The melanin inhibition effect of the fungal extract increased with increasing concentration. The fungal extract, at 100 ppm, had greater melanin inhibition effect than both arbutin and kojic. At 10 ppm the fungal extract showed very slight inhibition effect to melanin.

In mammals, colour of skin and hair is caused by melanin synthesis and distribution. Although melanin plays a crucial protective role in human skin by absorbing free radicals and shielding from UV light, abnormal changes of melanin synthesis such as hypermelanotic or hypomelanotic can result in a vast number of skin disease and disorders [26]. Skin whitening products are commercially available for the purpose of obtaining a lighter skin and they are also used for clinical treatment of pigmentary disorders such as melasma or postinflammatory hyperpigmentation [27]. These products contain whitening agents which act at various levels of melanin production in the skin. Many of the skin whitening agents are known as competitive inhibitors of tyrosinase, the key enzyme in melanogenesis. Azelaic acid, kojic acid, arbutin and aloesin are often used as positive skin whitening agents [27].

This study has shown that *P. hepiali* extract has the ability to significantly restrict production of melanin. However, the study did not determine the actual active ingredient(s) responsible for this effect.

3.6. Effect on Skin Roughness/Texture

Figure 6 shows results of an evaluation on effect of three different fungal extracts on human skin texture using replica method. The improvement percentage on roughness of the skin by *P. hepiali* was higher than wild *O. sinensis* and *L. edodes*. However, the values obtained for *P. hepiali* and *O. sinensis* were not significantly different. As pointed out by Schrader and Bielfeldt [28] skin roughness is a very important parameter in the characterization of cosmetic skin properties. Although the actual active ingredient(s) responsible for the effect on skin texture was not determined, the results imply that these fungal species have potential to be used in the cosmetics industry.
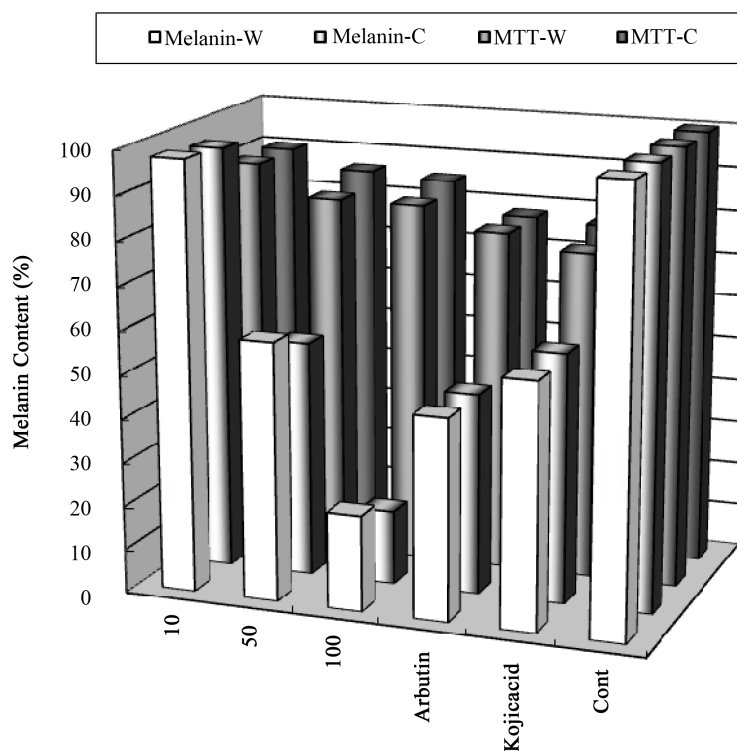

Figure 5. Effect of hot water extracts of *P. hepiali* on content of melanin produced by B16 melanoma cells. The height of each bar represents the mean of five replicates.
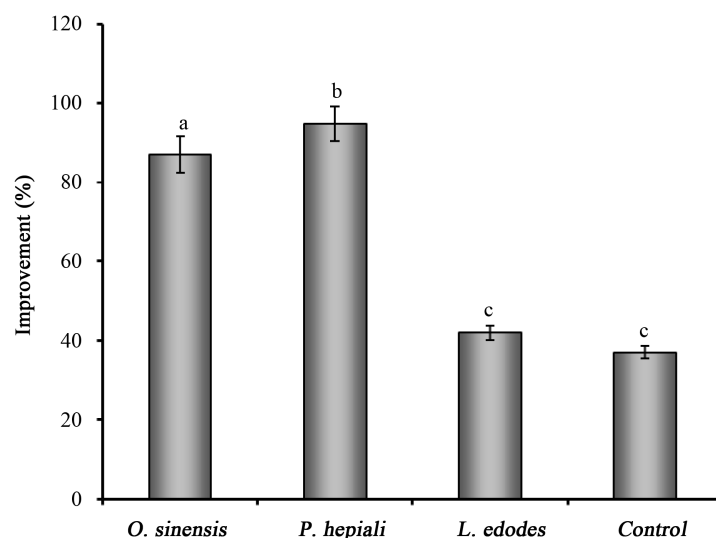
Figure 6. Improvement effect on human skin roughness by using replica method. The error bars are representing standard deviation (n = 5). Bars not sharing the same letter represent means that are significantly different (p < 0.05).

4. Conclusion

Results on restriction of platelet aggregation induced by arachidonic acid and PAF showed that both *O. sinensis* and *P. hepiali* have higher restriction than *Lentinula edodes*. The control treatment did not display any restriction. The inhibition on platelet aggregation by *P. hepiali* was greater than that by *O. sinensis*. The restriction by *O. sinensis*, *P. hepiali* and *L. edodes* on platelet aggregation were slightly higher in arachidonic acid-induced aggregation than that in PAF-induced aggregation. *Paecilomyces hepiali* showed a higher inhibition effect on IL-8 gene expression than *O. sinensis* as well as *L. edodes*. Its effect was almost equal to the action of the positive control, hydrocortisone. As regard to anti-mutagen test to IQ, the restriction percentages displayed by both *O. sinensis* and *P. hepiali* were not significantly different. Both *P. hepiali* and *O. sinensis* have been shown to have anti-mutagenic activity. The hot water extract of *P. hepiali* has also been shown to have skin whitening effect and ability to improve the texture of the human skin. This study has demonstrated that both *P. hepiali* and *O. sinensis* have potential to be used in the cosmetic industry and also in treatment of cancer and inflammatory conditions.

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