

# Cumulative Production of Bioactive Rg3, Rg5, Rk1, and CK from Fermented Black Ginseng Using Novel *Aspergillus niger* KHNT-1 Strain Isolated from Korean Traditional Food

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*Keywords:* *Aspergillus niger*, soybean, ginseng, ginsenosides, processing, Ginseng Lateral root (GLR), Ginseng Main root (GMR), human keratinocyte cell line (HaCaT) cells, B16BL6 (Murine melanoma) cell line

## Abstract:

Ginseng is an ancient herb widely consumed due to its healing property of active ginsenosides. Recent researchers were explored to increase its absorption and bioavailability of ginsenosides at the metabolic sites, due to its pharmacological activity. The purpose of this study was to investigate the isolation and characteristics of components obtained by a shorter steaming cycle (seven cycles) of white ginseng to fermented black ginseng, using a novel strain of *Aspergillus niger* KHNT-1 isolated from fermented soybean. The degree of bioactive of Rg3 increased effectively during the steaming process, and biotransformation converted the color towards black along active ginsenosides. Glycol moiety associated with C-3, C-6, or C-20 underwent rapid biotransformation and hydrolysis, such as Rb1, Rb2, Rc, Rd ? Rg3, F2, and was converted to CK. Dehydration produces Rg3 ? Rk1, Rg5. Rh2 ? Rk2; thus, converted fermented black ginseng was solvent-extracted, and the isolated components were identified by TLC, HPLC, and quantification by LCMS. The unique composition obtained during this process with Rk1, Rg3, Rg5, and CK is nontoxic to HaCaT cell line up to 200 ug/mL for 24 h and was found to be effective in B16BL6 cell lines, in a dose- and time-dependent manner. Thus, it is a suitable candidate for nutraceuticals and cosmeceuticals.

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## Article

# Cumulative Production of Bioactive Rg3, Rg5, Rk1, and CK from Fermented Black Ginseng Using Novel *Aspergillus niger* KHNT-1 Strain Isolated from Korean Traditional Food

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**Abstract:** Ginseng is an ancient herb widely consumed due to its healing property of active ginsenosides. Recent researchers were explored to increase its absorption and bioavailability of ginsenosides at the metabolic sites, due to its pharmacological activity. The purpose of this study was to investigate the isolation and characteristics of components obtained by a shorter steaming cycle (seven cycles) of white ginseng to fermented black ginseng, using a novel strain of *Aspergillus niger* KHNT-1 isolated from fermented soybean. The degree of bioactive of Rg3 increased effectively during the steaming process, and biotransformation converted the color towards black along active ginsenosides. Glycol moiety associated with C-3, C-6, or C-20 underwent rapid biotransformation and hydrolysis, such as Rb1, Rb2, Rc, Rd → Rg3, F2, and was converted to CK. Dehydration produces Rg3 → Rk1, Rg5. Rh2 → Rk2; thus, converted fermented black ginseng was solvent-extracted, and the isolated components were identified by TLC, HPLC, and quantification by LCMS. The unique composition obtained during this process with Rk1, Rg3, Rg5, and CK is nontoxic to HaCaT cell line up to 200 ug/mL for 24 h and was found to be effective in B16BL6 cell lines, in a dose- and time-dependent manner. Thus, it is a suitable candidate for nutraceuticals and cosmeceuticals.

**Keywords:** *Aspergillus niger*; soybean; ginseng; ginsenosides; processing; Ginseng Lateral root (GLR); Ginseng Main root (GMR); human keratinocyte cell line (HaCaT) cells; B16BL6 (Murine melanoma) cell line

## 1. Introduction

An ancient herbal from East Asia, *Panax ginseng* Meyer (family Araliaceae) gained its importance for its medicinal property. Ginseng is a very critical oriental plant that is attributed to higher medicinal effects in curing many diseases, including heart disease, cancer, diabetes, and autoimmune disease; it also supports the central nervous system and contains stress-relieving and antioxidant capabilities [1]. *Panax ginseng* has been categorized as white ginseng, red ginseng, black ginseng, and fermented ginseng. Each ginseng has different actives based on bioconversion. This bioconversion leads to two classifications of ginseng, based on its active components namely protopanaxatriol (PPT) and protopanaxadiol (PPD). The biological activities of PPT and PPD depend on the number and position of sugar moieties linked to the aglycone [2]. This deglycosylation rate

determines the effect of ginsenosides on human metabolism. Due to this importance of different ginsenosides deglycosylation, compound k (CK) and Rh1 are more detected in the circulatory system [3]. To neutralize this bioconversion in human metabolism, it is essential to convert to the necessary ginsenosides before administrating to the human body [4]. Physical methods such as steaming, sulfur fumigation, and microwave treatment were adopted for this approach; however, such treatment was not found in its application in industrial commercialization, except for the steaming process. In the chemical method, though, processes like acid hydrolysis and alkaline hydrolysis are much simpler and easy to handle. However, it is very hard to control the side reactions and exercise hydrolysis of glycosylation for selective enrichment of the ginsenosides. The third important method of transformation in a biological process is using the enzymatic conditions that have their unique significance in sustaining important actives of ginsenosides [5]. This biotransformation includes various industrial-grade or food-grade agents, such as enzymatic hydrolysis, intestinal bacteria for selective conversions to mimic biological conditions, Endophytic bacteria, Edible bacteria, and soil microbes, etc. Enzymatic conversions are considered safe, are commercially utilization, and are edible biotransformation mechanisms [6]. Thus, the food-grade fungus has gained its importance and is considered safe for successful selective transformations [7]. White ginseng needed energy for penetration, whereas the penetration abilities are higher for red, and the highest is for fermented black ginseng [8]. However, it is mainly due to the selectivity and bioactivity of the ginsenosides present in it. Bioconversion of ginseng significantly increases the number of minor ginsenosides and their biological activity from 0.30% up to 35%. Compound K is one of the most important ginsenosides, because it has a higher degree of biofunctional activity, and  $\beta$ -Glucosidase derived from *Aspergillus usamii* KCTC 6954 was applied for the bioconversion of ginsenoside Rb1 to compound K, which shows comparatively higher bio-activity. A very unique ginsenoside Rf was bio-converted into 20-protopanaxatriol (PPT(S)), through glycosidase from *Aspergillus niger* [9]. *Aspergillus niger* was isolated from fermented soybean, which was applied to decontaminate AFB1 (Aflatoxin B1) and cope with its toxic effects [10]. Considering the significant importance of *Aspergillus niger*, we isolated the strain from fermented soybean for the conversion of ginsenosides. *Aspergillus niger* belongs to the species *Aspergillus*, which was first reported almost three centuries ago and is almost as important as *Penicillium* and *Fusarium* species for human food and medicinal uses. *Aspergillus niger* is a very important mold for bioconversion of many medicinal and food materials [11]. Bioconversion, or biotransformation, is the process of converting the biological or organic materials and matters, i.e., plant and animal wastes, to useful and beneficial products, including green energy, foods, medicinal materials, enzymes, and microbes. The FDA declared *Aspergillus niger* as a GRAS (generally recognized as safe) fungi, and it is utilized to increase the yield for various biotransformations. The isolated strain from *Aspergillus niger* is named KHNT-1(National Center for Biotechnology Information(NCBI) accession number: MT804610), and the biotransformation was made due to the deglycosylation at C3, C6, and C20 positions. Herein we report biotransformation of ginsenosides, using KHNT-1 microbe isolated from fermented soybean with a shortened fermentation cycle. This microbe was isolated from the edible soybean, and it was isolated by extraction for the selective strain and amplified for PCR. The isolated strain from *Aspergillus niger* is named KHNT-1(NCBI Accession number: MT804610), and the biotransformation was made due to the deglycosylation at C3, C6, and C20 positions.

## 2. Materials and Methods

### 2.1. Plant Materials

About 5.0 kg of fresh ginsengs were collected from KeumSan, Korea, and fermented soybean (Meju) used for the isolated microbe was commercially procured from popular Korean traditional food.

## 2.2. Chemicals

All media were supplied from Difco, MB Cell (Gangnam-gu, Seoul, Korea). Methanol and butanol were supplied by Samchun Pure Chemical Co. Ltd. (Gyeonggi-do, Korea); PDA (Potato Dextrose Agar) and PDB (Potato dextrose broth) were received from Difco, MB cell (Gangnam-gu, Seoul, Korea). The human keratinocyte cell line (HaCaT) and B16BL6 (Murine melanoma) cell lines were collected from the Korean cell line bank (Seoul, Korea). RPMI 1640 culture medium (Gen DEPOT Inc., Barker, TX, USA) was used for B16BL6 grown with fetal bovine serum (10%) and penicillin (1%), at a temperature of 37 °C, in a humidified incubator with 5% CO<sub>2</sub> atmosphere. For normal cell line culture, Dulbecco's modified eagle's medium (DMEM) (Gibson- BRL, Grand Island, NY, USA) with 10% FBS (fetal bovine serum) 1% penicillin/Streptomycin (p/s), (WEIGENE Inc., Daegu, Korea) was used.

## 2.3. Phylogeny Construction

Based on the similarity searches in NCBI with the 16S rRNA sequence of the novel strain, the closest organisms were chosen for the phylogenetic analysis. The phylogenetic tree was constructed by using the maximum likelihood method, with MEGA X (Molecular Evolutionary Genetic Analysis, version 10.1). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Both PCR sequencing and amplification were analyzed by an automated DNA sequencing system (Applied Biosystems, Foster City, CA, USA) at Macrogen, Inc., Seoul, Korea. The obtained PCR product was initially sequenced and then subjected to a homology search by using an online tool, BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## 3. Isolation of KHNT-1

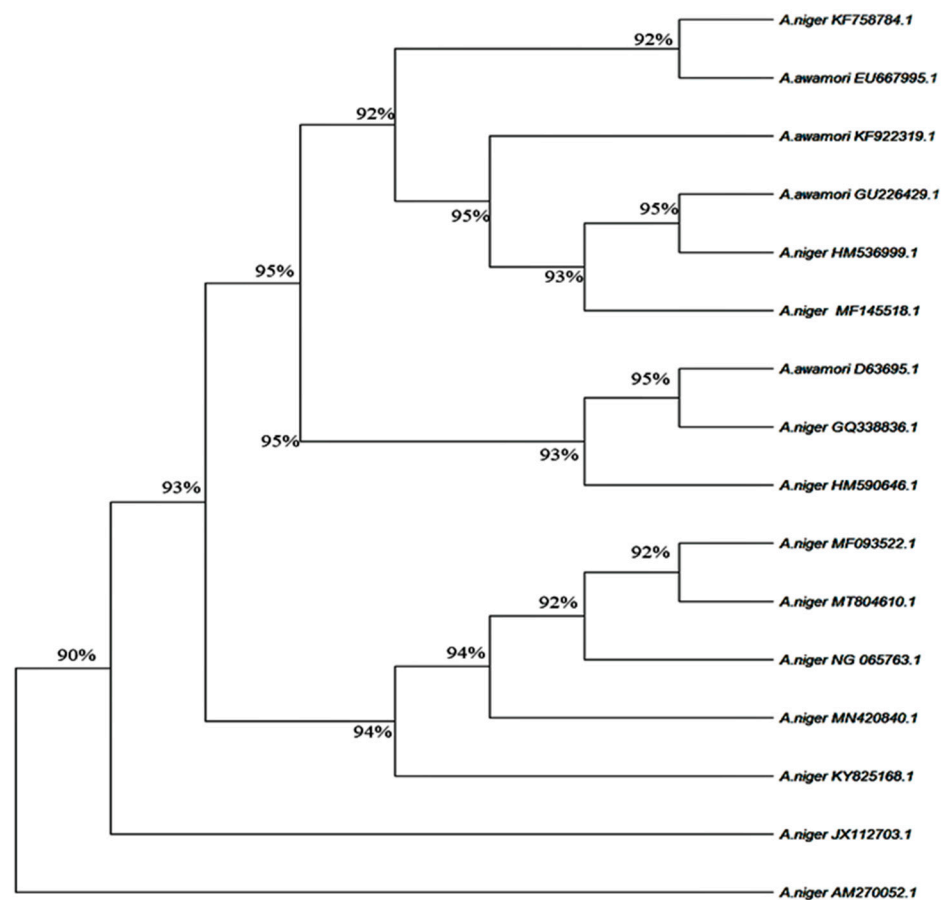
A fermented soybean powder was collected from the Republic of Korea. Aqueous extract of the dried fermented soybean was collected, using 10 mL of water. After serial dilution from the extract, 100 µL of the extract was incubated, using Potato Dextrose Agar (PDA) plates. These plates were carefully incubated for 2 days, at 30 °C; after incubation, the different colony morphologies were selected, and a single colony was further subcultured on fresh PDA, with the same conditions. The colonies were stored and mixed with PDB containing 30% (v/v) glycerol and maintained at −70 °C. The strain was deposited into the Korean Collection, for further sequencing, and the isolated novel strain was designated as KHNT-1. KHNT-1 corresponds with *Aspergillus niger*.

### 3.1. Sequencing and Naming

The selected strain was identified by amplifying the Internal transcribed spacer (ITS) region of 18 S rDNA with universal primers. The obtained strain is designated as *A. niger*\_MT804610.1.

### 3.2. Phylogenetic Tree Analysis

Figure 1 shows the phylogenetic relationship of the 16S ribosomal RNA gene, partial sequence (1693 bp) of the isolated strain, and other strains related to *A. niger*\_MT804610.1 in the GenBank database. The tree results showed that newly isolated species formed a sub-cluster with *A. niger*\_MF093522.1 with 92% bootstrap support. The same observation was made in the neighbor-joining tree method as well (data not shown).



**Figure 1.** Phylogenetic tree of *A. niger*.

### 3.3. Preparation of Ginseng Extract

Dried materials were solvent extracted as per the standard procedures and solvent evaporated to dryness, to obtain the extract containing active components.

#### 3.3.1. Characterization of the Isolated Ginsenosides

Fermented black ginseng was extracted and isolated as per the standard procedure. Thus, the obtained concentrate was confirmed for its identification by TLC, HPLC, and mass analysis, as per the below methods.

#### Identification by Thin-Layer Chromatography (TLC)

TLC was performed on silica gel 60 F254 plates.  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (65:35:10 *v/v/v*, high dense phase) was used as a mobile phase. The eluted plate was detected by spraying with 10%  $\text{H}_2\text{SO}_4$  and dried at an elevated temperature until the spots were visualized [12].

#### Detection by HPLC for Ginsenosides

Analysis of ginsenosides by high-performance liquid chromatography determined against the standard provided. An interesting observation was that the isolated extract contained various bioactives such as Rb1, Rg3, Rg5, Rk1, and CK. The HPLC separation was carried out on an Agilent 1260 series HPLC system (Palo Alto, CA, USA). This experiment employed a C18 (250 mm  $\times$  4.6 mm, ID 5 mm) column, using HPLC-grade water (Solvent A) and HPLC-grade acetonitrile (Solvent B) mobile phases, as well as a 5  $\mu\text{L}$  injection volume with a flow rate of 0.6 mL/min and the following gradient: A/B ratios of 81:19 for 0–10 min, 71:29 for 11–24 min, 60:40 for 25–27 min, 44:56 for 28–29 min, 30:70 for 30–31.4 min, 10:90 for 31.5–34 min, and 81:19 for 34.5–40 min, having column temperature of 45 deg. The sample was detected at a wavelength of 203 nm [13,14].

### Mass Analysis

Further confirmation of the isolated ginsenosides was further analyzed by using Mass spectrometry. Mass analysis was carried out on Waters TQ-D system with UPLC conditions having BEH C18 (2.1 × 100 mm, 1.7 μM), with the column temperature being 45 °C, using Mobile Phase A: 0/1% formic acid in water; Mobile Phase B: Acetonitrile with a flow rate of 0.3 mL/min. The following gradient was used: A/B ratios of 80:20 for 0–0.10 min, 68:32 for 2 min, 67–33 for 7 min, 48:52 for 20 min, 47:53 for 23 min; 20:80 for 26 min, 0:100 for 26.90 min; 80:20 for 27 min, and 80:20 for 30 min, with ESI (Electro spray ionization) positive and negative ionization mode, as shown in Table 1.

**Table 1.** LC (Liquid chromatography)/MS (Mass spectrometry) conditions.

Instrument	Waters Tq-D System		
Column	Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm)		
Column temp	45 °C		
Injection volume	2 μL		
Flow rate	0.3 mL/min		
Mobile phase A	0.1% Formic acid in water		
Mobile phase B	Acetonitrile		
Gradient program	Time	A(%)	B(%)
	Initial	80	20
	0.10	80	20
	2.00	68	32
	7.00	67	33
	20.00	48	52
	23.00	47	53
	26.00	20	80
	26.90	0	100
	27.00	80	20
30.00	80	20	
Ionization mode	ESI positive and negative		
Capillary voltage	3.3 kV		
Source temp	120 °C		
Desolvation temp	300 °C		
Gas flow	Desolvation 600 L/h, Cone 50 L/h		
Scan type	MRM (Multiple reaction monitoring) mode		
Collision gas flow	0.14 mL/min		

### 3.4. Cell Culture

#### Cell Viability Assay

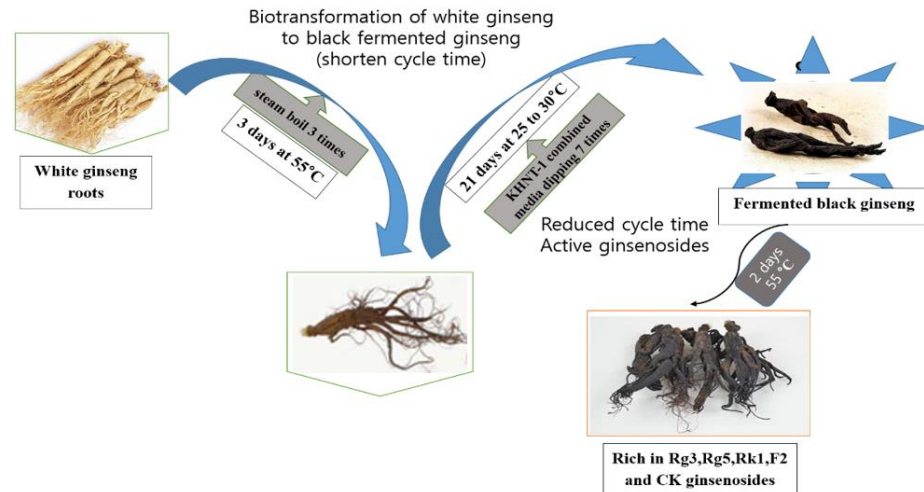
The seeding concentration for each used cell line, HaCaT, was an aneuploid keratinocyte cell line; B16BL6 was mouse melanoma cells was 1 × 10<sup>4</sup> cell/well. Totals six concentrations (Control, 100, 200, 250, 300, 350, 400 μg/mL) were set for both cell line and incubated for 24 and 48 h at 5% CO<sub>2</sub> atmosphere. After the treatment, the cell line was incubated further 37 °C with 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide solution (Life Technologies, Eugene, OR, USA about 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT (5 mg/mL, PBS) for incubation about 4 h at 37 °C conditions. Mostly the viable cell would convert the MTT solution to purple-colored

formazan. Further, this insoluble formazan was dissolved by DMSO with 100  $\mu$ L each well, and reading was taken at 570 nm with an Enzyme-Linked Immunosorbent Assay (ELISA) reader (Bio-Tek, Instruments, Inc., Winooski, VT, USA).

#### 4. Results and Discussions

##### 4.1. Conversions of White Ginseng to Fermented Black-Colored Ginseng

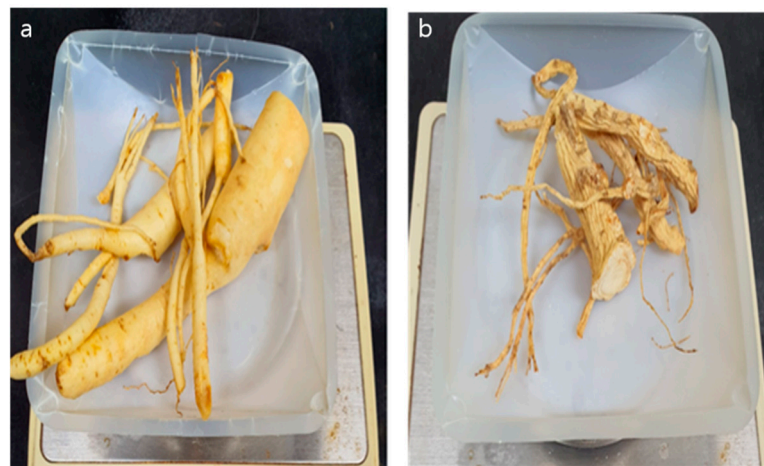
A schematic illustration of the enzymatic biotransformation was created, using the novel *Aspergillus niger* KHNT-1 (Figure 2).



**Figure 2.** Illustration of biotransformation process.

##### Pre-Drying of White Ginseng

White ginseng obtained from the source was dried at 55 deg, for three days, to remove the moisture. The weight was almost reduced to 1/3 from the original, after drying (Figure 3a,b). During this preliminary drying process, we did not observe a change in the color.



**Figure 3.** White ginseng preliminary process: (a) before drying and (b) after drying.

##### 4.2. Fermentation of Ginseng

The process of converting the white ginseng to black ginseng requires a steam process, followed by a fermentation process, to convert to fermented black ginseng [15,16]. We performed steaming about three times, for 1 h, and the drying process was carried out before performing the fermentation process seven times, with 1 min dipping in the culture media and drying. During the fermentation, the steam-dried ginseng was dipped into the *A. niger*

containing PDB media at equal intervals over, 25 days, at 25 to 30 deg. During dipping, the dried white ginseng slowly turned black over a period. The visual transformation is represented below (Figure 4a (1–10)). This process confirms the conversion of white ginseng to black-colored ginseng with a normal treatment process without any harsh conditions. To understand the efficacy of the black-colored fermented ginseng, we further checked the conversion at the lateral part and main part of each ginseng, and, interestingly, the conversion was uniformed throughout the sample (Figure 4b).



**Figure 4.** (a) Transformation of fermentation process. (b) Main and lateral part after transformation.

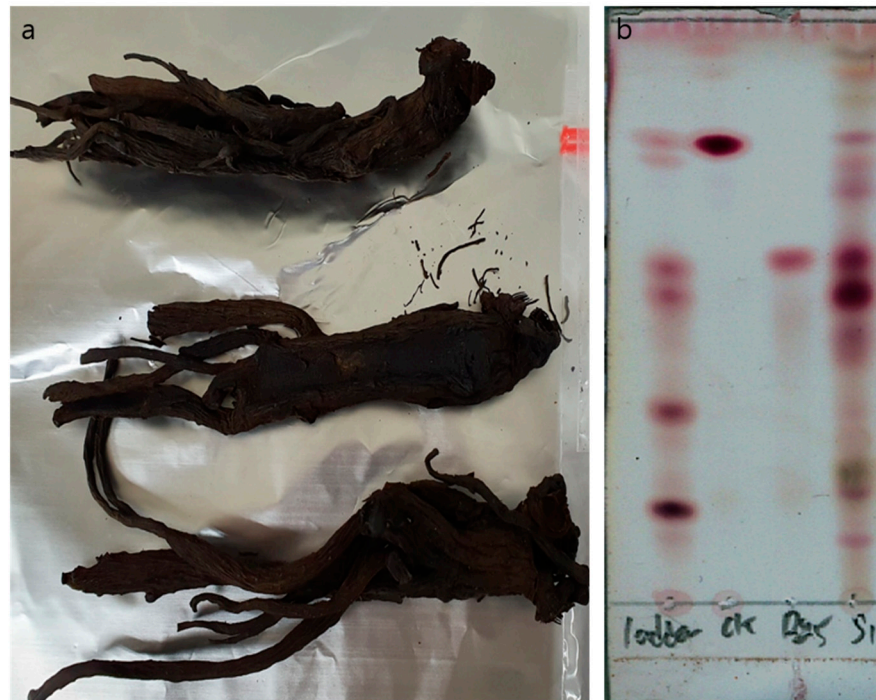
#### 4.3. Extraction of Ginsenosides

A total of 1.0 g of dried parts of ginseng was treated, separately, to compare the active components present in it for high bioactivity. The dried materials were soaked in an 80% (*v/v*) methanol solution, in water, at 70 °C, for 1 h, and the extracts were separated by filtration and further by centrifugation. This process was repeated twice, for better recovery, and then the extracted solvents were combined and evaporated to almost dryness. Then, ~300 mg of crude product was obtained. The optimization of the extraction process with solvent screening was not performed for this report, as much attention was focused on the interest in the identification of actives, due to the biotransformation process using the novel strain. The residue was further dissolved in distilled water and extracted, using water-saturated n-butanol. The butanol layer was then collected separately and evaporated, to obtain saponin-rich fractions. Thus obtained saponin fractions were characterized by TLC, HPLC, and mass, for further characterization of the individual ginsenosides contents for further identification [5].

#### 4.4. Identification by Thin-Layer Chromatography

The fermented black ginseng (Figure 5a) extracts of the butanol fraction were further identified by using TLC. TLC was performed on silica gel 60, using  $\text{CHCl}_3$ -MeOH- $\text{H}_2\text{O}$  (65:35:10 *v/v/v*, lower phase) as a mobile phase. The eluted plate was detected by spraying 10%  $\text{H}_2\text{SO}_4$  and dried at an elevated temperature, until the spots were visualized (Figure 5b). The eluted fractions confirmed the presence of ginsenosides [17].





**Figure 5.** (a) Dried fermented black ginseng. (b) Ginsenosides confirmation by TLC.

#### 4.5. Identification of the Components by HPLC

To further quantify and confirm the components, HPLC was performed with standards. HPLC represents clear evidence for the different components. The extracted butanol residue was further characterized by using HPLC with a standard condition and methods. The eluted samples were compared with the standard ginsenosides (Figure 6). Each sample was dissolved in HPLC-grade methanol (1 g/5 mL), filtered using a 0.45  $\mu\text{m}$  filter, and then used for high-performance liquid chromatography (HPLC) analysis. Based on the HPLC chromatograms the fractions from both the lateral (Figure 7a) and the main part (Figure 7b), they are very similar, and it suggests the uniformity of the biotransformation using KHNT-1. For making more quantification purposes, we decided to combine both the extracts and continue for further characterizations and efficacy understanding.

To understand the actual contents of the ginsenosides, we performed LC–mass spectrometer for the residue, to confirm the exact actives present after biotransformation and quantify the actives isolated after the biotransformation process.

#### 4.6. Identification by Mass Spectrometer

The isolated samples were spot-detected by TLC and further confirmed by HPLC, using the reference standards with standard methods. Interestingly, the present method shows the co-elution of Rg5 and CK with the same retention time. However, there is a distinct spot in the standard TLC method. To evaluate the importance of each component, LC–MS was performed as per the below-mentioned conditions. The mass and retention factors with standards clarify each fraction of Rg5, Rg3, Rk1, CK, and F2 (Figure 8). The below data confirm the individual mass, as in the table below (Table 2). Interestingly, *A. niger* KHNT-1 is able to transform the selective ginsenosides to the most active ginsenosides, like F2, Rg3, CK, and Rg5 [11], and was confirmed by mass spectrometric method with reference standards retention times.

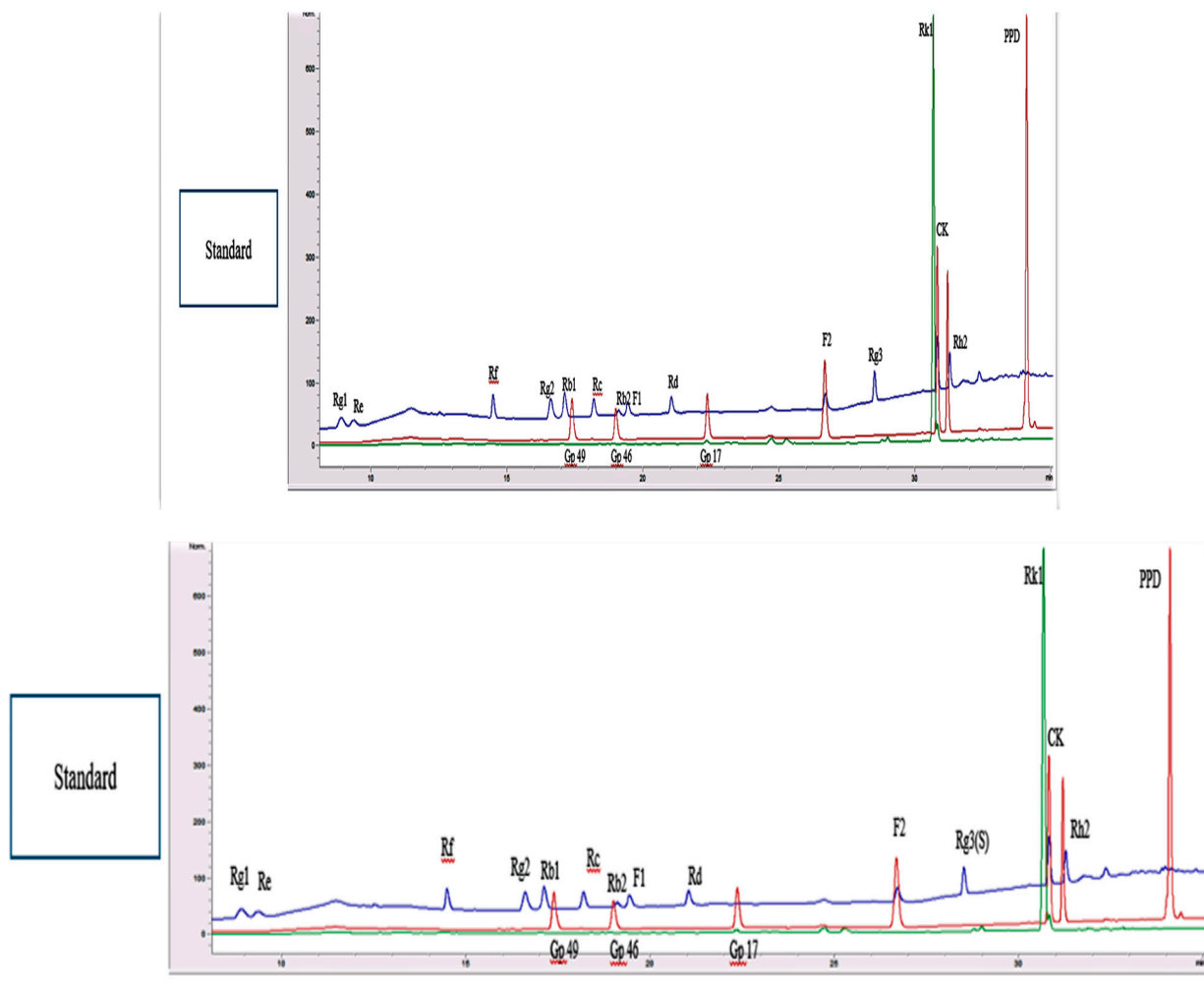


Figure 6. HPLC analysis of ginsenosides.

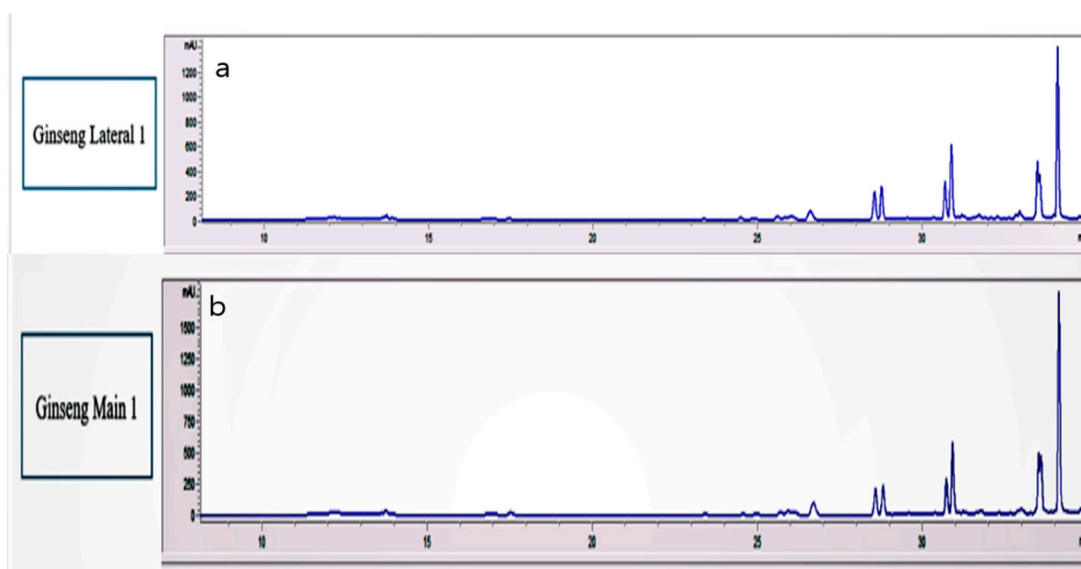


Figure 7. HPLC chromatograms of extract: (a) lateral part and (b) main part.

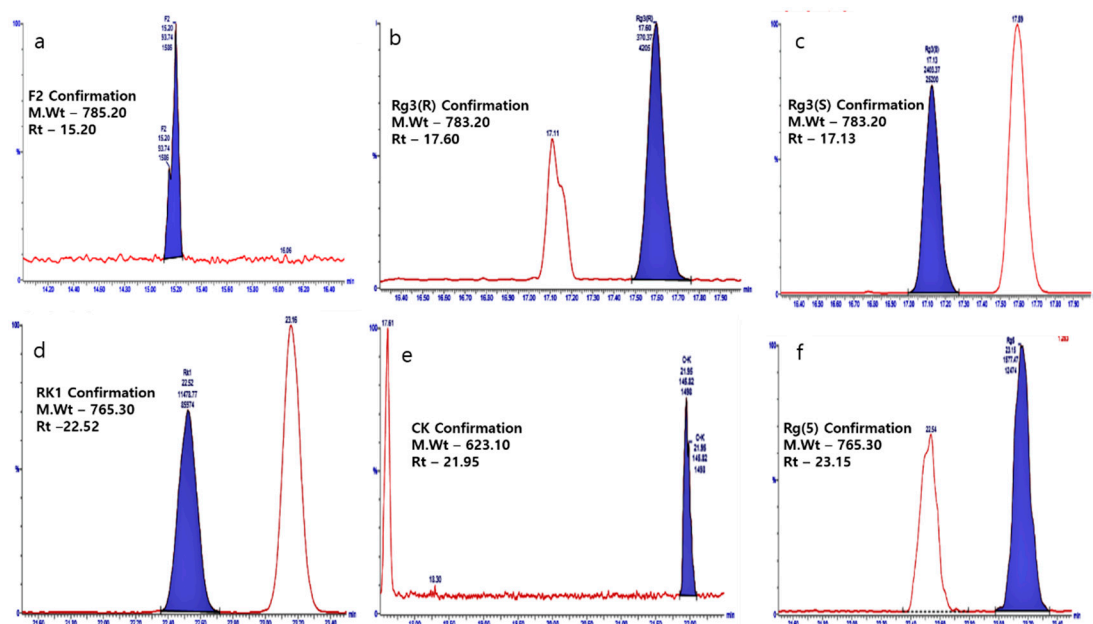


Figure 8. Mass analysis of (a) F2, (b) Rg3, (c) Rg3(S), (d) Rk1, (e) CK1, and (f) Rg5.

Table 2. Retention time and molecular weight.

Compound	Molecular Weight	Retention Time (lcms) in Min
F2	785.20	15.20
Rg3(R)	783.20	17.13
Rg3(S)	783.20	17.60
RK1	765.30	22.52
CK	623.10	21.95
Rg5	765.30	23.15

The identification of mass analysis confirms each isolated active from the fermented black-colored ginseng. Interestingly, by combing the results, we confirm the actives have more of Rg3, Rg5, RK1, and CK. These interesting results encouraged us to check the content of each activity, and the results match our identification results. The individual contents are listed in the table below (Table 3).

Table 3. Quantification details of the extracted ginsenoside.

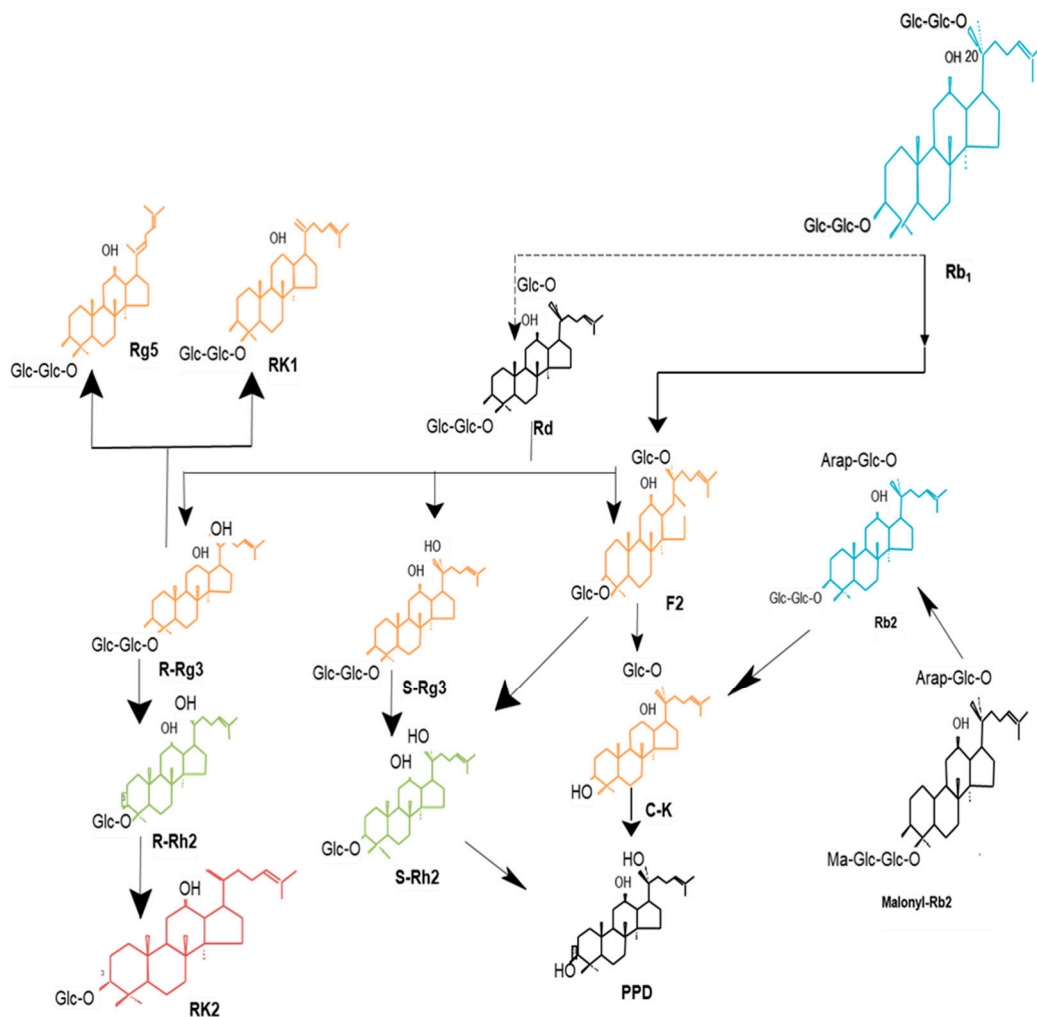
Compound	Fermented Black Ginseng Extract
	Content mg/kg
F2	63.23
Rg3(S)	11,171.21
Rg3(R)	8754.86
CK	38.13
RK1	9193.77
Rg5	15,217.90

Recent research and reports and three repetitions of the steaming process convert white ginseng to red ginseng, which eventually converts Rg3, Rg5, and Rg1; and during the continuation of the process, Rg1 converts to Rg3, which further transforms to Rg5 and Rk1 [5,18,19]. The degree of bioactivity of Rg3 has increased efficiency during the steaming process. During our fermentation process, the white ginseng after the initial steam treatment conducted three times, and upon fermentation process, the color of the ginseng turned to black, and the contents remained intact. Based on the above test results, Rg3, Rg5,

and Rk1 were abundant, notably, along with CK. Generally, the CK will be formed during the biotransformation of the ginsenosides [20]. However, interestingly, our new fermented ginseng has a significant amount of CK, along with very active ginsenosides like Rg3, Rk1, and Rg5. This process enables us to perform selective biotransformation. This biotransformation influences the better penetration ability to reach the nucleus for cell metabolism [21]. This influences the cell penetration for high bioavailability to reach the site. This process enables us to reach the target site for more bioactive ginsenosides, without affecting its high active ingredients. This way, the terminal glucose presented at C-20 of Rb1 was slowly hydrolyzed by the influence of KHNT-1 strain. It confirms the presence of the newly identified strain which ably transfers the bio-enzymatic pathway for the conversion of ginsenosides by selectively hydrolyzing the glycon moiety at different sites. Therefore, metabolite 2 was identified as 3-O-[ $\beta$ -D-glucopyranosyl]-20-O-[ $\beta$ -D-glucopyranosyl]-20(S)protopanaxadiol, also known as ginsenoside F2. Therefore, the glucose connected at the C-3 site of the aglycon of metabolite 2 was hydrolyzed, further indicating that the glucopyranosyl moiety of metabolite 3 was connected to the C-20 site of aglycon [22,23]. Therefore, metabolite 3 was 20-O-[ $\beta$ -glucopyranosyl]-20(S)-protopanaxadiol, also known as compound K.

#### 4.7. Bioconversion Pathway

The process of hydrolysis of ginsenoside Rb1 was catalyzed by strain KHNT-1 and analyzed by using TLC, HPLC, and mass analysis. These changes were interestingly followed by the earlier reported process and thereby confirm that this newly isolated KHNT-1 is effective for this enzymatic conversion. As represented by HPLC and TLC, the concentration of Rb1 was reduced and transformed into CK and F2. However, the kinetics of the conversions were not investigated, as we were more focused on the color conversion rate. This biotransformation coincides well with the earlier findings of the following intermediate of F2 [24]. This biotransformation suggests the KHNT-1 strain has excellent potential for hydrolysis of  $\beta$ -glucosidase activity; this hydrolysis mostly occurs at 20-C,  $\beta$ -(1  $\rightarrow$  6) glucoside, 3 C, $\beta$ -(1  $\rightarrow$  2) glucoside, and 3-C,  $\beta$ -glucose of ginsenoside Rb1 [25]. Figure 9 represents the typical bioconversion pathway. In this investigation, we majorly focused on the biotransformation of white ginseng to fermented black-colored ginseng for the efficacy understanding. In this study, we primarily focused on the efficacy understanding of the fermented black-colored ginseng from white ginseng, using the new isolated KHNT-1 strain. For more efficacy improvement, we are in the process of investigating further biotransformation concerning temperature, as the enzyme tends to lose its energy-release property and denaturation at higher temperatures [26]. For initial screening, we performed at the normal temperature of initial observations. For the initial conversion and understanding, we carried out at a normal temperature and pH of 6.0–6.5 for understanding the activity of the material biotransformation. This research provides scope for future advancements to optimize for pH, temperature, and enzymatic activities. Overall, we are more focused on the biotransformation and the activity of the components. Due to the time limits, we could not investigate the transformation kinetics and other intermediate details further. The ginsenoside compound K is a promising natural product that could be used for the treatment of numerous human pathologies. Herein we have achieved the transformation of the ginsenosides involving multiple active components, like Rg3, Rg5, and CK. In this study, it was concluded that *Aspergillus niger* KHNT-1 efficiently transforms the ginsenoside to the most active components.



**Figure 9.** Bioconversion pathway of ginsenosides in the fermented black ginseng.

#### 4.8. *In Vitro* Cytotoxicity/Cell Viability Assays

In this study, normal cells (HaCaT) and B16BL6 (Murine melanoma) cells were treated with samples isolated from lateral root and main root in different concentrations (Control, 100, 150, 200, 250, 300, 350, and 400  $\mu\text{g}/\text{mL}$ ) for 24 and 48 h. B16BL6 cells are useful models for the study of metastasis and solid tumor formation; hence, cytotoxicity analysis was determined by using MTT assay [27]. Due to the presence of similar active ginsenosides, both Ginseng Main root (GMR) and Ginseng Lateral root (GLR) showed almost the same cytotoxic effects on the B16BL6 cell line (Figure 10) and showed little difference in the normal HaCaT cell line (Figure 11), in a dose-dependent manner, up to 48 h. Both GLR and GMR isolated samples significantly induce cell toxicity due to their penetration ability and targetability. The HaCaT cell line was found to be safe up to the concentration of 200  $\mu\text{g}/\text{mL}$  after 24 and 48 h treatment for GMR, and it is completely safe up to 400  $\mu\text{g}/\text{mL}$ . However, interestingly, the B16BL6 cell line showed the effect of this action from 150  $\mu\text{g}/\text{mL}$ . Both GLR and GMR showed similar behavior. This ginsenoside content and the preliminary efficacy property have evidenced that the biotransformation has uniformly occurred throughout the root during the bioconversion process. Though we anticipated the difference in the property behavior at two different sites, due to the localization of the ginsenosides, the results from the visual observation and characterization of the isolated ginsenosides were coinciding at two different parts. These cytotoxicity results further confirm that biotransformation behavior is uniform, even concerning the presence of the active ginsenosides for their efficacy behavior.

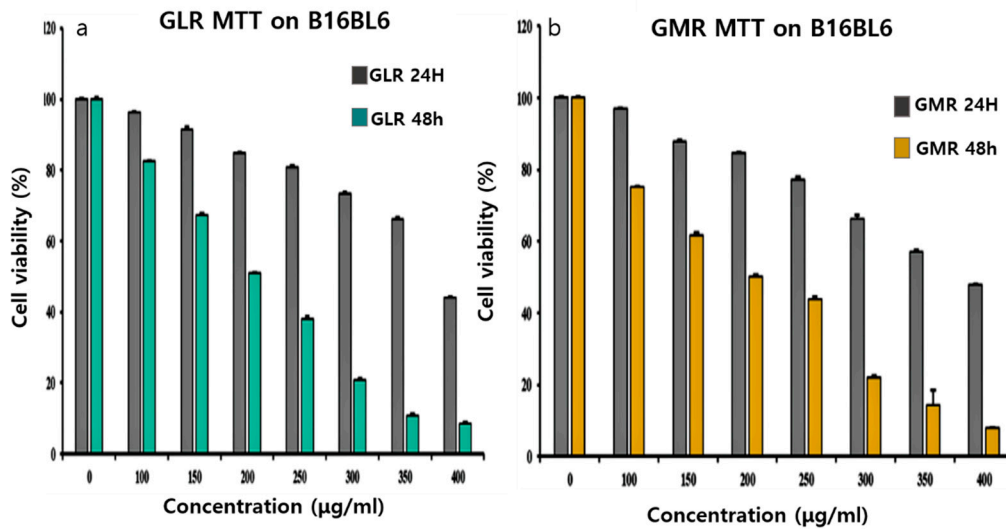


Figure 10. B16BL6 cytotoxicity at 24 and 48 h (a) Ginseng lateral root (GLR) toxicity and (b) Ginseng main root (GMR) toxicity.

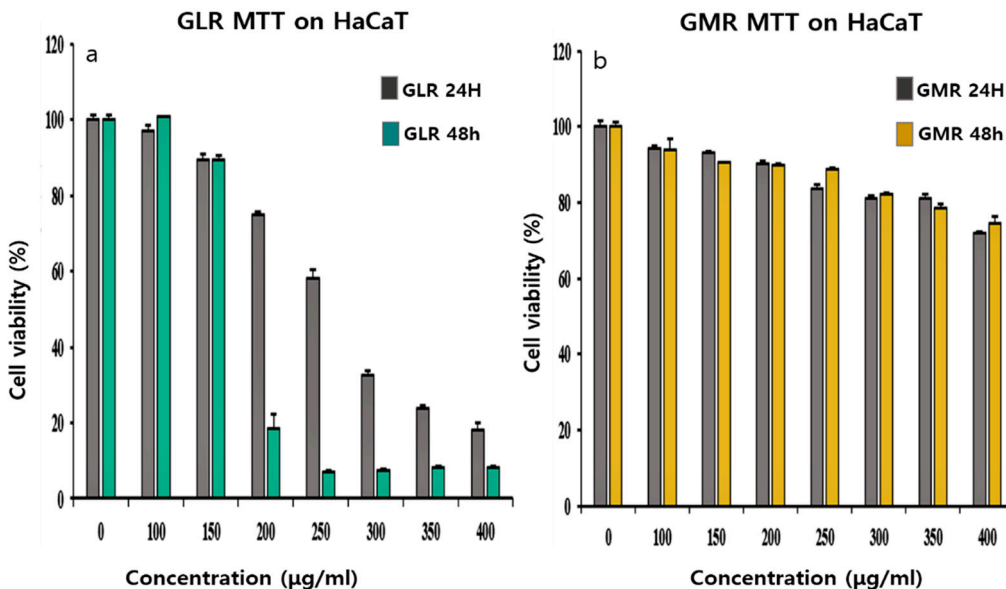


Figure 11. HaCaT cytotoxicity at 24 and 48 h (a) GLR toxicity and (b) GMR toxicity.

## 5. Conclusions

The enzymatic biotransformation of minor ginsenosides plays a critical role in any conversion with a precise and repeatable process. The utilization of this process is to speed up the conventional natural transformation for increased productivity. This study confirms that the process is considered consistently safe and hence found its uniqueness in industrial applications. Newly isolated strain *Aspergillus niger* KHNT-1 (NCBI Accession number: MT804610) effectively converts into minor ginsenosides. Various black ginseng have Rg5 and RK1 as a major component, and fermented black ginseng is known for having CK as a chief component in it. However, the newly isolated strain ably produces Rg3, Rg5, and CK, having a chief component with a shorter time of seven times steam-dry, instead of nine times steaming, without any other harsh conditions. With various techniques like TLC, HPLC, and mass analysis, the quantification and identification of the actives of each ginsenoside of fermented black-colored ginseng extract authenticate the presence of Rg3, Rg5, RK1, and CK. This conversion obtained at a shorter time has provided increased efficiency and utilized a new method for converting the minor ginsenosides.

These compositions of ginsenosides from lateral to main part possess a homogenetic accumulation of the bio-actives. The unique composition obtained during this process with Rk1, Rg3, Rg5, and CK are nontoxic to HaCaT cell line up to 200 ug/mL for 24 h and found effective in B16BL6 cell lines in a dose- and time-dependent manner. This safe bioactive containing extract provides a way to investigate the kinetics of the bioconversion pathway and thereby evaluate the increase in ginsenosides. This unique biotransformation provides scientific advancements and receives scientific attention for new functional nutraceutical, cosmeceutical, and pharmaceutical applications like antitumor, immunomodulatory or genoprotective, and other pandemic diseases.

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