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Original Article

BIOTRANSFORMATION OF DEHYDROABIETIC ACID WITH MICROBIAL CELL CULTURES AND α-GLUCOSIDASE INHIBITORY ACTIVITY OF RESULTING METABOLITES

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ABSTRACT

Dehydroabietic acid (DHA, **1**), a natural occurring diterpene resin acid, is an abundant resin acid in conifers, representing a natural wood protectant. The aim of this study was to use microbial cell cultures as tools for modification of **1** in order to obtain value-added functional derivatives. A scaled-up biotransformation of **1** by filamentous fungus *Cunninghamella elegans*, *Rhizopus stolonifer*, *Gibberella fujikuroi*, and *Cephalosporium aphidicola* were conducted for the first time. Three hydroxylated metabolites; 1β-hydroxydehydroabietic acid (**2**); 15-hydroxy dehydroabietic acid (**3**); and 16-hydroxy dehydroabietic acid (**4**). The structure of the hydroxylated metabolites were elucidated by 1-D (^{1}H, ^{13}C) and 2-D NMR (COSY, HMBC, HMQC, NOESY) techniques and MS analyses. Dehydroabietic acid (**1**) and their transformed products **2**-**4** exhibited a promising α-Glucosidase inhibitory activity. Compound **1** showed 38 times more active than the standard α-Glucosidase inhibitor, deoxynojirimycin. Compound **1** and its transformed metabolites **2**-**4** also showed significant antibacterial activities.

Keywords: Microbial transformation, Dehydroabietic acid, Antibacterial activities, α-Glucosidase.

INTRODUCTION

Dehydroabietic acid (Abieta-8,11,13-trien-18-oic acid, **1**), a natural occurring diterpenic resin acid, is an abundant resin acid in conifers, representing a natural wood protectant. It is also one of the constituents found in byproducts of the kraft chem. pulping industry. Dehydroabietic acid (DHA, **1**) and its derivatives has been found to have properties of enhancing the inhibition activity of anticancer drug in various cells, such as hepatocellular carcinoma cells, cervical carcinoma cells, and breast cancer cells. It has been shown that DHA treatment decreases plasma glucose and insulin levels with plasma triglyceride (TG) and hepatic TG levels. DHA is also a useful food-derived compound for treating obesity-related diseases [1-6].

Microbial transformation has been useful tool for *in vitro* drug metabolism studies [7-8]. The advantages of using microorganisms such as bacteria and fungi for the production of metabolites are that the cultures are relatively easy to maintain and grow, and that scale-up to produce milligram or gram amounts is readily accomplished. In addition, a variety of metabolites could be obtained by microbial transformations, from which more active metabolites might be found. In recent years, many research works frequently reported to modify the structures of natural products for finding some novel biotransformed reactions, or some new chemical entities with the proper drugability [8-33]. Moreover, microorganisms such as fungi and bacteria can provide better yields of the metabolites when compared with *in vivo* metabolism studies, producing enough material for toxicological and biological assays. Fungi of the genus *Cunninghamella* stand out amongst other genera because can catalyze reactions mimicking the mammalian metabolism. *Cunninghamella* species are capable of metabolizing a wide variety of xenobiotics using both phase I and phase II reactions, oftentimes giving rise to new compounds [8-23]. The microbial-oriented oxidation of terpene under mild conditions appears as an attractive alternative as compared to the traditional chemical methods, have an elevated regio- and enantioselectivity, and do not generate toxic waste products, and the products obtained can be labeled as "natural"source [24-33].

In present work, biotransformation of Dehydroabietic acid (**1**) by filamentous fungus *Cunninghamella elegans* TSY 0865, *Rhizopus stolonifer* ATCC-10404, *Gibberella fujikuroi* ATCC-10704 and *Cephalosporium aphidicola* IMI-68689 were carried out for the first time (Scheme 1). Three hydroxylated transformed products were isolated and identified in the biotransformation process of **1**. In addition, the α-glucosidase inhibitory activity and antibacterial activities of metabolites **2**-**4** were evaluated (Table 1 and 2).

MATERIALS AND METHODS

General

Dehydroabietic acid (**1**) was purchased from sigma-aldrich (USA). Melting points were determined on a Yanaco MP-S3 apparatus. UV spectra were measured on a Shimadzu UV 240 spectrophotometer. JASCO DIP–360 Digital polarimeter was used to measure the optical rotations in chloroform by using 10 cm cell tube. FTIR-8900 Spectrophotometer was used to record IR spectra in $CHCl_3$. The ^{1}H-NMR and 2D NMR spectra were recorded on a Bruker Avance III 500 spectrometer, while ^{13}C-NMR spectra were recorded on Bruker Avance III 500 spectrometer operating at 125 MHz using $CDCl_3$ as solvent. chemical shifts were reported in δ (ppm), relative to $SiMe_4$ as internal standard, and coupling constants (J) were measured in Hz. The HREI MS were measured on Jeol HX 110 mass spectrometer. TLC was performed on Si gel precoated plates (PF_{254}, 20 × 20, 0.25 mm, Merck, Germany). Ceric sulphate in 10% H_2SO_4 spraying reagent was used for the staining of compounds on TLC. All reagents used were of analytical grades.

Fungi and culture conditions

Microbial cultures of the *Cunninghamella elegans* TSY 0865 was grown on Potato dextrose agar (PDA) at 25□ C and stored at 4□C. *Cunninghamella elegans* TSY 0865 medium was prepared by adding Glucose (10.0 g), peptone (5.0 g), KH_2PO_4 (5.0 g), yeast extract (5.0 g), NaCl (5.0 g) and glycerol (10 mL) into distilled water (1 L) and maintained pH at 5.6.

Microbial cultures of the *Rhizopus stolonifer* ATCC-10404 was grown on Potato dextrose agar (PDA) at 25□ C and stored at 4□C. Medium

for *Rhizopus stolonifer* was prepared by mixing Glucose (20.0 g), Peptone (5.0 g), Yeast extract (3.0 g) and KH_2PO_4 (5.0 g) into distilled water (1 L) and maintained pH at 5.6.

Microbial cultures of the *Gibberella fujikuroi* ATCC-10704 was grown on Potato dextrose agar (PDA) at 25□ C and stored at 4□C. Medium for *Gibberella fujikuroi* was prepared by mixing Glucose (80.0 g), NH_4NO_3 (1.0 g), KH_2PO_4 (5.0 g), $MgSO_4.7H_2O$ (1.0 g) and *Gibberella trace* element solution (2.0 mL) into distilled water (1 L) and maintained pH at 5.6.

Microbial cultures of the *Cephalosporium aphidicola* IMI-68689 was grown on Potato dextrose agar (PDA) at 25□C and stored at 4□ C. Medium for *Cephalosporium aphidicola* was prepared by mixing Glucose (50.0 g), KH_2PO_4 (1.0 g), $MgSO_4.7H_2O$ (2.0 g), Glycin (2.0 g), KCl (1.0 g) and *Gibberella trace* element solution (2.0 mL) into distilled water (1 L) and maintained pH at 5.6.

general stage ii fermentation and extraction procedure

The fungal media were transferred into 250 mL conical flasks (100 mL each) and autoclaved at 120□ C. Seed flasks were prepared from three-day old slant and fermentation was allowed for two days on a shaker at 25□ C. The remaining flasks were inoculated from seed flasks. After one days, Dehydroabietic acid (**1**) (1 g) was dissolved in 20 mL of acetone and transferred in each flask and the clear solution was evenly distributed among the 40 flasks, containing 24-h-old stage-II cultures. Fermentation was carried out for 12 days on a rotatory shaker (200 rpm) at 25° C. For TLC analysis, the content of one flask each was harvested every second day. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (1.5 L), and the filtrate was extracted with CH_2Cl_2 (12 L). The resulting brown gummy extracts (1.48, 1.62, 1.49, and 1.51 g for *R. stolonifer*, *C. elegans*, *G. fujikuroi* and *C.aphidicola,* respectively) were purified by repeated column chromatography (petroleum ether/EtOAc gradients). From these extracts, following compounds were isolated: **2** (8.1 mg); PE/ EtOAc (81:19), **3** (11.3 mg); PE/ EtOAc (82:18), and **4** (13.8 mg); PE/ EtOAc (85:15).

1β-hydroxy dehydroabietic acid (2):

amorphous material (8.1 mg); $[\alpha]^{25}{}_{D:}$ +14° (*c* = 0.24, $CHCl_3$); R_f: 0.5 (Pet. Ether/EtOAc 82:18); EI-MS *m/z* (rel. int., %): *m/z* 316 [M^+] (24), 302 (59), 218 (21), 170 (14), 154 (67), 86 (32), 70 (61), 43 (100); HREI-MS (mol. formula, calcd value): *m/z* 316.1528 ($C_{20}H_{28}O_3$, 316.1536); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: See [24]; ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: See [25].

15-hydroxydehydroabietic acid (3):

white solid (11.3 mg); $[\alpha]^{25}{}_{D:}$ +31° (*c* = 0.24, $CHCl_3$); R_f: 0.5 (Pet. Ether/EtOAc 82:18); EI-MS *m/z* (rel. int., %): *m/z* 316 [M^+] (62), 302 (12), 218 (61), 170 (38), 154 (52), 86 (62), 70 (48), 43 (100); HREI-MS (mol. formula, calcd value): *m/z* 316.1479 ($C_{20}H_{28}O_3$, 316.1492); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: See [25], ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: See [26].

16-hydroxydehydroabietic acid (4):

amorphous material (13.8 mg); $[\alpha]^{25}{}_{D:}$ +68° (*c* = 0.24, MeOH); R_f: 0.5 (Pet. Ether/EtOAc 82:18); EI-MS *m/z* (rel. int., %): *m/z* 316 [M^+] (62), 302 (19), 218 (51), 170 (19), 154 (37), 86 (52), 70 (100); HREI-MS (mol. formula, calcd value): *m/z* 316.1528 ($C_{20}H_{28}O_3$, 316.1536); ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: See [26]; ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: See [27].

α-Glucosidase enzyme inhibition assay

α-Glucosidase (E.C.3.2.1.20) enzyme inhibition assay has been performed according to the slightly modified method of Matsui *et al*. α-Glucosidase (E.C.3.2.1.20) from *Saccharomyces* sp. Was purchased from Wako Pure Chemical Industries Ltd. (Wako 076-02841). The inhibition was measured spectrophotometrically at pH 6.9 and at 37° C using 0.7mM p-nitrophenyl α-D glucopyranoside (PNP-G) as a substrate and 250 m units/mL enzyme in 50mM sodium phosphate buffer containing 100mM NaCl. 1- Deoxynojirimycin (0.425mM) and acarbose (0.78mM) were used as positive controls. The increment in absorption at 400nm due to the hydrolysis of PNP-G by α-glucosidase was monitored continuously with the spectrophotometer (Molecular Devices, CA, USA) [15].

Estimation of IC_{50} values

The concentrations of the test compounds, which inhibited the hydrolysis of PNP-G by α-glucosidase by 50% (IC_{50}), were determined by monitoring the effect of increasing the concentration of these compounds on the inhibition values. The IC_{50} values were then calculated by using EZ-Fit enzyme kinetics program (Perrella Scientific Inc., Amherst, MA, USA) [15].

Zone of inhibition assay

Test sample (3 mg) was taken and dissolved in 3 mL of DMSO (1 mg/mL). Molten nutrient agar (45 mL) was poured on the sterile petri plates and was allowed to solidify. Bacterial lawn was made on these nutrient agar plates by dispensing 7 mL sterile soft agar containing 100 mL of cultures of the test organisms. Wells were dugged with the help of 6 mm sterile metallic borer at appropriate distances, then 100 μL of sample was poured into each well and the plates were incubated at 37º C for 24 hours. The results in terms of zones of inhibition were recorded. Imiperium was used as a positive control, while DMSO was used as a negative control. All the chemicals used were of analytical grades (Sigma, USA) [20].

RESULTS AND DISCUSSION

Fermentation of dehydroabietic acid (**1**) for 12 days with *Cunninghamella elegans* TSY 0865, *Rhizopus stolonifer* ATCC-10404, *Gibberella fujikuroi* ATCC-10704 and *Cephalosporium aphidicola* IMI-68689 resulted three regio- and stereoselective hyroxyl products, **2**-**4** (Scheme 1). The HREI MS of metabolite **2** exhibited the molecular ion (M^+) at *m/z* 316.1536, corresponding to the formula $C_{20}H_{28}O_3$, which indicated that a new oxygen functionality was introduced during fermentation period. Metabolite **2** was found to be 1β-hydroxy derivative of **1**, based on ^{1}H and ^{13}C NMR signals at δ_H 3.92/δ_C 76.8. Hydroxylation at C-1 position was further supported by COSY and HMBC interactions. The β-orientation of C-1 OH group was deduced on the basis of NOESY correlations between H-1 (δ 3.92) and H-5 (δ 2.20). The structure of known metabolite **2** (1β-hydroxydehydroabietic acid) was further deduced by comparison with the reported data (Gouiric *et al*., 2004). Metabolite **2** was previously obtained by the fermentation of **1** with *Fusarium oxysporum, F. moniliforme* and *Aspergillus niger* [25].

Metabolite **3** was obtained as a white powder (Scheme 1). Its molecular formula was determined as $C_{20}H_{28}O_3$ according to the HREI MS data (*m/z* 316.1479: calcd for $C_{20}H_{28}O_3$, 316.1492), indicated that one oxygen had been incorporated into the molecule, as compared to **1**. Metabolite **3** was found to be 15-hydroxy derivative of **1**, based on ^{13}C NMR signals at δ 72.3. The position of the newly introduced hydroxyl at C-15 position was inferred from the HMBC coupling of C-16 and C-17 methyl protons with C-15 (δ 72.3). The structure of known metabolite **3** (15-hydroxydehydroabietic acid) was further deduced by comparison with the reported data [26].

Metabolite **4** was obtained as an amorphous material (Scheme 1). The HREI MS of metabolite **3** exhibited the molecular ion (M^+) at *m/z* 316.1528, corresponding to the formula $C_{20}H_{28}O_3$, indicating an introduction of a new oxygen in the molecule, probably as OH during fermentation period. Compound **4** was found to be C-16 hydroxy derivative of **1**, based on NMR signals at δ_H 3.64/δ_C 68.7 and further confirm by comparison with the reported data [27]. The position of the newly introduced hydroxyl at C-16 position was inferred from the HMBC coupling of H-15 and C-17 methyl protons with C-16 (δ 68.7).

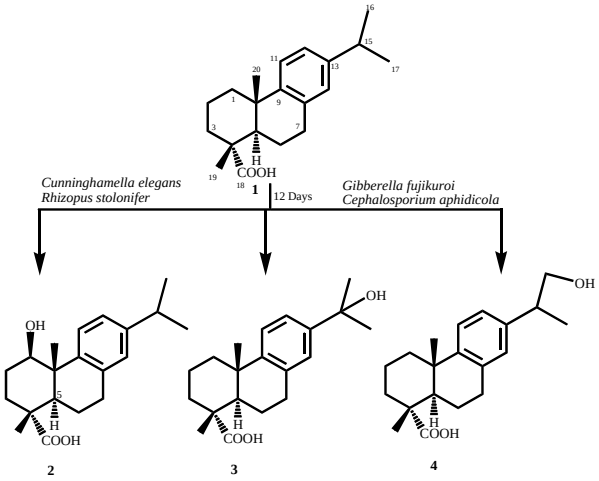
Dehydroabietic acid (**1**) and its transformed products **2**-**4** were subjected to α-glucosidase Inhibitory Activity. According to the results, the dehydroabietic acid (**1**) showed a promising inhibitory effect, which is 38 times more than the standard deoxynojirimycin. The activity of **2** was also found to be much better than the transformed products **2**-**4** (Table 1). This may be due to binding of substrate **2** with the enzyme binding site through hydrophobic

interactions. Introduction of hydroxyl groups through transformation such as in metabolites **2**-**4**, increase the hydrophilicity, which may cause formation of the hydrogen bonding with surrounding buffer system, thus the hydrophobic interactions with the binding site is distorted, thus a decrease in the overall α-glucosidase inhibitory activities was observed. Among the transformed products, compound **4** showing more inhibitory activity than the compound **2** probably due to hydroxylation at isopropyl side chain causing less steric hindrance, which help in the binding with the enzyme.

Table 1: α-Glucosidase Inhibitory Activity of Compounds 1-4

Compound	IC_{50} (μM ± S.E.M)
1	11±01
2	130±15
3	99±43
4	81±90
Acarbose	780±20
Deoxynojirimycin	425.6±14

Dehydroabietic acid (**1**) and its transformed products **2**-**4** were tested for their antibacterial activity against two Gram-positive bacteria including *Bacillus subtilis* and *Staphylococcus aureus,* in addition to four Gram-negative bacteria (*Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Salmonella typhi*) using previously reported zone of inhibition test method [19]. All metabolites **2**-**4** exhibited antibacterial activity but compound **4** demonstrated significant antibacterial activities against various strains maybe due to the presence of OH group at C-16 position (Table 2).



Scheme 1: Fermentation of dehydroabietic acid (1) by *Cunninghamella elegans*, *Rhizopus stolonifer*, *Gibberella fujikuroi*, and *Cephalosporium aphidicola*.

Table 2: Antibacterial Activities of Compounds 1-4

Compound	*Escherichia coli*	*Bacillus subtilis*	*Shigella flexnari*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Salmonella typhi*
1	15	17	15	15	Inactive	17
2	Inactive	11	11	Inactive	14	Inactive
3	Inactive	10	10	Inactive	12	Inactive
4	19	20	12	Inactive	16	20
Imipenem	30	33	27	33	24	25

CONCLUSIONS

In summary, the present research on the biotransformation of dehydroabietic acid (**1**) by *Cunninghamella elegans*, *Rhizopus stolonifer*, *Gibberella fujikuroi*, and *Cephalosporium aphidicola* have been conducted for the first time. Fermenation of **1** for 12 days with fungal strains yielded three hydroxylated transformed products **2**-**4**. Structures of all new metabolites were elucidated by using spectroscopic techniques. All compounds **1**-**4** were then subjected to α-glucosidase Inhibitory Activity and antibacterial activities. Compound **1** showed promising inhibitory effect against α-glucosidase, which is 38 times more than the standard deoxynojirimycin. All metabolites **2-4** exhibited antibacterial activity but compound **4** demonstrated significant antibacterial activities against various strains maybe due to the presence of OH group at C-16 position.

CONFLICT OF INTERESTS

Declared None

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