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Effects of Bioactive Composition in *Oryza sativa* L. cv. KDML105 Bran Extract on Gene Expression Related to Hair Cycle in Human Hair Follicle Dermal Papilla Cells

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Abstract: The aim of this study is to identify the effects of KDML105 bran extract on gene expression involving the hair cycle in HFDPCs and investigate its bioactive constituents, antioxidant, and anti-inflammatory activities. The content of tocopherols, γ-oryzanol, phytic acid, and phenolic compounds was quantified by liquid chromatography. Free fatty acids were determined using gas chromatography. Antioxidant capacities were estimated by DPPH, ABTS, and metal chelating assay. The nitric oxide (NO) production was determined by Griess reaction. Gene expression was measured by semi-quantitative RT-PCR. The major compounds in the extract were α- and γ-tocopherol, phytic acid, γ-oryzanol, chlorogenic acid, *o*-coumaric acid, palmitic acid, oleic acid, and linoleic acid, giving its antioxidant capacities. The nitrite level in lipopolysaccharide-induced macrophages (2.76 ± 0.13 μM) was significantly mitigated by the extract (0.81 ± 0.11 μM). Additionally, *SRD5A2* and *TGFB1* expressions in HFDPCs were downregulated, whereas *CTNNB1* and *VEGF* genes were upregulated after treatment with the extract. KDML105 extract ameliorated oxidative stress and NO production. According to the gene expression study, KDML105 bran extract may be involved in the induction and maintenance of the anagen phase and angiogenesis in the hair growth pathway. Therefore, KDML105 bran extract might be a promising source of anti-hair loss substances.

Keywords: androgen; anti-hair loss; antioxidant; by-product; hair growth promotion; jasmine rice; KDML105; rice bran; VEGF; WNT/β-catenin

1. Introduction

Rice (*Oryza sativa* L.) is a major dietary staple, which is consumed by more than half of the population globally [1]. The rice cultivar of Khao Dawk Mali 105 (KDML105), also known as Thai jasmine rice, is non-colored and non-glutinous rice, which is widely grown in Thailand [2]. Rice bran, a layer between the kernel and husk, is generated during milling of roughly 10% of whole grain rice [3]. In developing countries, this by-product is underutilized as animal feed or burned as a low-cost fuel, resulting in air pollution and environmental consequences [4]. Rice bran has been known as a potential source of several compounds, such as phenolic acids, flavonoids, essential fatty acids, and vitamin E, which may benefit human health [1,5]. Nevertheless, these compositions are extensively influenced by the types of rice cultivars and the efficiency of the milling system [5]. This rice milling by-product could be a cost-effective source for use in nutraceutical, pharmaceutical, and cosmeceutical applications for skin, hair, and scalp disorders [6].

Pattern hair loss (PHL), or androgenetic alopecia, is a progressive and non-scarring hair loss that manifests as a receding frontal hairline in men and diffuse hair thinning in women. Each hair follicle passes through four stages: anagen (growth), catagen (regression), telogen (rest), and exogen (hair shedding) phases [7]. Genetic factors associated with PHL increase the likelihood of abnormal scalp sensitizing to androgens [7]. Excessive activation of the androgen pathway through 5α-reductase enzymes, which convert testosterone to more potent androgen or dihydrotestosterone (DHT), causes follicular miniaturization and a shortened anagen phase [8]. A previous study reported that the PHL group has high serum levels of oxidants and inflammatory mediators, including nitric oxide (NO), suggesting that inflammation and oxidative stress play casual roles in PHL [9].

It is broadly recognized that vascular endothelial growth factor (VEGF) is linked to angiogenesis during the anagen phase, facilitating the supply of nutrients and oxygen-rich blood to hair follicles [10]. On the other hand, transforming growth factor beta 1 (TGF-β1) is a paracrine mediator from hair follicle dermal papilla cells (HFDPCs), which is associated with hair growth deceleration by facilitating premature catagen entry [11]. The expression of TGF-β1 was found to be higher in balding hair follicles [12]. Additionally, activation of the canonical WNT/β–catenin pathway is required for hair follicle development and hair cycling [13]. The activity of β-catenin in HFDPCs reportedly implicates the prolongation of the anagen phase and augments hair growth [14]. In addition to this pathway, the development and regeneration of hair follicle stem cells (HFSCs) is closely related to the sonic hedgehog pathway [15]. A previous study demonstrated that protein expression of sonic hedgehog (SHH) and β-catenin regulates hair follicle development and anagen induction in the hair cycle [16].

Currently, only minoxidil and finasteride are conventionally approved drugs for PHL treatment [7]. The use of finasteride, a 5α-reductase blocker, is restricted to men [8]. However, minoxidil is a common medicine for treating PHL in both genders, which acts through multiple pathways by stimulating *VEGF* expression and inducing WNT/β-catenin signaling [8,14,17]. Side effects associated with these drugs have been reported, including skin irritation, burning, impotence, decreased libido, and breast enlargement [18]. These limitations contribute to an increasing interest in herbal sources of hair growth-promoting substances, which could be further developed as an alternative treatment and non-invasive option for hair regeneration.

The regulatory potential of rice bran extract, including KDML105 bran extract, on gene expression involving hair growth promotion in HFDPCs has not been established. If it is shown that bran could be used as a substitute source for anti-hair loss compounds, the disposal problem with rice by-products may also be reduced, and the farmer's income may increase. Considering the aforementioned aspects, the purposes of this study were to identify bioactive constituents of KDML105 bran extract, as well as to investigate antioxidant activities, anti-inflammation, and effects of the extract on gene expression related to the maintenance and induction of the anagen phase for hair growth promotion.

2. Materials and Methods

2.1. Chemicals and Reagents

Anthrone; 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); 2,2-diphenyl-1-picrylhydrazyl (DPPH); bovine serum albumin; caffeic acid; catechin; chlorogenic acid; *o*-coumaric acid; *p*-coumaric acid; diclofenac sodium; epicatechin; (−)-epigallocatechin gallate (EGCG); ethylenediaminetetraacetic acid (EDTA); ferrous chloride; ferulic acid; Folin-Ciocalteu phenol reagent; gallic acid; *p*-hydroxybenzoic acid; kaempferol; lipopolysaccharide (LPS); naringenin; naringin; potassium persulfate; phytic acid; quercetin; rosmarinic acid; rutin; sulforhodamine B (SRB); trolox; and α-, β-, γ-, and δ-tocopherols were from Sigma Chemical (St. Louis, MO, USA). Fatty acid methyl esters (FAMEs) standard mixtures were purchased from Restek Corporation (Bellefonte, PA, USA). Dutasteride, finasteride, minoxidil, and purmorphamine were purchased from Wuhan W&Z Biotech (Wuhan, China). Agarose gel and Tris/acetic acid/EDTA (TAE 50X) were from Bio-Rad Laboratories (Hercules, CA, USA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were acquired from Gibco Life Technologies (Thermo Fisher Scientific, Waltham, MA, USA). The Follicle Dermal Papilla Cell Growth Medium Kit was ordered from Promo Cell GmbH, Heidelberg, Germany. The penicillin/streptomycin solution was from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). Acetic acid, dimethyl sulfoxide (DMSO), ethanol, trichloroacetic acid, and other chemical compounds were purchased from RCI Labscan (Bangkok, Thailand). All other chemicals were of analytical grade.

2.2. Extraction

The bran of *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) was obtained from an organic farm of Saleekam Trading Co., Ltd. (Chiang Mai, Thailand) in February 2020. The voucher specimen of rice bran (PNPRDU63028) was deposited at the Pharmaceutical and Natural Products Research and Development Unit, Faculty of Pharmacy, Chiang Mai University. Rice bran was macerated in 95% *v*/*v* ethanol for 24 h with a ratio of 1:3 (bran: ethanol). The solution was double filtered through Whatman filter paper, then was vacuum evaporated at 50 °C by an evaporator (Hei-VAP value, Heidolph, Schwabach, Germany).

2.3. Bioactive Compound Estimation of KDML 105 Bran Extract

2.3.1. Determination of Tocopherols

Oil was extracted using the Soxhlet method from rice bran. Hexane was used to dissolve the oils to a final concentration of 1 mg/mL, and then the mixture was filtered using a 0.45 m syringe filter. Tocopherols (α-, β-, γ-, and δ-isoforms) were quantified by the previous method [19]. High-performance liquid chromatography (HPLC) connected with a fluorescence detector (RF-20A; Shimadzu Corporation, Kyoto, Japan) was utilized. Each isoform of tocopherol was separated on a normal-phase Inertsil SIL-100A column (5 µm, 4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan). The mobile phase was 0.6% *v*/*v* propan-2-ol in hexane at flow rate 1 mL/min. Fluorometric detection of all peaks was performed at an excitation wavelength of 298 nm and an emission wavelength of 325 nm.

2.3.2. Determination of γ-Oryzanol

Oil was extracted from rice bran using the Soxhlet method. Dichloromethane was used to dissolve the oils to a final concentration of 1 mg/mL, and then the mixture was filtered using a 0.45 m syringe filter. A Shimadzu HPLC system comprising a UV-Vis detector with a diode array detector (SPD-20A; Shimadzu, Kyoto, Japan) and an Ultra C18 column (5 µm, 4.6 250 mm; Restek, PA, USA) was used to analyze γ-oryzanol in the extract [19]. The UV detector was set at 330 nm. A mobile phase system that consisted of methanol, acetonitrile, dichloromethane, and acetic acid (50:44:3:3) was run at 1.4 mL/min.

2.3.3. Determination of Phytic Acid and Phenolic Compounds

The sample was extracted with 95% v/v ethanol and evaporated at 40 °C using an evaporator (Hei-VAP value, Heidolph, Schwabach, Germany). To produce a final concentration of 1 mg/mL, the samples were diluted with ethanol and then filtered through a 0.22 µm membrane [20]. As reported by previous studies [19,21], phenolic compounds were quantified using liquid chromatography-mass spectrometry (LC-MS). An Agilent 1260 Infinity II series chromatography and an Agilent 6130 electrospray ionization quadrupole mass spectrometer (Agilent Tech., Santa Clara, CA, USA) were used. The Ultra C18 column (5 µm 4.6 × 250 mm; Restek, Bellefonte, PA, USA) was used for separation. The mixture of 0.2% v/v acetic acid in 5% v/v MeOH is a mobile phase A, while the mixture of mobile phase B consists of 0.2% v/v acetic acid in 50% v/v acetonitrile. The flow rate was set at 0.5 mL/min. A linear elution gradient was programmed as follows: 10–20% B (0–45 min), 20–55% B (45–85 min), 55–100% B (85–97 min), 100% B (97–110 min), and re-equilibration for 10 min using the initial condition. The sample (20 µL) was injected into the preheated column at 40 °C. Nitrogen was a nebulizer and an auxiliary gas. The mass spectrometer was programmed as follows: a negative selected ion-monitoring mode, a capillary voltage of −3.5 V, a voltage detector of 1.35 V, a dry gas flow rate of 12 L/min, a nebulizing gas flow rate of 1.5 L/min, and a dissolving line temperature of 250 °C. Full scan spectra ranged from 100 to 1200 m/z at a rate of 250 ms/spectrum.

2.3.4. Determination of Free Fatty Acids

The sample was first methylated to produce fatty acid methyl esters (FAMEs) [19]. Free fatty acids were determined using gas chromatography (GC-2030; Shimadzu, Kyoto, Japan) connected with a wall-coated, fused, wax capillary column (0.25 mm × 100 m × 0.25 µm, RT-2560; Restek, Bellefonte, PA, USA) [22,23]. The injector volume of the sample was 1 µL. Further, the temperature of the flame ionization detector was set at 250 °C. Helium was the carrier gas. The oven temperature program was increased from 50 to 220 °C at a rate of 10 °C/min and held constant for 35 min, then increased from 200 to 230 °C at a rate of 5 °C/min and maintained constant for 20 min. Chromatograms were processed via Lab Solutions (Shimadzu, Kyoto, Japan). By comparing the peak retention periods to the respective FAMEs mixture standard (Food Industry Fame Mix, RESTEK, Bellefonte, PA, USA), the fatty acid composition was determined. Individual fatty acids were quantified in terms of g/100 g of extract.

2.4. Determination of Antioxidant Activities

2.4.1. 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Scavenging Assay

The DPPH radical scavenging capacity of the extract was measured using the previous method [24]. In a 96-well plate, 0.1 mM DPPH solution was mixed with trolox (0.02–0.4 mg/mL) or extract solution. After 30 min of incubation in the dark, absorbances were recorded at 515 nm by a microplate reader (EZ Read 400 Flexi, Biochrom, Cambridge, UK). The results are expressed as milligrams of trolox equivalents per gram of extract (mg TE/g extract).

2.4.2. 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) Scavenging Assay

The ABTS assay was carried out using the previously reported method [25]. The stock solution of $ABTS^{\bullet+}$ comprised ABTS solution (7 mM) and potassium persulfate solution (2.45 mM). After 16 h of incubation, the stock solution was diluted with ethanol to obtain the working solution with an absorbance of 0.700 ± 0.020. Different concentrations of trolox (0.02–0.4 mg/mL) or extract solution were mixed with the working solution, incubated for 6 min, and analyzed for absorbances at 734 nm by a microplate reader (EZ Read 400 Flexi, Biochrom, Cambridge, UK). The results are shown in milligrams of trolox equivalents per gram of extract (mg TE/g extract).

2.4.3. Metal Chelating Assay

The described method [26] was used to test ferrous ion chelating activity. Ferrous chloride (2 mM) was reacted with different concentrations of EDTA (0.02–0.4 mg/mL) or extract solution for 10 min. Ferrozine (5 mM) was added, and absorbances at 515 nm were measured using a microplate reader (EZ Read 400 Flexi, Biochrom, Cambridge, UK). The results were expressed in milligrams of EDTA equivalents per gram of extract (mg EDTAE/g extract).

2.5. Cell Viability Assay

RAW 264.7 macrophage cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic 100× solution. Human hair follicle dermal papilla cells (HFDPCs: Promo Cell GmbH, Heidelberg, Germany) were grown in Follicle Dermal Papilla Cell Growth Medium Kit supplemented with a 1% antibiotic-antimycotic 100× solution. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

The effects of KDML105 bran extract and reference standards (diclofenac sodium (DF), minoxidil, finasteride, dutasteride, and purmorphamine) on cell viability were estimated by the sulforhodamine B assay, as described in a previous study [27]. The highest concentration (0.1 mg/mL) that provided cell viability greater than 80% was considered non-cytotoxic and was chosen for further testing (Figure S1).

2.6. Anti-Inflammation via Nitric Oxide Inhibition

To determine the NO level in the culture medium, the Griess reaction colorimetric assay kit (Invitrogen, Thermo Fisher Scientific, Inc., Eugene, OR, USA) was used [28]. NO was indirectly quantified by measuring nitrite, which is the final inert product of NO [29]. A positive control was DF, which has been shown to lower NO levels in LPS-stimulated RAW246.7 cells [29,30]. Since DF decreased the levels of iNOS protein and mRNA expression, NO synthesis was also decreased [29].

RAW 264.7 macrophage cells were seeded (10^4 cells/well) into a 96-well plate, incubated for 24 h to obtain monolayer cells, then pretreated with DF (0.1 mg/mL), KDML105 bran extract (0.1 mg/mL), and solvent (blank). After pretreatment for 2 h, cells were incubated with LPS. The positive control was a group of cells that did not receive any pretreatments, but were still stimulated to produce NO by LPS. Regarding the blank, cells were pretreated with the solvent in DMEM without LPS. Each supernatant solution was reacted with the Griess reagent mixture, incubated for 30 min, and measured for absorbance at 570 nm. The nitrite concentration was calculated using the standard curve equation of reference standard sodium nitrite.

2.7. Semi-Quantitative Reverse Transcription and Polymerase Chain Reaction Analysis

KDML105 bran extract was compared to reference standard compounds (minoxidil, finasteride, dutasteride, and purmorphamine) at the same concentration of 0.1 mg/mL. RNA was extracted from HFDPCs using the E.Z.N.A.® Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA). The concentration of the total RNA was determined using the Qubit™ 4 fluorometer (Invitrogen, Carlsbad, CA, USA) and the Qubit™ RNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). The RNA solution was stored at 20 °C until it was used. Semi-quantitative RT-PCR was performed to assess gene expression levels as previously described with minor modification [31]. The MyTaq™ One-Step RT-PCR Kit was used to generate complementary DNA (Bioline, Memphis, TN, USA). The primer sequences are presented in Table 1**.** The expression of the gene of interest relative to the internal control gene (*GAPDH* gene) in the treated sample was compared to the vehicle-treated control. The results are expressed as a fold change in gene expression.

Table 1. List of forward and reverse primer sequences.

Gene	Forward Sequence	Reverse Sequence	Accession Number
SRD5A1	AGCCATTGTGCAGTGTATGC	AGCCTCCCCTTGGTATTTTG	NM_001047.4
SRD5A2	TGAATACCCTGATGGGTGG	CAAGCCACCTTGTGGAATC	NM_000348.4
SRD5A3	TCCTTCTTTGCCCAAACATC	TCCTTCTTTGCCCAAACATC	NM_024592.5
SHH	AAAAGCTGACCCCTTTAGCC	GCTCCGGTGTTTTCTTCATC	NM_000193.4
SMO	GAAGTGCCCTTGGTTCGGACA	CCGCCAGTCAGCCACGAAT	NM_005631.5
GLI1	GCAGGGAGTGCAGCCAATACAG	GAGCGGCGGCTGACAGTATA	NM_005269.3
CTNNB1	CCCACTAATGTCCAGCGTTT	AACCAAGCATTTTCACCAGG	NM_001330729.2
TGFB1	GCCCTGGACACCAACTATTG	GTCCAGGCTCCAAATGTAGG	NM_000660.7
VEGF	CTACCTCCACCATGCCAAGT	GCGAGTCTGTGTTTTTGCAG	NM_001025366.3
GAPDH	GGAAGGTGAAGGTCGGAGTC	CTCAGCCTTGACGGTGCCATG.	NM_001289745.3

Finasteride and dutasteride, 5α-reductase inhibitors, suppressed *SRD5A* expression in previous studies [31,32]. Purmorphamine, an agonist of the smoothened receptor, has been reported to enhance the gene expression of mediators in the sonic hedgehog pathway [33,34]. Furthermore, previous studies used minoxidil, a vasodilator, as a positive control for assessing the expression of the *CTNNB1*, *VEGF*, and *TGFB1* genes [35,36]. Thus, these standard compounds were used as positive controls in this study.

2.8. Statistical Analysis

The results are given as a mean ± standard error of the mean. The GraphPad Prism version 9.4.0 for MacOS (GraphPad Software, San Diego, CA, USA) was used for statistical comparisons. The statistical differences between the means of pairs were determined using one-way analysis of variance followed by Tukey's test. A *p*-value below 0.05 was deemed statistically significant. Experiments were carried out in triplicate.

3. Results

3.1. Bioactive Constituents of KDML105 Bran Extract

The appearance of KDML105 bran extract is a pale-yellow paste with a greasy texture. The bioactive compounds of KDML105 bran extract, including tocopherol derivatives, phytic acid, phenolic compounds, and fatty acids, were estimated (Table 2). The bioactive constitution of tocopherols was quantified using HPLC. The γ-tocopherol content (Figure 1) was highest, followed by α-tocopherol, β-tocopherol, and δ-tocopherol.

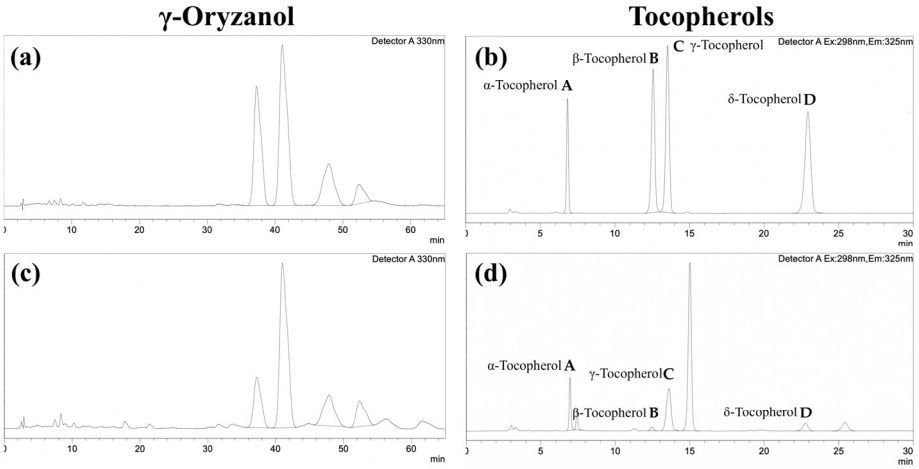

Figure 1. High performance liquid chromatography (HPLC) chromatograms of (**a**) standard γ-oryzanol, (**b**) standard tocopherols, (**c**) γ-oryzanol in *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) bran extract, and (**d**) tocopherols in KDML105 bran extract.

Table 2. The contents of bioactive compounds in KDML105 bran extract.

Group	Bioactive Compounds	Content (mg/100 g extract)
Phenolic compounds	Caffeic acid	2.21 ± 0.00
	Chlorogenic acid	10.28 ± 0.01
	Epigallocatechin gallate	5.50 ± 0.04
	Ferulic acid	2.87 ± 0.00
	Gallic acid	1.52 ± 0.00
	Hydroxybenzoic acid	4.94 ± 0.01
	Naringin	2.77 ± 0.00
	o-Coumaric acid	9.46 ± 0.02
	p-Coumaric acid	6.14 ± 0.00
	Quercetin	4.13 ± 0.01
	Rosmarinic acid	1.17 ± 0.00
	γ-Oryzanol	10.39 ± 2.17
Phytic acid	Phytic acid	19.26 ± 0.01
Tocopherols	α-Tocopherol	40.19 ± 0.04
	β-tocopherol	2.30 ± 0.01
	γ-tocopherol	48.01 ± 0.00
	δ-tocopherol	0.81 ± 0.08
Group	**Bioactive compounds**	**Content (g/100 g extract)**
Saturated fatty acids	Arachidic acid	1.06 ± 0.32
	Behenic acid	0.25 ± 0.00
	Heneicosylic acid	1.29 ± 0.01
	Lignoceric acid	0.45 ± 0.00
	Myristic acid	0.27 ± 0.02
	Palmitic acid	19.67 ± 0.18
	Stearic acid	2.17 ± 0.02
Monounsaturated fatty acids	Oleic acid	42.62 ± 0.40
Polyunsaturated fatty acids	Eicosadienoic acid	0.11 ± 0.00
	Linoleic acid	31.62 ± 0.29
	α-Linolenic acid	0.48 ± 0.00
	γ-Linolenic acid	0.02 ± 0.00

Note: All values are mean ± SD.

The LC-MS analysis of phytic acid and phenolic compounds in KDML105 bran extract is shown in Table 2 and Figure 2. KDML105 bran extract contains phytic acid of approximately 19.26 ± 0.01 mg/100 g extract. The most abundant phenolic compounds in KDML105 bran extract were γ-oryzanol (10.39 ± 2.17 mg/100 g extract; Figure 2) and chlorogenic acid (10.28 ± 0.01 mg/100 g extract), followed by *o*-coumaric acid, *p*-coumaric acid, epigallocatechin gallate, hydroxybenzoic acid, quercetin, ferulic acid, naringin, caffeic acid, gallic acid, and rosmarinic acid.

The fatty acid profile of KDML105 bran extract, including saturated, monounsaturated, and polyunsaturated fatty acids, was identified (Table 2). Among all types of fatty acids, oleic acid was the major fatty acid in KDML105 bran extract, with a content of 42.62 ± 0.40 g/100 g extract. On the other hand, palmitic acid (19.67 ± 0.18 g/100 g extract) was the predominant saturated fatty acid, followed by stearic, heneicosylic, and arachidic acids and small amounts of other saturated fatty acids. Regarding the polyunsaturated group, it is noticeable that KDML105 bran extract contained the highest linoleic acid content. Overall, the total content of fatty acids was ranked in descending order as follows: monounsaturated > polyunsaturated > saturated fatty acids.

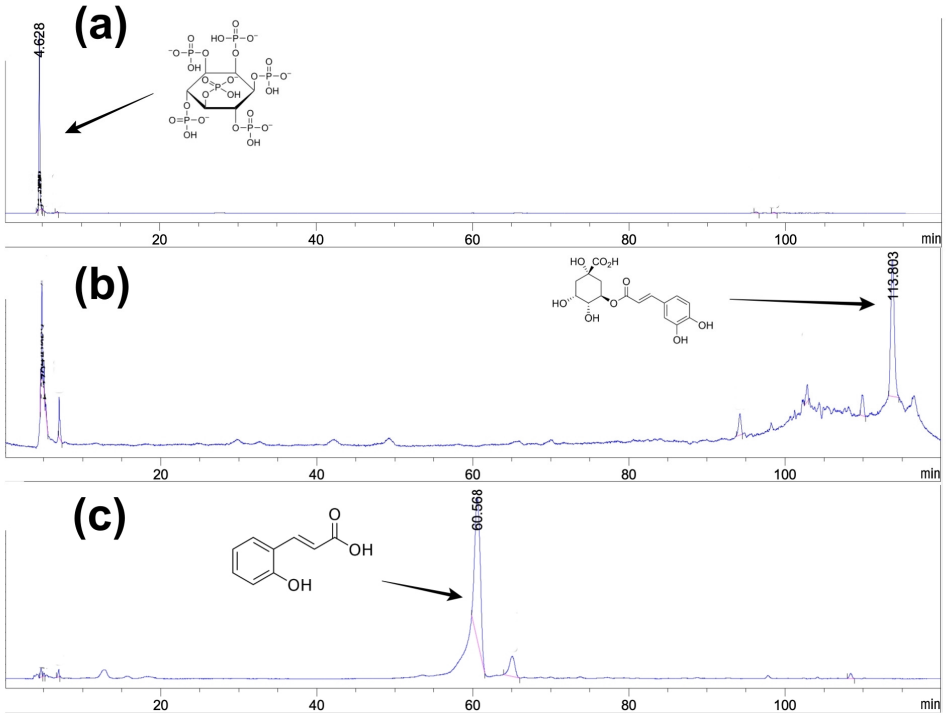

Figure 2. Liquid chromatography-mass spectrometry (LC-MS) chromatograms of (**a**) phytic acid, (**b**) chlorogenic acid, and (**c**) *o*-coumaric acid in *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) bran extract.

3.2. Antioxidant Activities

The antioxidant capabilities of KDML105 bran extract were assessed using DPPH, ABTS, and metal chelating assay. The DPPH radical scavenging capacity of KDML105 bran extract was equivalent to 5.45 ± 0.24 mg TE/g extract, whereas it could stabilize the radical form by ABTS oxidation with the potency of 5.56 ± 1.03 mg TE/g extract. For the ferrous iron chelating ability, KDML105 bran extract presented a great value of 95.10 ± 0.03 mg EDTAE/g extract.

3.3. Anti-Inflammation via Nitric Oxide Inhibition

Nitric oxide (NO) production in RAW 264.7 macrophage cells was induced via lipopolysaccharide (LPS), the inflammatory trigger derived from *Escherichia coli*. The metabolite nitrite could indirectly predict NO levels in the culture medium. As shown in Figure 3, the nitrite concentration of the LPS-stimulated group without any pretreatment was considerably higher than that of the solvent-treated control group without LPS (blank). There was no significant difference between the nitrite level of the pretreatment with 0.1 mg/mL of diclofenac sodium (0.36 ± 0.01 μM) and a blank (0.40 ± 0.06 μM). In addition, the attenuation of NO production was observed in the pretreatment of KDML105 bran extract (0.81 ± 0.11 μM), which was significantly lower than in the LPS-stimulated group (2.76 ± 0.13 μM).

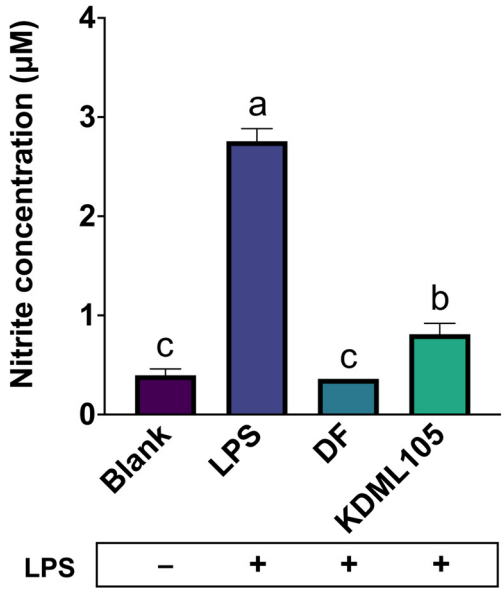

Figure 3. The nitrite level (μM) in RAW 264.7 murine macrophages after lipopolysaccharide (LPS) stimulation for 24 h. Diclofenac sodium (DF) or *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) bran extract was pretreated at 0.1 mg/mL to inhibit nitrite production, and these pretreatments were compared to the solvent-treated group without LPS (blank) and the LPS-stimulated group (+LPS). Different alphabets represent significant differences ($p < 0.05$) between groups.

3.4. Effect of KDML105 Bran Extract on Gene Expression

HFDPCs were exposed to KDML105 bran extract and the reference compounds, such as finasteride, dutasteride, purmorphamine, and minoxidil, at the same concentration of 0.1 mg/mL for 24 h. The regulatory consequences of KDML105 bran extract for genes involved in the pathogenesis of PHL, including the *SRD5A1*, *SRD5A2*, *SRD5A3*, *SHH*, *SMO*, *GLI1*, *CTNNB1*, *VEGF*, and *TGFB1* genes, are demonstrated in Figure 4.

Regarding the androgen pathway, genes encoding steroid 5α-reductase types 1, 2, and 3, which are *SRD5A1* (Figure 4a), *SRD5A2* (Figure 4b), and *SRD5A3* (Figure 4b) genes, were investigated. In our study, it was noticed that finasteride and dutasteride dramatically downregulated all types of *SRD5A* genes compared to the control group. On the other hand, the suppression effects of KDML105 bran extract on *SRD5A1* and *SRD5A3* genes were slightly observed, with a fold change of 0.83 ± 0.04 and 0.88 ± 0.01, respectively. *SRD5A2* expression was substantially downregulated by KDML105 bran extract.

The sonic hedgehog signal transduction cascade involves proteins, namely, the sonic hedgehog molecule (SHH), smoothened (SMO), and glioma-associated oncogene 1 (GLI1), which are encoded by *SHH*, *SMO*, and *GLI1* genes, respectively. In HFDPCs, KDML105 bran extract slightly upregulated *SHH*, *SMO*, and *GLI1* (Figure 4d–f), compared to the control group, with fold changes of 1.09 ± 0.01, 1.19 ± 0.03, and 1.25 ± 0.01, respectively. Purmorphamine substantially enhanced the expressions of *SHH* (fold change of 1.63 ± 0.06) and *SMO* (fold change of 1.33 ± 0.06), but only marginally altered *GLI1* expression (fold change of 1.13 ± 0.03).

The *CTNNB1* gene, which encodes β-catenin and is a critical component of the canonical WNT/β-catenin signaling pathway, was manifestly upregulated by KDML105 bran extract (fold change of 3.51 ± 0.41), compared to the control and minoxidil-treated groups (fold change of 1.15 ± 0.04, Figure 4g).

Additionally, KDML105 bran extract increased *VEGF*, a gene encoding VEGF, with a fold change of 2.73 ± 0.28, compared to the control group (Figure 4h). Indeed, the mRNA expression of *VEGF* was distinctly upregulated by minoxidil, with a fold change of 9.30 ± 0.30. On the other hand, mRNA expression of the gene encoding TGF-β1, a *TGFB1*

gene, was diminished by minoxidil (fold change of 0.56 ± 0.04) and KDML105 bran extract (fold change of 0.76 ± 0.13), as compared to the control group (Figure 4i).

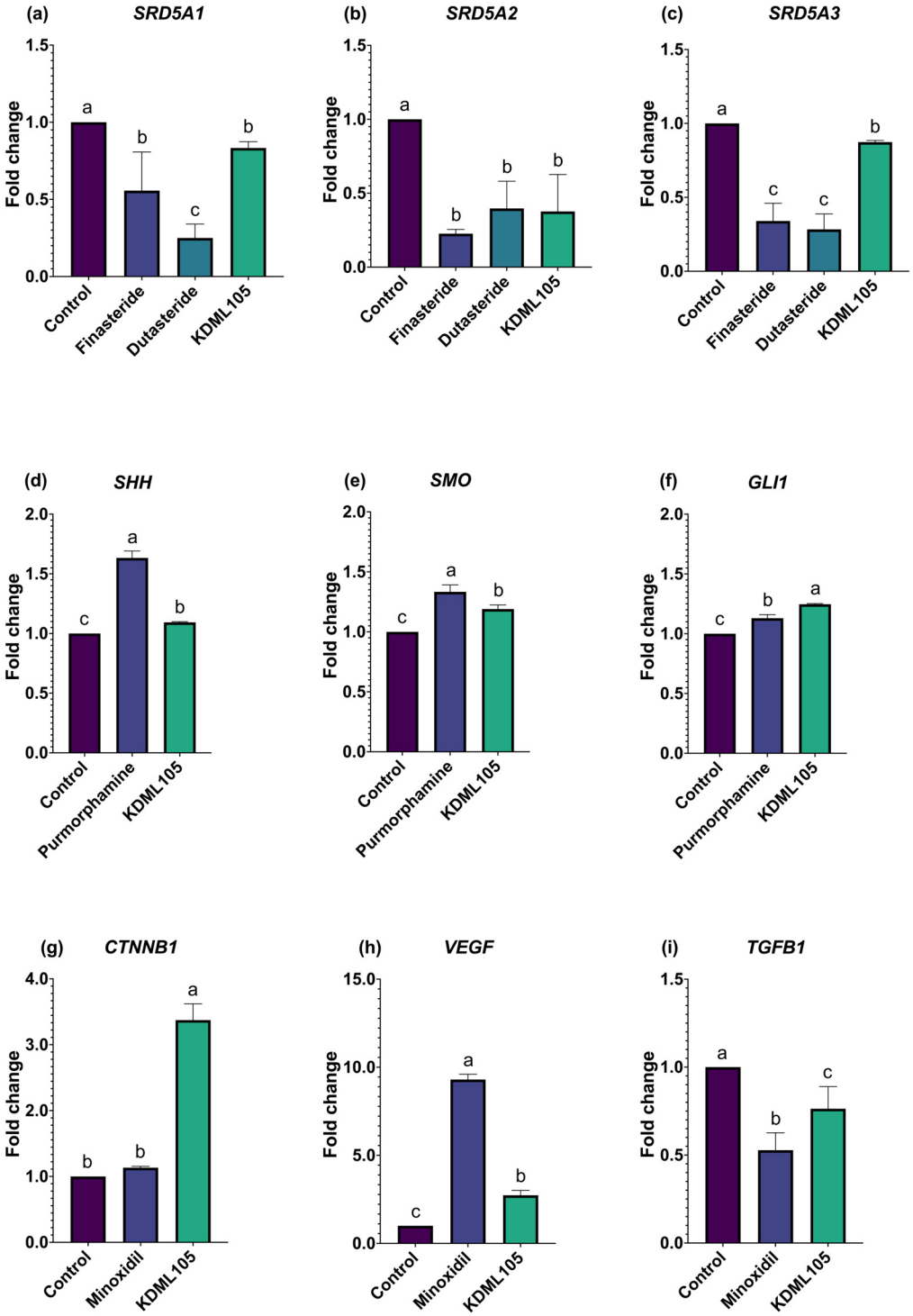

Figure 4. Gene expression profiling of human hair follicle dermal papilla cells (HDCPCs) in response to *Oryza sativa* L. cv. Khao Dawk Mali 105 (KDML105) bran extract was compared to the vehicle-treated control and standard reference compounds, including finasteride, dutasteride, purmorphamine, and minoxidil, at the same concentration of 0.1 mg/mL. Examined genes relating to hair growth consist of (**a**) *SRD5A1*, (**b**) *SRD5A2*, (**c**) *SRD5A3*, (**d**) *SHH*, (**e**) *SMO*, (**f**) *GLI1*, (**g**) *CTNNB1*, (**h**) *VEGF*, and (**i**) *TGFB1* genes. Different alphabets represent significant differences ($p < 0.05$) between groups.

4. Discussion

Hair cycling mediates a dynamic interaction between dermal and epidermal cells, especially HFDPCs, which is an integral regulator of hair follicle development and maintenance of the anagen phase [7]. HFDPCs activates HFSCs, resulting in proliferation and modification of these stem cells into derived matrix cells of keratinocytes, subsequently forming the hair shaft and other hair follicle components [37]. The anagen phase is induced and maintained by several pathways, including the WNT/β-catenin and sonic hedgehog pathways [13]. Excessive androgen stimulation, chronic inflammation, and oxidative stress, on the other hand, can disrupt the telogen–to–anagen transition or promote anagen–to–catagen transition, thereby delaying hair growth [27,37,38].

Excessive ROS accumulation in HFDPCs beyond its antioxidative capacity causes premature senescence [39]. HFDPCs derived from PHL scalps are more sensitive to cellular oxidative stress, as well as external stress, such as pollution and UV radiation, than non-balding cells [40]. This contributes to the reduction in the cell proliferation and migration of HFDPCs, which consequently affects hair follicle development and hair growth [40]. Antioxidant-rich treatments may reduce the oxidative stress associated with hair loss and premature hair follicle aging [8].

Isomers of α- and γ-tocopherol are predominantly present in KDML105 bran extract. A previous comparative study also found that the highest abundance of tocopherols in KDML105 bran and other bran of non-colored rice varieties were α- and γ-tocopherols, whereas minor tocopherols in those raw materials were β- and δ- tocopherols [19].The hydroxyl group on the chromanol ring of tocopherols that were found in KDML105 bran extract transfers its hydrogen atom to scavenge free radicals, giving a resonance-stabilized vitamin E radical [41] and contributing to the scavenging activity of the extract.

Phytic acid, or phytate phosphorus, a potent iron chelator, is mainly detected in rice grain and bran fractions [42]. The negative charge on the phytic acid structure in KDML105 bran extract can facilitate the chelation ability, resulting in an insoluble salt or phytate [43]. γ-Oryzanol, a mixture of steryl ferulates, mainly resides in bran [42]. A study reported that γ-oryzanol enhances the activities of antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase [41]. Moreover, γ-oryzanol behaves as a radical scavenger in both hydrophilic and lipophilic conditions, which means its antioxidant ability is stronger than that of tocopherol derivatives [41,42]. Based on previous results, chlorogenic acid and *o*-coumaric acid were found in the bran of all Thai rice varieties [19]. The number of hydroxyl groups on a phenolic ring, the location of the hydroxy substitution, and the presence of other function groups are responsible for the free radical scavenging capacity [41]. Our results indicate that KDML105 bran extract is rich in phytic acid, γ-oryzanol, chlorogenic acid, and *o*-coumaric acid, which may contribute to the abilities of the extract in scavenging and chelating and could be beneficial for reducing oxidative stress in PHL-hair follicles.

The fatty acid composition of the KDML105 bran extract in this study is in accordance with the results of previous studies, in which the major fatty acids in rice bran of several rice varieties were oleic acid, linoleic acid, and palmitic acid [19,44–46]. However, the fatty acid content can be affected by the cultivar, growth area, weather conditions, and post-treatment process [46]. Unsaturated oleic acid enhanced oxidative stress resistance in *Caenorhabditis elegans*, which could regulate by the Forkhead transcription factor DAF-16 [47]. Although the antioxidant properties of the oleic acid, linoleic acid, and palmitic acid found in KDML105 bran extract were highlighted [48,49], and these fatty acids could be able to mitigate oxidative stress linked to hair loss, additional cell-based antioxidant assays are required to confirm the extract's antioxidant potential on HFDPCs.

Nitric oxide synthase (iNOS) is activated by noxious circumstances, for instance, injury, infection, oxidative stress, and androgen [50,51]. Consequently, large amounts of NO are produced and released by perifollicular macrophages [52], resulting in inflammatory aggravation and surrounding tissue damage [51,53]. Interestingly, stimulating HFDPCs with DHT increased the NO level by three-fold, which is mediated by iNOS [50]. Perifollicular

inflammation and inflammatory infiltration are characterized as histological features of PHL [38]. In this study, KDML105 bran extract reduced NO production in RAW267.4 cell lines. This result is consistent with previous studies, which observed effects of different local rice varieties of Thailand on LPS-induced NO formation [54]. Chlorogenic acid and coumaric acid, two phenolic compounds found in KDML105 bran, have been shown to inhibit iNOS expression and reduce NO production in cultured macrophages [55]. A recent study by Ma et al. [56] revealed that γ-oryzanol efficiently diminished NO production and mitigated oxidative stress via inhibiting the MAPK signaling pathway and activating the Nrf2 signaling pathway [56]. Furthermore, Müller et al. discovered that the expressions of iNOS, cyclooxygenase, and interleukin-6 (IL-6) in macrophages were downregulated by oleic acid [57]. The content of chlorogenic acid, coumaric acid, γ-oryzanol, and oleic acid in KDML105 bran extract may be responsible synergistically for reducing NO production.

Androgens, particularly DHT, induce an early anagen–to–catagen transition of the hair follicle [37]. The activities of 5α-reductases and expression of their genes (*SRD5A1*, *SRD5A2*, and *SRD5A3*) were found to be upregulated in androgen-sensitive hair follicles of PHL [58,59]. According to our findings, KDML105 bran extract greatly reduced *SRD5A2* expression in HFDPCs, and slightly downregulated the expression of *SRD5A1* and *SRD5A3* genes. The type and concentration of phytochemicals in KDML105 bran extract may have different effects on the transcription level of *SRD5A* genes [60]. A previous study indicated that tocopherols (especially β- and γ-isoforms) and unsaturated fatty acids (oleic acid and γ-linolenic acid) significantly diminished mRNA expression of *SRD5A2* in DU-145 prostate cancer cells [31]. Additionally, the expression of *SRD5A* in HFDPCs was attenuated by chlorogenic acid, a major constituent of coffee berry extract, in a dose-dependent manner [61]. Tocopherols, oleic acid, and γ-linolenic acid in KDML105 bran extract could synergistically downregulate *SRD5A2* expression. It is also reported that the activities of 5α-reductases were inhibited by bioactive constituents that can be found in KDML105 bran extract, namely, quercetin, epigallocatechin gallate, oleic acid, linoleic acid, and myristic acid [62], resulting in inhibiting the conversion of testosterone to DHT and ameliorating progressive miniaturization.

Activation of the WNT/β–catenin pathway is required for hair follicle regeneration and hair growth. The accumulation of β-catenin is involved in hair follicle regeneration and regulates the expression of genes responsible for hair follicle development [13,17]. Crosstalk between the androgen and WNT/β–catenin pathways has been demonstrated, in which DHT may disrupt translocation of β-catenin into nuclease, mediating by glycogen synthase kinase-3β (GSK-3β) [13]. The failure of hair follicle formation could be caused by β-catenin ablation [12]. A study found that *Prunus mira* nut oil upregulated the mRNA and protein expression of β-catenin in C57BL/6 mice [63]. The major bioactive compounds of *P. mira* nut oil, including α-tocopherol, oleic acid, and linoleic acid, are likely to have a role in inducing hair follicles into the anagen [63]. Likewise, our results indicate that the *CTNNB1* expression was obviously upregulated by KDML105 bran extract. The presence of α-tocopherol, oleic acid, and linoleic acid in KDML105 bran extract may contribute to promoting the expression of β-catenin and help to the extend the anagen phase.

The sonic hedgehog pathway modulates hair follicle formation, cell proliferation, and the initiation of anagen [16]. This pathway is activated by the binding of the SHH molecule to its receptor, followed by the stimulation of the SMO molecule and transcription factor GLI1, contributing to the transcription of hair growth-related genes [64,65]. The protein expression of GLI1 and SHH was detected in the matrix, HFDPCs, and outer root sheath cells of the hair follicle during the anagen phase [66]. It has been reported that the absence of SHH or SMO perturbs hair follicle growth and hair cycling [64,65]. Moreover, a study reported that red ginseng oil containing linoleic acid and β-sitosterol reversed androgen-induced suppression of hair growth in C57BL/6 mice by increasing the protein expression of *SHH*, *SMO*, and *GLI1* [67]. Furthermore, the topical application of tocopheryl acetate promoted hair growth in C57BL/6 mice and upregulated the mRNA expression of SHH [68]. However, the expression of *SHH*, *SMO*, and *GLI1* was only marginally improved

by the KDML105 bran extract in 24 h. The replicate study might be conducted over a longer period to observe the effect of KDML105 bran extract on the expression of these genes.

Several growth factors that can control the development and cycle of the hair follicle have been identified so far. TGF-β1 is known as a catagen inducer in the hair cycle [11]. DHT causes the release of hair growth suppressors, such as TGF-β1, IL-6, and dickkopf-1 (DKK-1) [39]. TGF-β1 attenuates epithelial cell growth and also enhances androgen sensitivity in bald HDPC [11]. The PHL-affected group had an increase in the TGF-β1 level and a reduction in the *VEGF* level compared with the control group [69]. Additionally, a previous study showed that mRNA and protein expressions of *VEGF* in HFDPCs were highest in the anagen phase and then declined from catagen to telogen [10]. Choi et al. [70] demonstrated that rice bran supercritical CO_2 extract, containing particularly linoleic acid and γ-oryzanol, induced the hair follicles of C57BL/6 mice to an anagen stage by upregulating the *VEGF* gene and downregulating the *TGFB1* gene [70]. In our study, KDML105 bran extract elevated *VEGF* expression, and on the other hand, diminished *TGFB1* expression in HFDPCs. KDML105 bran extract, a source of linoleic acid and γ-oryzanol, could promote blood flow and accelerate hair growth during the anagen phase.

5. Conclusions

Our findings revealed that KDML105 bran extract contained several antioxidant compounds, particularly α-, γ- tocopherol, phytic acid, γ-oryzanol, chlorogenic acid, *o*-coumaric acid, palmitic acid, oleic acid, and linoleic acid, which supported its antioxidant and anti-inflammatory activities. Notably, KDML105 bran extract downregulated mRNA expression of *SRD5A2* and *TGFB1* genes in HFDPCs, which could delay progressive follicular miniaturization and catagen transition. In addition, KDML105 bran extract enhanced *CTNNB1* and *VEGF* expression, possibly assisting in the induction and maintenance of the anagen phase, as well as angiogenesis, in hair follicles, contributing to hair growth promotion. However, additional research is needed to confirm the regulatory potential of KDML105 bran extract on the hair cycle. The results of this study suggested that KDML105 bran extract may be a potential source for hair growth-promoting substances.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020295/s1, Figure S1. Cell viability of the KDML105 bran extract by the sulforhodamine B (SRB) assay: (a) hair follicle dermal papilla cells (HFDPC); (b) RAW 264.7 murine macrophages.

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