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Phenolic Glycosides Isolated from Safflower (*Carthamus tinctorius* L.) Seeds Increase the Alkaline Phosphatase (ALP) Activity of Human Osteoblast-like Cells

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Abstract The chemical compositions of the seeds of the safflower (*Carthamus tinctorius* L.) plant were evaluated to determine possible compound having proliferative effects on human osteoblast cells. Three-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test and alkaline phosphatase (ALP) activity were used to assess the effects of the isolates on the human osteoblast-like line (Saos-2). Activity guided fractionation led to the isolation of ALP activating lignin and alkaloid glycosides through the extraction of the seeds, solvent partitioning and repeated silica gel and octadecyl silica (ODS) column chromatographic separations. The data from Nuclea Magnetic Resonance (NMR), Mass (MS), and Infrared (IR) analyses enabled the determination of the chemical structure and characterization of two compounds as a tracheloside and an N-(*p*-coumaroyl)-serotonin mono-*β*-D-glucopyranoside. These two compounds showed respectively 149.2±4.2 and 138.9± 3.5% ALP activity compared to the control when evaluated at a concentration of 100 μg/mL.

Keywords: *Carthamus tinctorius* L., compositae, tracheloside, N-(*p*-coumaroyl)-serotonin mono-*β*-D-glucopyranoside, alkaline phosphatase (ALP), human osteoblast-like line

Introduction

The safflower plant, *Carthamus tinctorius* L., has long been used for a variety of purposes, including as a source of edible fats, food colorants, for natural pigments and as a Chinese medicine (1). There were have been occasional reports regarding coloring agents (safflower yellow) and physiological functions e.g., lowering plasma and hepatic lipids (2) and the recovery from bone fractures (3). Various glycerides and lipids are reportedly as the main components of the plant (4-6). Especially important is linoleic acid, which is in 75% of this plant's whole seed and is known to be effective in diminishing cholesterol levels in the blood, and to treat such circulatory system ailments as arteriosclerosis and hyperlipemia (7). Only a few reports claim that it is the lignin and serotonin compounds of the plant act to prevent osteoporosis or treat bone fractures (4, 8), The screening of several hundred plant extracts, using 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) testing and alkalinephosphatase (ALP) assay of human osteoblast-like cells (Saos-2) has shown significant activity in the safflower seeds (9, 10). Therefore, we have conducted a study of the seeds of safflower in an attempt to identify the principal components responsible for the proliferation of the Saos-2.

Bone is a tissue maintained through continuous osteogenesis and osteolysis regulated by osteoblasts and osteoclasts, respectively (11). Osteoporosis is a common metabolic bone disease usually the result of an imbalance between osteoblast and osteoclast cell activities which are influenced by multiple physical and physiologic factors. Therefore, any remedy for osteoporosis would theoretically involve modifying osteoblast activity to facilitate osteogenesis (bone formation), or inhibiting osteoclast activity to suppress osteolysis (bone resorption) (12) or some combination of the two processes. ALP is an enzyme present in the liver (LALP), bone (BALP), and intestines (IALP) (13). The BALP is specific to the maturation of osteoblasts (14) and therefore, BALP is a useful marker for osteoblast cell activation (15-17). In this study, the MTT assay, which indicates differentiation and proliferation of Saos-2, and the ALP activity detection assay, was applied to extracts of safflower seeds after extract fractionation by solvent partitioning and column chromatography. Two phenolic glycosides were isolated as the principal active components facilitating osteogenesis of Saos-2. This paper describes the procedures for the isolation and structure determination of the active compounds and their effects on the MTT and ALP activities of Saos-2.

Materials and Methods

Plant materials The safflower (*Carthamus tinctorius* L.) was supplied from the Woorihonghwain Farm Corporation (Gyeongbuk, Korea) and identified by Prof. Dae Keun Kim, Woosuk University, Jeonju, Korea. A voucher specimen (KHU03201) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University, Suwon, Korea.

Chemicals The human osteoblast-like cell line (Saos-2), was obtained from the Korean Cell Line Bank (KCLB,

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Seoul, Korea). Dimethylsulfoxide (DMSO), MTT, penicillin, streptomycin, and the RPMI-1640 medium were purchased from Sigma (St. Louis, MO, USA) and fetal bovine serum (FBS) was purchased from JRH Bio Science (Lenexa, KS, USA).

Isolation of active compounds from the seeds of safflower The dried and powdered seeds of safflower (3.8 kg) were extracted three times at room temperature with 80% aqueous methanol (MeOH,). The extracts were partitioned with water (2 L), ethyl acetate (EtOAc, 3×20 L) and *n*-butanol (*n*-BuOH, 3×20 L) in succession. The EtOAc extract (54 g) was applied to a silica gel column (6 ×50 cm) and eluted with *n*-hexane : EtOAc (5:1) and monitored by thin layer chromatography (TLC) yielding 15 fractions (CTE1 to CTE15). Fraction CTE11 (933 mg) was subjected to silica gel chromatography (4×30 cm) and eluted with chloroform ($CHCl_3$):MeOH (7: 1yielding 20 fractions (CTE11-1 to CTE11-20). The CTE11-5 fraction (171 mg) was purified by ODS chromatography (3×30 cm) using MeOH:H_2O (1:2) as eluent to ultimately produce compound **1** (81 mg). Also, CTE11-13 (701 mg), a second major fraction, was chromatographed on another silica gel column (5×45 cm) using $CHCl_3$:MeOH (4:1), and eight fractions were collected (CTE11-13-1 to CTE-11-13-8). Fraction CTE11-13-5 (254 mg) was purified by octadecyl silica (ODS) chromatography (3×30 cm) using MeOH: H_2O (1:2) as eluent to yield compound **2** (92 mg).

Identification of the active compounds from the seeds of safflower The melting points of the isolated compounds were determined on a Fisher-John apparatus and not corrected. Optical rotations were measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan) to determine the stereochemistry of the compounds. FAB-MS was recorded on a JEOL JMSAX 505-WA (Tokyo, Japan) and these data provided molecular weight determinations for each compound. The IR spectra were run on a Perkin Elmer Spectrum One FT-IR spectrometer (Perkin Elmer, Beaconsfield, Buckinghamsshire, England) to enable detection of any functional groups of the compounds. The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using a Varian Unity Inova AS 400 FT-NMR spectrometer (Varian, Paloalto, CA, USA). These ^{1}H and ^{13}C NMR data provided some chemical environments in which each nuclear was placed on the site.

MTT test The Saos-2 cells were grown in RPMI-1640 medium containing 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. The cells were maintained in a humidified incubator at 37°C and in a 5% CO_2 atmosphere. Human osteoblast-like cell proliferation was evaluated using an established MTT assay protocol (16). Cells were plated at a density of 2×10^4 cells per 96-well plate in 100 μL of medium and cells plated then added to samples (10^{-1} to 10^{-8} mg/mL) containing 1% DMSO. After incubation for 48 hr at 37°C, 0.5 mg/mL MTT was added to each well and incubation continued for 4 hr at 37°C. Formazan crystals were dissolved in DMSO and the absorbance was determined at 550 nm using a microplate reader (SPECTRA Max 340PC, Sunnyvale, CA, USA).

ALP activity assays ALP activities were determined using *p*-nitrophenyl phosphate as the substrate by the method of Bowers and McComb (10). The ALP reagent was obtained from Thermo Electron (Louisville, CO, USA). The Saos-2 cells were cultured as in for the MTT test described previously. After incubating for 48 hr at 37°C, the cells were washed with PBS and then lysed with 1% Triton X-100. The lysates were sonicated for 15 sec and centrifuged at 14,000×g for 20 min at 4°C. The clear supernatant was used to measure ALP activity which was determined at 405 nm using a microplate reader (SPECTRA Max 340PC).

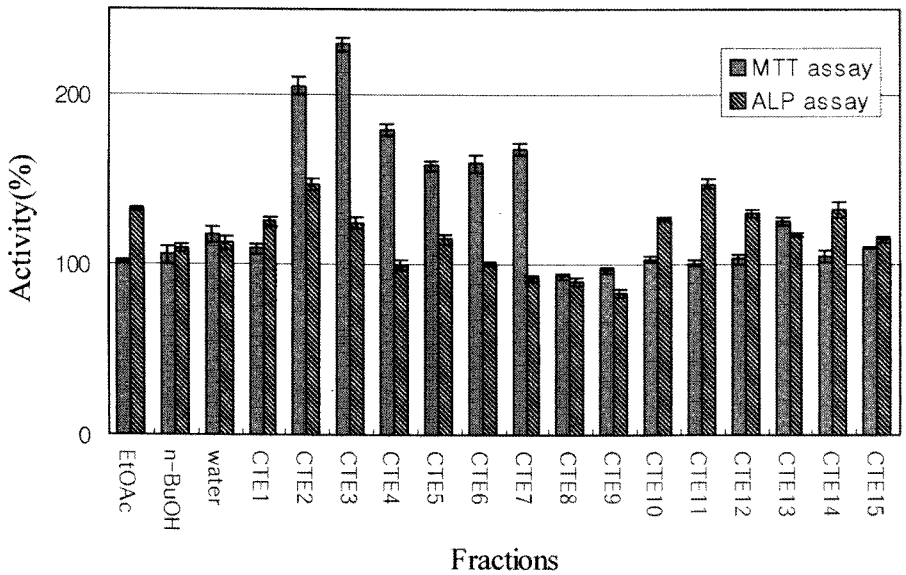


Fig. 1. Activity of fractions from the EtOAc layer of the safflower seed on human osteoblast-like cells (Saos-2). The values indicate the means of three replications of the experiment for the activity of each fraction (100 μg/mL) compared to control.

Results and Discussion

The MeOH extracts of the seeds of safflower showed an activation effect on BALP (bone alkaline phosphatase), a significant biomarker for osteoblast cell activation. Therefore, activity-guided fractionation for the MeOH extracts of the seed of safflower was conducted.

The MeOH extracts were partitioned into EtOAc, *n*-BuOH, and H_2O layers through solvent fractionation. Among these three layers, the EtOAc fraction showed the lowest activity by the MTT assay (101.2±1.2%), indicating that the EtOAc fraction should have little or no cytotoxicity on the Saos-2 and most likely the highest activity in the ALP assay (132.3±2.7%). Hence, is speculated that the EtOAc fraction may be more effective in stimulating bone formation than would the other fractions. (Fig. 1)

The EtOAc fraction was subjected to further chromatography on silica gel which yielded 15 sub-fractions (CTE1-CTE15), which were evaluated for their effects in the MTT and ALP assays. Fractions CTE2 and CTE11 exhibited higher ALP activities (147.4±3.3 and 147.6±2.9 %, respectively) than the control (145%), (Fig. 1). Fraction CTE2, however, showed very high cytotoxic activity (204.7±4.9%), which may indicate that the increase is not due to increased ALP activity but rather cell proliferation. A GC-MS analysis showed that CTE2 and CTE3 were lipids, and thus no further analyses were carried out. Cytotoxic activity (101.0±2.2%) of fraction CTE11 in the MTT assay indicated minimal or no cytotoxicity to the cells. Successive SiO_2 column chromatography of fraction CTE11 yielded 20 sub-fractions (CTE11-1 - CTE11-20) with CTE11-5 and CTE11-13 possessing the highest ALP activities, 146.1±3.9 and 133.2±3.1%, respectively. Cytotoxicity to the cells according to the MTT assay results would be minimal, i.e., 106.3±1.6 and 104.3±1.9% were related to these two subfractions (Fig. 2). The TLC chromatograms showed both subfractions to have one main component and slight impurities. Therefore, repeated ODS and SiO_2 column chromatography of CTE11-5 and CTE11-13 provided the purified compounds indicated as **1** and **2**, respectively.

Compound **1**, colorless crystals, showed absorbance bands due to hydroxyl (3450 /cm), lactones (1770 /cm), and olefins (1610 /cm) in the Infrared (IR) spectrum ($CHCl_3$-MeOH) and a molecular ion peak $[M+H]^+$ at *m/z* 551 in the positive Fast atom bambardment Mass (FAB/MS). In the ^{1}H-Nuclear magnetic resonance (NMR) spectrum (400 MHz, pyridine-d_5), six olefin methine signals (δ_H 7.59, 7.15, 7.04, 6.91, 6.90, 6.86) originated in the benzene ring; one oxygenated methylene signal (δ_H 4.30, 4.16) was observed. One methine (δ_H 2.73) and two methylene signals (δ_H 3.63, 3.31, 3.24, 2.94) were also observed. The observance of one oxygenated methine signal (δ_H 5.66) of an anomeric proton and a number of oxygenated methine and methylene signals verified the presence of a molecule of sugar. In the ^{13}C-NMR (100 MHz, pyridine-d_5), 27 signals consisting of one lactone (δ_C 179.5), four olefin oxygenated quaternary (δ_C 149.4, 149.4, 147.9, 146.5), two olefin quaternary (δ_C 131.8, 129.7), and six olefin methine

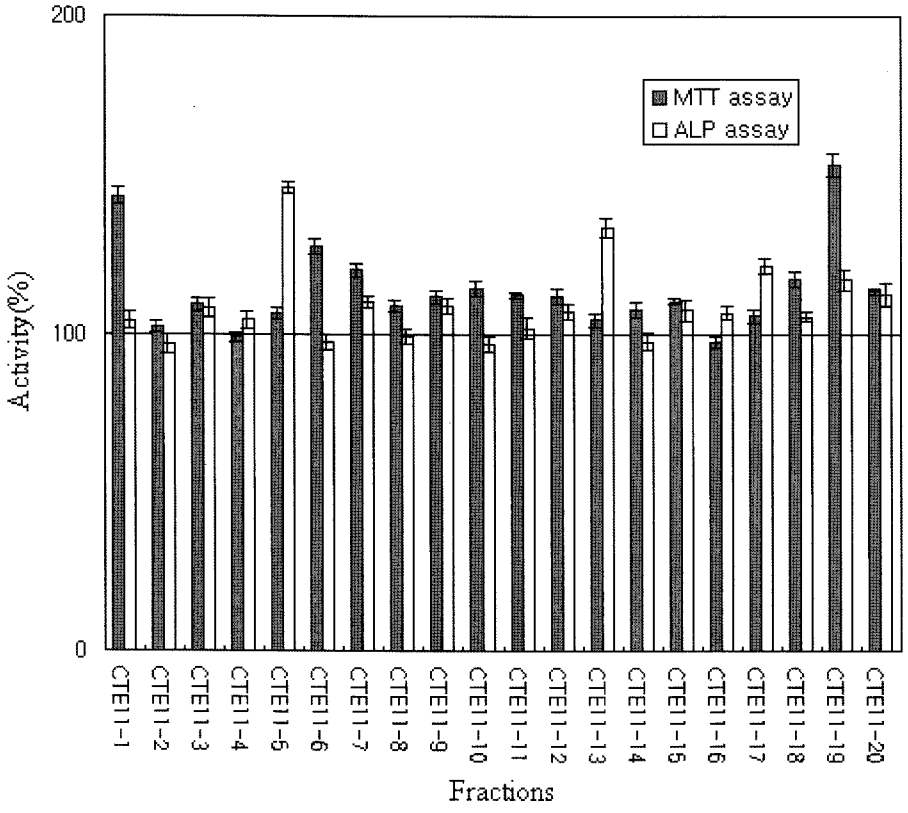


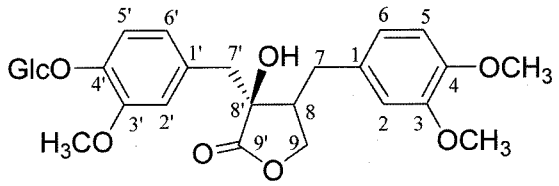
Fig. 2. Activity of subfractions from the EtOAc E11 fraction of the seed of safflower on human osteoblast-like cells (Saos-2). The values indicate the mean's of three replications of experiment for the activity of each fraction (100 μg/mL) on that of control.

(δ_C 122.8, 120.6, 115.4, 112.7, 114.6, 112.1) were observed. In addition, one oxygenated quaternary (δ_C 76.1), one oxygenated methylene (δ_C 70.4), one methine (δ_C 43.6), two methylene (δ_C 41.1, 31.3), and three oxygenated methyl (δ_C 55.5, 55.4, 55.4) signals were observed. Through the comparison of the chemical shifts of sugar and those cited in the literature (18), the sugar in the compound was identified with certainty as a β-D-glucose. Thus, these results led to the conclusion that compound **1** is a lignin glucoside composed of two phenyl propanoids and compound **1** was identified as 8*S*,8'*S*-4',8'-dihydroxy-3,4,3'-trimethoxy-lignan-olide(9,9')-4'-β-D-glucopyranoside (tracheloside) given comparisons using the physical and spectral data with that identified from the literature (19) (Fig. 3).

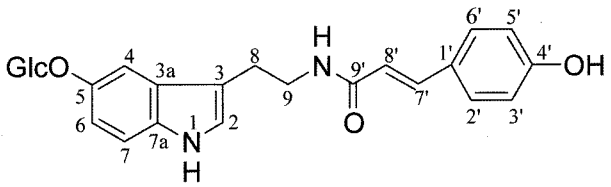
Compound **2**, colorless crystals, showed absorbance bands due to amine and hydroxyl (3750-3100 /cm) and olefin (1660 /cm) in the IR spectrum (CH_3OH) and a molecular ion peak $[M+1]^+$ at *m/z* 485 in the positive FAB/MS. When compound **2** was further developed by silica gel TLC, the spot showed an orange colorization in response to spraying with dragendorff's reagent and subsequent heat, indicating the presence of an alkaloid in the molecular structure. In the ^{1}H-NMR spectrum (400 MHz, CD_3OD), two olefin methines of trans-form (δ_H 7.41, 6.35), six olefin methines originating from a benzene ring (δ_H 7.38, 7.33, 7.19, 7.01, 6.93, 6.74), one N-methylene (δ_H 3.52), and one methylene (δ_H 2.90) signal were observed. Also, the observation of one oxygenated methine signal (δ_H 4.87) of an anomeric proton and a number of oxygenated methine and methylene signals demonstrated the presence of a molecule of sugar. In the ^{13}C-NMR (100 MHz, CD_3OD), 25 signals consisting of one N-ketone (δ_C 169.1), two olefin oxygenated quaternary (δ_C 160.2, 152.6), four olefin quaternary methines (δ_C 134.3, 128.9, 127.5, 113.1), ten olefin methines (δ_C 141.6, 130.4, 130.4, 124.5, 118.3, 116.6, 116.6, 114.1, 112.5, 106.6), and two methylene (δ_C 41.4, 26.4) signals were observed, leading to the conclusion that compound **2** is serotonin-like. Compound **2** was ultimately identified as N-(*p*-coumaroyl)-serotonin mono-β-D-glucopyranoside through comparison of the physical and spectral data with literature citations (20) (Fig. 3).

The ALP activity responses of the Saos-2 to compounds **1** and **2** were 149.2±4.2 and 138.9±3.5% at a concentration of 100 μg/mL (Table 1). Although compounds **1** and **2** showed relatively weaker effects on ALP than the well known ALP activating hormone estradiol (E2) (14), the fact that the compounds could in theory improve a physiological disorder caused by osteoporosis and identified in an edible natural source could prove significant. The compounds have comparatively high ALP activity with little or no cytotoxicity to the human osteoblast-like cells. It suggested that the compounds could improve mineralization in mechanism of bone formation against osteoporosis (21, 22).

Although safflower has been well known to Chinese medicine as an effective natural source for the recovery of bone mass or osteoporosis, no study on the principal components of the plant that might demonstrate the relevant bioactivity has been reported. In summary, active compounds likely related to bone formation derived from the plant seeds of safflower were sought, isolated and characterized through activity-guided fractionation. Two active compounds were precisely identified NMR, MS,



tracheloside (**1**)



N-(*p*-coumaroyl)-serotonin mono-β-D-glucopyranoside (**2**)

Fig. 3. Chemical structure determination for compounds 1 and 2 isolated from the seed of safflower. 8S,8'S-4',8'-dihydroxy-3,4,3'-trimethoxy-lignan-olide(9,9')-4'-β-D-glucopyranoside (tracheloside, 1): colorless crystals (CHCl3-MeOH); m.p. 168-170°C; $[\alpha]_D$ = -60.0° (c=0.2, pyridine); pos. FAB/MS *m/z*: 551 $[M+1]^+$, 532, 370; IR_ν ($CHCl_3$-MeOH, 1/cm) 3450, 1770, 1610; 1H-NMR (400 MHz, pyridine-d5, δ) 7.59(1H, d, J=8.5 Hz, H-5'), 7.15(1H, d, J=2.0 Hz, H-2'), 7.04(1H, dd, J=8.5, 2.0 Hz, H-6'), 6.91(1H, d, J=8.1 Hz, H-5), 6.90(1H, d, J=1.7 Hz, H-2), 6.86(1H, dd, J=8.1, 1.7 Hz, H-6), 5.66(1H, d, J=7.2 Hz, H-1"), 4.30(1H, dd, J=9.2, 8.4 Hz, H-9a), 4.16(1H, dd, J=8.4, 7.6 Hz, H-9b), 3,79(3H, s), 3.74(3H, s), 3.73(3H, s), 3.63(1H, d, J=13.2 Hz, H-7'a), 3.31 (1H, d, J=13.2 Hz, H-7'b), 2.73(1H, m, H-8); 13C-NMR (100 MHz, pyridine-d5, δ) 178.7(C-9'), 149.4(C-3), 149.4(C-3'), 148.0(C-4), 146.5 (C-4'), 131.8(C-1'), 129.7(C-1), 122.8(C-6'), 120.7(C-6), 115.4(C-5'), 114.6(C-2'), 112.7 (C-2), 112.1(C-5), 101.7(C-1"), 78.3(C-5"), 78.0(C-3"), 76.1(C-8'), 74.3(C-2"), 70.6(C-4"), 70.4 (C-9), 61.7(C-6"), 55.5(OCH3), 55.4(OCH_3), 55.4(OCH_3), 43.6(C-8), 41.1(C-7'), 31.3(C-7). N-(p-coumaroyl)-serotonin mono-β-D-glucopyranoside (2): colorless crystals ($CHCl_3$-MeOH); m.p. 228-230°C; $[\alpha]_D$ = –24.7° (c= 0.2, $CHCl_3$); pos. FAB/MS *m/z*: 485 $[M+1]^+$, 484, 334; IR_ν (CH_3OH, 1/cm) 3750-3100, 1660; 1H-NMR (400 MHz, CD_3OD, δ) 7.41(1H, d, J=16.2 Hz, H-7'), 7.38(1H, s, H-4), 7.33 (2H, d, J= 7.8 Hz, H-2', 6'), 7.19(1H, d, J=8.8 Hz, H-7), 7.01(1H, s, H-2), 6.93(1H, d, J=8.8 Hz, H-6), 6.74(2H, d, J=7.8 Hz, H-3', 5'), 6.35 (1H, d, J=16.2 Hz, H-8'), 4.87(1H, d, J=7.6 Hz, H-1"), 3,52(2H, br s, H-9), 2.90(2H, br s, H-8); 13C-NMR (100 MHz, CD_3OD, δ) 169.1(C-9'), 160.2(C-4'), 152.6(C-5), 141.6(C-7'), 134.3(C-7a), 130.4 (C-2', 6'), 128.9(C-1'), 127.5(C-3a), 124.5(C-2), 118.3(C-8'), 116.6 (C-3', 5'), 114.1(C-6), 113.1(C-3), 112.5(C-7), 106.6(C-4), 104.0 (C-1"), 78.0(C-5"), 77.9(C-3"), 75.1(C-2"), 71.6(C-4"), 62.6(C-6"), 41.4(C-9), 26.4(C-8).

Table 1. Activity of tracheloside (1) and N-(*p*-coumaroyl)-serotonin mono-β-D-glucopyranoside (2) from the seeds of safflower on human osteoblast-like cells (Saos-2)[1)]

	1 (100 μg/mL)	**2** (100 μg/mL)
MTT assay	104.9±2.7%	105.6±3.1%
ALP assay	149.2±4.2%	138.9±3.5%

[1)]The values indicate the mean's of three replications of the experiment for the activity of each fraction compared to that of control.

and IR for the first time, as reported.

Acknowledgments

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