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α-Glucosidase inhibitory fatty acids from *Morchella fluvialis* mushroom

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ABSTRACT

Morchella fluvialis, a morel mushroom, is one of the most famous edible mushrooms all over the world. Interest in this mushroom is steadily increasing due to its organoleptic properties and nutritional value. The methanolic extract of *M. fluvialis* showed α-glucosidase inhibitory and antioxidant activities in an assay system. Therefore, the purification and characterisation of bioactive metabolites and evaluation of biological activity were conducted. Fractionation of the *M. fluvialis* extract resulted in the isolation of nine compounds, namely, three fatty acids, (9*Z*,12*Z*)-octadecadienoic acid (linoleic acid, 1), (9*Z*,12*Z*)-3-hydroxyoctadecadienoic acid (2) and (6*Z*,9*Z*)-13-hydroxyoctadecadienoic acid (3); four sterols, stellasterol (4), ergosterol peroxide (5), ergosterol (6) and brassicasterol (7); one sugar alcohol, arabitol (8); and nicotinamide (9). Among them, compounds 2–3 and 7 were first reported from *Morchella*. In addition, compound 1 exhibited potent α-glucosidase inhibition, with an IC_{50} value of 14.8 µM. The content of compound 1, the major compound, was 1.2 mg · g^{-1} extract, as quantitated by HPLC analysis, which was lower than the IC_{50} value of compound 1. Therefore, *M. fluvialis* can benefit from diabetes and related diseases through the synergistic effect of linoleic acid (1) and other ingredients.

Keywords: α-glucosidase, linoleic acid, *Morchella fluvialis*, morel

INTRODUCTION

Due to their organoleptic properties (e.g., texture and flavour) and nutritional value, wild mushrooms have been part of the human diet for decades. In reality, fungi have excellent value with large quantities of proteins and many essential amino acids and low lipid content (Zanes Furlani and Godoy, 2007). This natural matrix has ignited interest in the profusion of bioactive compounds (Ferreira et al., 2009). The compounds present in mushrooms are tocopherol (Heleno et al., 2010), phenolic compounds (Vaz et al., 2011) and organic acids (Barros et al., 2013), with nutraceutical potential (Barros et al., 2008). The researchers have studied various mushroom species to develop new therapies, which have demonstrated their bioactive properties (Lindequist et al., 2005).

Morchella mushroom (morels) is an edible ascomycetous mushroom consumed worldwide. They are highly appreciated by gastronomes for their desirable taste quality and are valued for their rich unique fragrance, delicate flavour and meaty texture in many cuisines (Tietel and Masaphy, 2018). The number of species has been the subject of taxonomic debate for years,

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while existing phylogenetic trees show the seventies of *Morchella* species worldwide. Various scientific studies on phylogeny, biogeography, taxonomy and nomenclature of this genus have been performed to classify new species worldwide (Rahgo et al., 2019). Thanks to their health-related advantages, these edible mushrooms, which are valued internationally, have been used in conventional medicine for centuries. Mushrooms are often considered functional in traditional medicine and have different medicinal qualities. Pharmacological and phytochemical studies of mushrooms have shown over recent decades that they contain a range of active metabolites containing desirable pharmacological activities, including antimicrobial (Martel et al., 2017), anti-inflammatory (Paterson and Lima, 2014), anti-obesogenic, anti-diabetic, antiviral, antioxidant, anticancer and immunomodulatory properties (Martel et al., 2017).

Diabetes mellitus is a chronic metabolic disease accompanied by an abnormal increase in plasma glucose levels due to the imbalanced development of insulin and/or insensitivity (Stojkovic et al., 2019). The inhibition of complex polysaccharide hydrolysis and restriction of glucose absorption are among the strategies for controlling type 2 diabetes (Saito et al., 1998). Intestinal α-glucosidase is an essential enzyme that catalyses the final step in carbohydrate digestion by converting carbohydrates into single monosaccharides (Ahn et al., 2020). Inhibition of α-glucosidase delays the digestion and absorption of carbohydrates by regulating postprandial hyperglycaemia, resulting in a hypoglycaemic impact. For the treatment of carbohydrate-mediated diseases such as diabetes, some α-glucosidase inhibitors, such as acarbose and voglibose, have been used (Joshi et al., 2015; Ríos et al., 2015). Consequently, natural products with α-glucosidase inhibitory activity will be helpful in the treatment and prevention of diabetes by regulating blood glucose levels (Ahn et al., 2020). Recently, various mushrooms have been reported to be effective in preventing and treating diabetes, and research on this is being actively conducted. Oxidative stress is known as one of the important factors that are related to the onset and progression of diabetes. It is caused by the excessive production of reactive oxygen species (ROS), which is considered a major mediator of diabetes (Giacco and Brownlee, 2010; Pitocco et al., 2013). Although the human body has an defence mechanism for protection against ROS generation, persistent oxidative stress caused by excessive ROS production exacerbates diabetes (Maritim et al., 2003; Rendra et al., 2019). Therefore, antioxidants are also used to treat diabetes. Natural products are good antioxidants and are widely used in the therapeutics of diabetes (Umeno et al., 2016).

In the course of research on the usefulness of morel mushrooms, the total extract of *M. fluvialis* was found to show α-glucosidase inhibitory and antioxidant activities in an assay system. Therefore, the isolation of active constituents was conducted, and the structures were identified by using a spectroscopic method. The biological activity of isolated compounds and the content in the extract were also investigated in this study.

MATERIALS AND METHODS

Experimental section

The fruiting bodies of *M. fluvialis* were collected from the local market at Yunnan, China, in April 2018. The mushroom was first identified by Prof. Hyun You Chang of the Korea National College of Agriculture and Fisheries. In addition, a DNA-based approach was used. In the process of DNA extraction, *Morchella* specimens were pulverised into a fine powder using a grinder. The extraction process was subsequently carried out using a commercially available kit (DNeasy® Plant Mini Kit, Qiagen Hilden, Germany) following the protocol provided by the manufacturer. The quantification of DNA was performed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Voucher specimens (CBNU2018-MF) were deposited in a specimen room of the herbarium of the College of Pharmacy, Chungbuk National University.

Extraction and isolation

Dried fruiting bodies of *M. fluvialis* (10.2 g) were subjected to two extractions using 80% MeOH, resulting in a methanolic extract weighing 1.4 g. Subsequently, the methanolic extract was diluted in H_2O and subjected to sequential partitioning with *n*-hexane, CH_2Cl_2, EtOAc and *n*-BuOH. The *n*-hexane fraction (0.2 g) underwent filtration using MPLC RP-Silica to characterise nine distinct fractions (HE1-HE9). HE4 was subjected to column chromatography over Sephadex LH-20 eluting with CH_2Cl_2-MeOH (1:1) to give three fractions (HE4A-HE4C). Compounds 1 (8.7 mg), 2 (2.5 mg) and 3 (3.1 mg) were obtained from HE4C by semi-preparative HPLC eluting with acetonitrile. A sample of an EtOAc fraction weighing 0.2 g was subjected to column chromatography using silica gel. The elution process involved a mixture of *n*-hexane and EtOAc, resulting in the separation of the sample into nine distinct fractions, labelled EA1 to EA9. The compound EA3 underwent column chromatography with Sephadex LH-20 as the stationary phase and a mixture of CH_2Cl_2 and MeOH in a 1:1 ratio as the eluent. This process resulted in the separation of EA3 into five distinct fractions, namely, EA3A-EA3E. Compounds 4 (2.8 mg), 5 (3.1 mg), 6 (5.3 mg) and 7 (3.4 mg) were obtained from EA3E by semi-preparative HPLC eluting with acetonitrile–water (90:10). Compounds 8 (0.7 mg) and 9 (0.7 mg) were obtained from EA3C by semi-preparative HPLC eluting with acetonitrile–water (80:20).

Development of HPLC methods for the quantitation of compound 1

For the determination of linoleic acid (1), the total extract was prepared by extracting the fruiting bodies

of *M. fluvialis* with 80% MeOH. *n*-Hexane, EtOAc and *n*-BuOH fractions were prepared from the total extract by partitioning successively with *n*-hexane, EtOAc and *n*-BuOH.

The HPLC analysis was conducted utilising a Waters HPLC system that was equipped with Waters 600Q pumps, a 996 photodiode array detector and Waters Empower software. The analysis employed a Phenomenex Gemini-NX 3µ C18 110A column with a dimension of 150 mm × 4.60 mm. The process of chromatographic separation was successfully achieved by employing a mixture of acetonitrile and water in a ratio of 85:15. The separation was conducted at a flow rate of 2.0 mL · min^{-1}. Calibration solutions were prepared using linoleic acid (1) at varying concentrations. The relationship between concentration and peak area was examined by employing the regression line derived from the least square analyses.

Determination of α-glucosidase inhibitory activity

The evaluation of the inhibitory effects of the samples on α-glucosidase activity was conducted utilising α-glucosidase derived from *Saccharomyces cerevisiae*. In brief, α-glucosidase (1.0 U · mL^{-1}) was added to the sample, and then it was incubated at 37 °C for 15 min. The enzyme reaction was performed at 37 °C for 20 min after adding 10 µL of *p*-nitrophenyl α-$_D$-glucopyranoside solution. Absorbance at 405 nm in a 96-well microplate reader was used to calculate the quantity of *p*-nitrophenol cleaved by the enzyme. The calculation of the percentage of α-glucosidase inhibition for the compounds was performed as follows: [(Absorbance without sample – absorbance with sample)/absorbance without sample] × 100. Acarbose was employed as a positive control in the study.

Statistical analysis

Statistical significance was assessed using one-way analysis of variance (ANOVA), where a significance level of $p < 0.05$ was deemed to indicate statistical significance.

RESULTS AND DISCUSSION

Characterisation of M. fluvialis

Morchella was first identified by morphological characteristics using DNA-based approaches (Figure 1). Morphologically, a yellow morel was similar to *M. esculenta*, but with a slender habit, a stipe longer than the pileus and a pileus that is normally conical, with the top slanted to one side. At the pileus base, there is no sinus. The pileus primary pits are spherical or elongated, with crests that stain orange when injured and are orientated somewhat lengthwise.

For additional DNA-based approaches, ITS sequencing for *Morchella* authentication was used. Because the ITS regions have different rates of evolution, they are common markers in evolutionary studies at different taxonomic levels (Balasubramani et al., 2010). PCR amplification was performed with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) for ITS, while EF1-983 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and EF1-1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used to amplify the translation elongation factor 1-α(*tef1*) gene.

The number of reports on this mushroom species is quite limited in the literature. Most of them especially focused on *M. esculenta*, but there are no record of *M. fluvialis.* Therefore, we revealed a new interesting finding of *M. fluvialis*, which suggests these species to be a good candidate for a highly nutritive source of mushrooms.

Evaluation of the biological activity of the extracts

For the investigation of fruiting bodies of *M. fluvialis* for the beneficial effects on metabolic diseases, α-glucosidase inhibitory and antioxidant activities

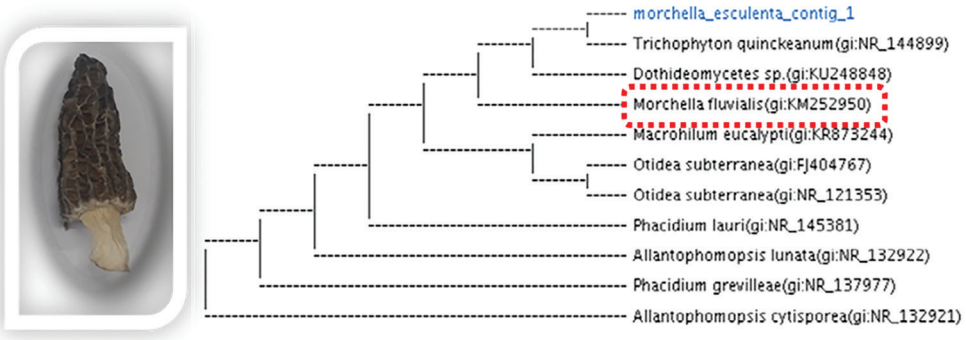

Figure 1. Characterisation of *Morchella fluvialis.* Phylogenetic analysis was conducted based on ITS1 and ITS4 sequence data. The length of the branches corresponds to the cumulative number of nucleotide substitutions in the DNA sequences under analysis. The sequences were obtained from GeneBank and EMBL databases, and their corresponding accession numbers are given within brackets.

of its methanolic extract were measured. As shown in Figure 2, the total extract of *M. fluvialis* showed α-glucosidase inhibitory and antioxidant activities. To characterise the active constituents, the total extract was further fractionated into *n*-hexane, EtOAc and *n*-BuOH fractions according to the polarity. Among the fractions, the *n*-hexane fraction showed the most potent α-glucosidase inhibitory activity. On the other hand, the EtOAc fraction exerted mild α-glucosidase inhibitory and antioxidant activities in an assay system (Figure 2).

Characterisation of compounds

For the characterisation of active constituents, the isolation of compounds from *n*-hexane and EtOAc-soluble fractions was conducted using chromatographic techniques (Supplementary Scheme S1). As a result, nine compounds (Figure 3) were isolated from *n*-hexane and EtOAc-soluble fractions. The structures of isolated compounds were determined as three fatty acids, linoleic acid (1), (9*Z*,12*Z*)-3-hydroxy-9,12-octadecadienoic acid (2) and (6*Z*,9*Z*)-13-hydroxy-6,9-octadecadienoic acid (3); four sterols, stellasterol (4), ergosterol peroxide (5), ergosterol (6) and brassicasterol (7); one sugar alcohol, arabitol (8); and nicotinamide (9) by spectroscopic analysis including ^{1}H, ^{13}C NMR and MS analyses (Supplementary Figures S1–S6), which were confirmed by the comparison of literature values (Dong et al., 2000; Jinming et al., 2001; Seo et al., 2009; Kim et al., 2011). Among them, compounds 2–3 and 7 were first reported from this mushroom.

Evaluation of the biological activity of the compounds

The biological activity of isolated compounds was evaluated (Table 1). Among the isolated compounds, compound 1 showed the most potent α-glucosidase inhibitory, with an IC_{50} value of 14.8 µM (4.2 µg · mL^{-1}). Compounds 2 and 3, fatty acid derivatives, also showed α-glucosidase inhibition by 53.1 and 32.9% at a concentration of 100 µM. However, sterol derivatives (4–6) showed weak α-glucosidase inhibitory activity. Related to antioxidant activity, all the isolated compounds exerted weak activities.

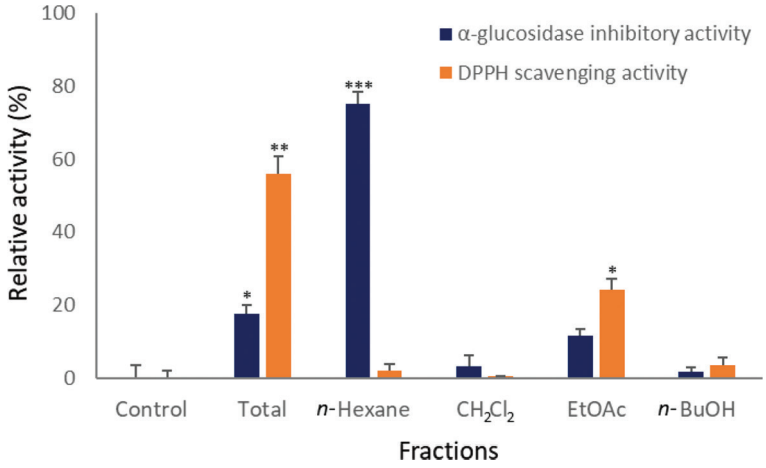

Figure 2. Effects of total extract and different fractions of *M. fluvialis* on α-glucosidase inhibitory and antioxidant activities. All data are presented as mean ± SEM (n = 3). *$p < 0.05$ and **$p < 0.01$, ***$p < 0.001$ indicates significant differences compared to the control group.

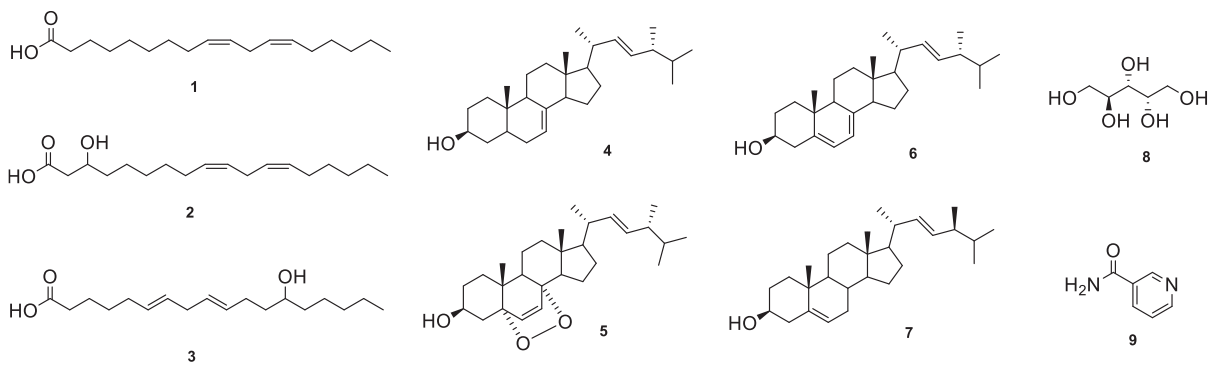

Figure 3. Chemical structures of compounds 1–9 isolated from *M. fluvialis*.

Quantitation of compound 1 in the extract of M. fluvialis

Compound 1 is the major compound of morel and showed potent α-glucosidase inhibitory activity. Therefore, the content in the total extract and each fraction were quantitated by HPLC analysis (Figure 4). The content of compound 1 was 1.2 mg · g^{-1} extract in the total extract of morel mushroom and was much higher as 15.9 mg · g^{-1} extract in the *n*-hexane fraction, which is consistent with α-glucosidase inhibitory activity. However, compound 1 was not detectable in EtOAc and *n*-BuOH-soluble fractions. These results showed that compound 1 is a major active compound for α-glycosidase inhibitory activity of *Morchella* mushroom.

Interestingly, the *n*-hexane-soluble fraction of morel contained 1.59% of compound 1 and showed inhibitory activity of 75% at a concentration of 200 μg · mL^{-1}. The amount of compound 1 contained in 200 μg · mL^{-1} of the *n*-hexane-soluble fraction was about 3.2 μg · mL^{-1}, which is lower than the IC_{50}, but exhibits more than 50% inhibitory effect. As mentioned earlier, morel contains several fatty acids with α-glucosidase inhibitory activity such as (9*Z*,12*Z*)-3-hydroxy-9,12-octadecadienoic acid (2) and (6*Z*,9*Z*)-13-hydroxy-6,9-octadecadienoic acid (3). From these results, it can be inferred that the α-glucosidase inhibitory activity of *Morchella* mushroom is synergistic with compound 1 and other compounds.

Table 1. α-Glucosidase inhibitory activity of compounds 1–6 from *M. fluvialis.*

Compound	α-Glucosidase inhibitory activity (100 μM)	IC_{50} (μM)
1	89.2 ± 3.4	14.8
2	53.1 ± 6.3	95.2
3	32.8 ± 6.6	>100
4	24.8 ± 6.5	>100
5	21.9 ± 0.3	>100
6	17.0 ± 0.3	>100
Acarbose*	76.4 ± 3.4	72.5

*Acarbose was used as the positive control.

Anti-diabetic potential of M. fluvialis

Previous studies on the strain of *Morchella* related to anti-diabetic activity have been mainly focused only on the organic acid profile of *Morchella*. Dietary fat, which is a large component of the typical diet and hence a tight feedback regulator, is required to maintain lipid homeostasis. Many studies have investigated the fatty acid content of a variety of mushrooms to better understand their nutritional roles in human diets (Lv et al., 2015). Polyunsaturated fatty acids, which are also present in mushrooms, play a crucial role in human nutrition and the prevention of diseases. Linoleic acid (1), a polyunsaturated fatty acid, has been consumed for the prevention of various diseases, such as cardiovascular and inflammatory diseases, as well as depression. A higher level of linoleic acid in diet is also suggested to contribute to insulin sensitivity (Lv et al., 2015). Consistent with previous research, linoleic acid (1) of morel mushroom showed potent inhibitory activity, with an IC_{50} value of 14.73 μM in an assay system. The present study also yielded another fatty acid derivative, (9*Z*,12*Z*)-3-hydroxy-9,12-octadecadienoic acid (2) and (6*Z*,9*Z*)-13-hydroxy-6,9-octadecadienoic acid (3), with α-glucosidase inhibitory activity. The observation highlights the importance of fatty acids and their synergic contribution to α-glucosidase inhibition activity.

CONCLUSIONS

In this study, *M. fluvialis* was characterised through the utilisation of phylogenetic analysis, marking the first instance of its identification. In addition, the chemical composition of *M. fluvialis*, as well as its potential α-glucosidase inhibitory and antioxidant properties, was investigated. Seven metabolites were purified from the fruiting bodies of *M. fluvialis* using various chromatographic techniques, and the structures were identified by extensive spectroscopic analysis including NMR and MS. Among the isolated compounds, compounds 2, 3 and 7 were first reported from *Morchella* species. Related to biological activity, compound 1 showed α-glucosidase inhibitory activity with an IC_{50} value of 14.8 μM, and the quantitation of

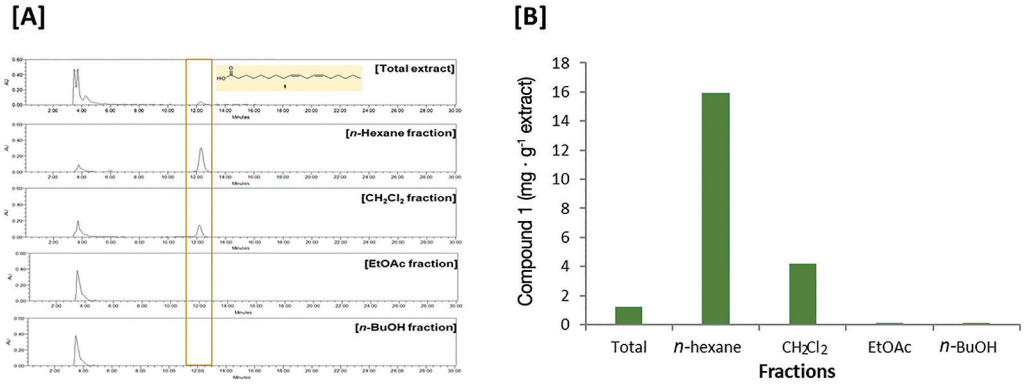

Figure 4. [A] HPLC chromatogram and [B] amount of compound 1 of total extract and different fractions of *M. fluvialis*.

compound 1 by HPLC analysis suggested the important role in α-glucosidase inhibitory activity in *M. fluvialis*. The findings of this study will provide valuable insights into the functional characterisation and bioactivity of *M. fluvialis*.

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AUTHOR CONTRIBUTIONS

A.T. and M.K.L. – conceptualisation. A.T., S.W.Y., S.H.R., B.Y.H., G.H.S. and H.Y.C – methodology. A.T. and M.K.L. – software. A.T., B.Y.H. and M.K.L. – validation. A.T., B.Y.H. and M.K.L. – formal analysis. A.T., S.L., H.H.L., S.W.Y., S.H.R., G.H.S. and M.K.L. – investigation. A.T. and M.K.L. – writing – original draft preparation. A.T. and M.K.L. – writing – review and editing. M.K.L. – supervision. M.K.L. – project administration. M.K.L. – funding acquisition. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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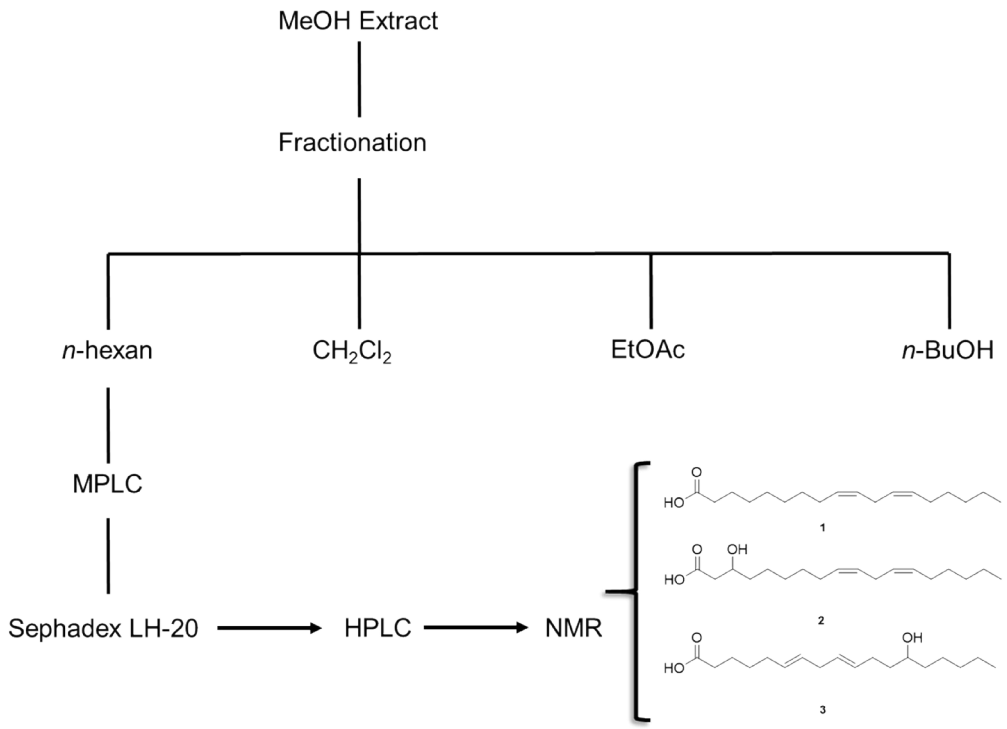
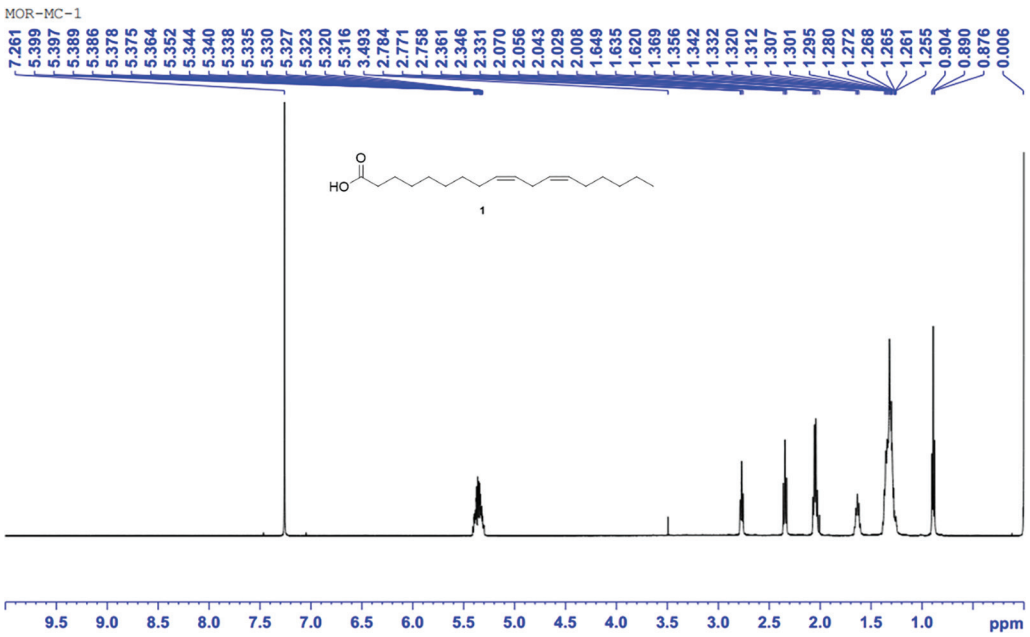
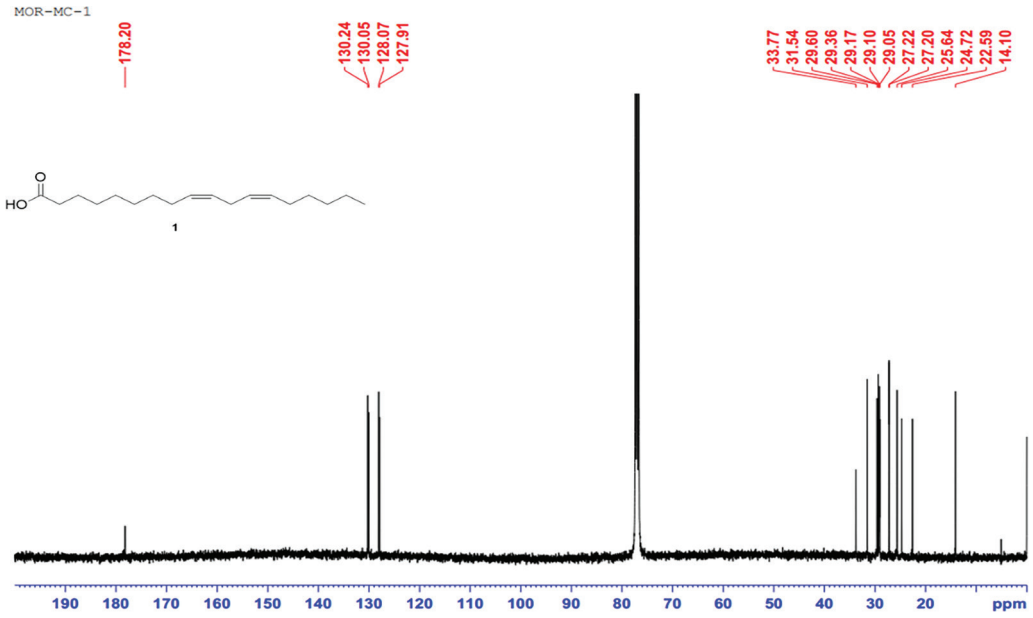
SUPPLEMENTARY MATERIALS



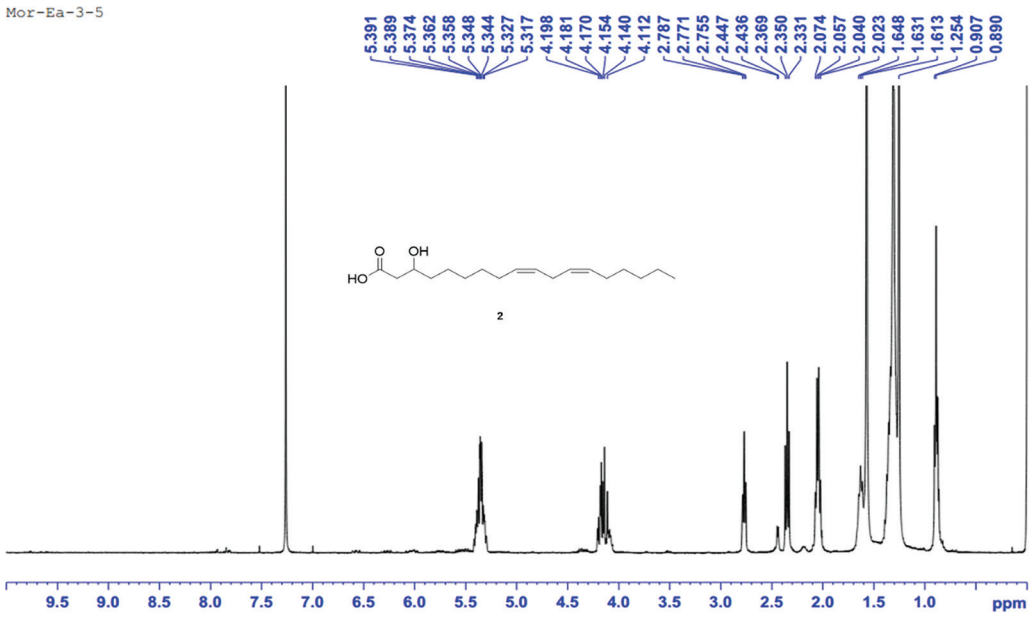
Supplementary Scheme S1. Extraction and isolation of sterols.



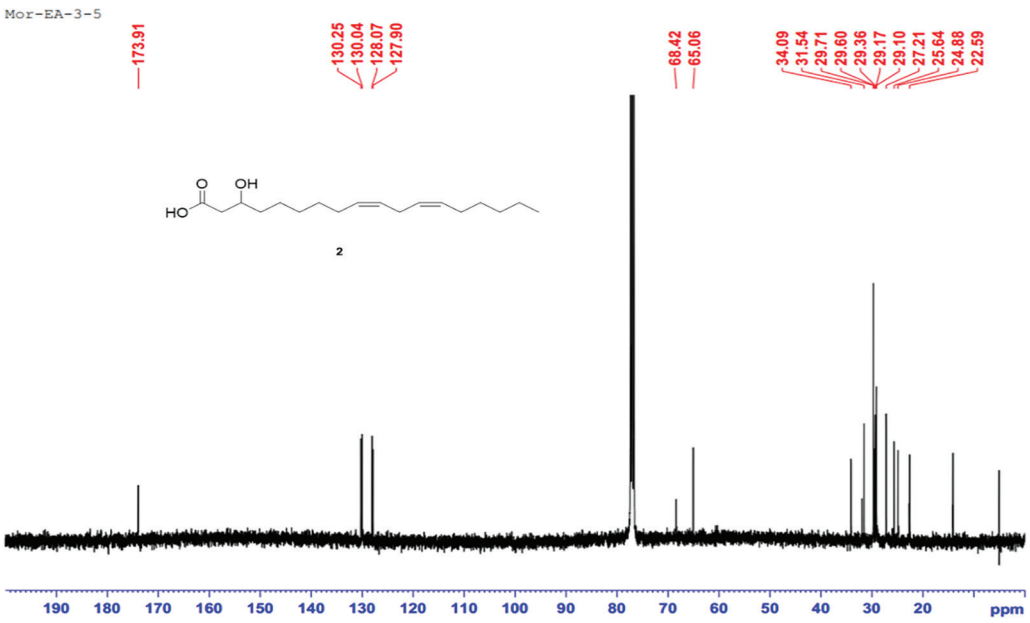
Supplementary Figure S1. ^{1}H NMR spectrum of compound 1 ($CDCl_3$, 500 MHz).



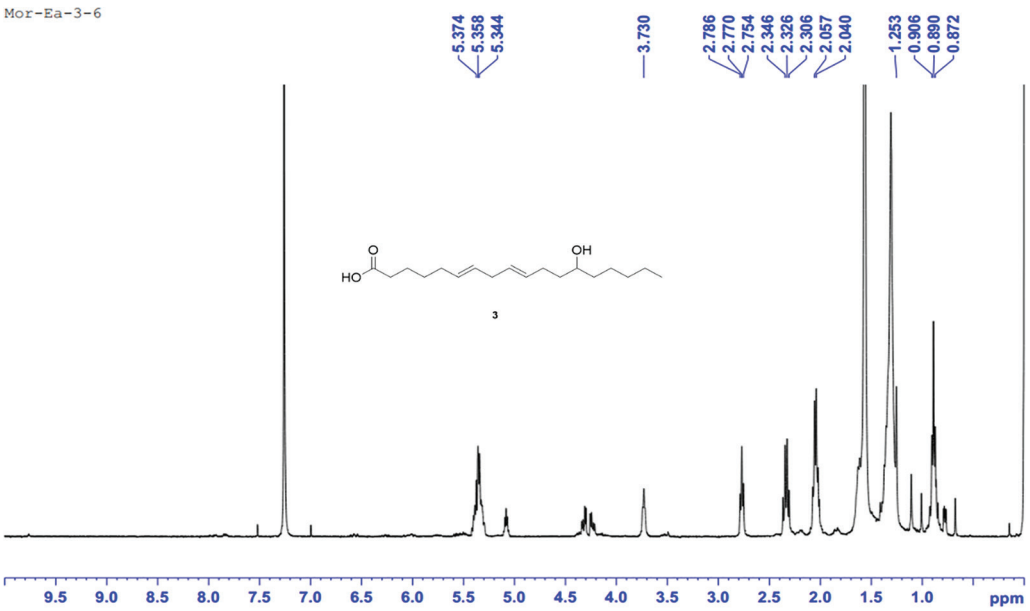
Supplementary Figure S2. ^{13}C NMR spectrum of compound 1 ($CDCl_3$, 125 MHz).



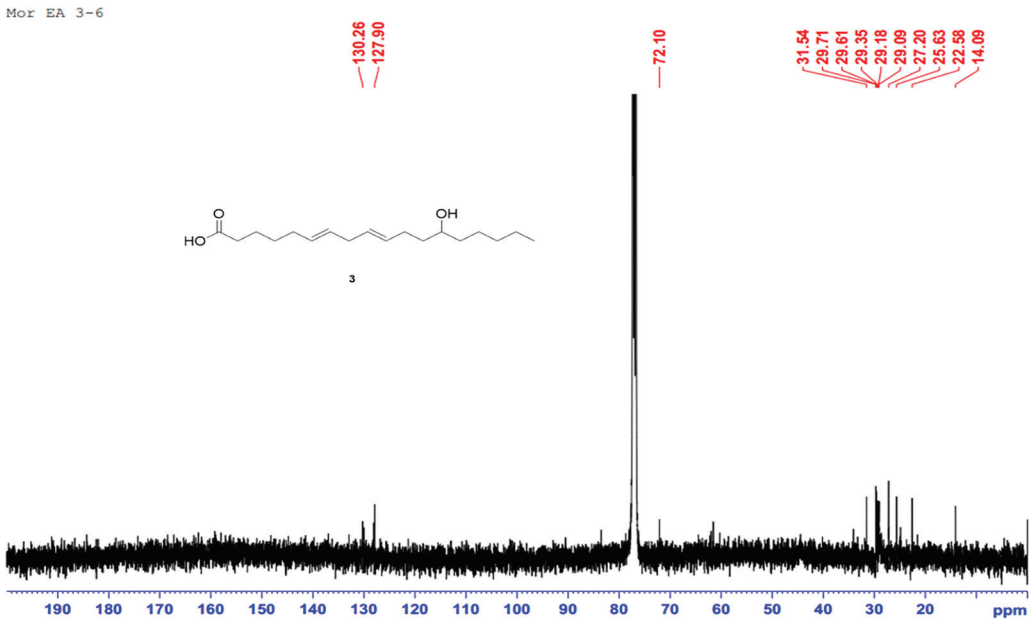
Supplementary Figure S3. ^{1}H NMR spectrum of compound 2 ($CDCl_3$, 500 MHz).



Supplementary Figure S4. ^{13}C NMR spectrum of compound 2 ($CDCl_3$, 125 MHz).



Supplementary Figure S5. ^{1}H NMR spectrum of compound 3 ($CDCl_3$, 500 MHz).



Supplementary Figure S6. ^{13}C NMR spectrum of compound 3 ($CDCl_3$, 125 MHz).