A lignan with glucose uptake activity in 3T3-L1 adipocytes from the stem bark of *Knema patentinervia*

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Abstract: A new naturally occurring dibenzylbutyrolactone lignan named isocubebinic ether has been isolated from *Knema patentinervia*. The structure was established by spectroscopic methods, which include Ultraviolet, Infrared, Nuclear Magnetic Resonance and Mass Spectrometry. The compound showed activity in the stimulation of glucose uptake by 3T3-L1 adipocytes.

Keywords: *Knema patentinervia*, lignan, glucose uptake, 3T3-L1 adipocytes.

INTRODUCTION

Members of the genus *Knema* are evergreen trees in the family Myristicaeae. In Malaysia this family is called "penarahan" meaning blood, referring to the red resin secreted from the bark (Taher *et al*., 2013). Previous study reported that *Knema laurina* exhibited acetylcholinesterase inhibitory activity (Akhtar *et al*., 2011). The extract of *Knema attenuata* showed potential larvicidal activity against *Aedes albopictus* and *A. stephensi* (Vinayachandra and Chandrashekar, 2011). Previous phytochemical studies reported that the genus contained stilbene and lignan metabolites (Gonzales *et al*., 1993; Rangkaew, 2009; Pinto, 1990). Lignan is a class of secondary metabolites possessing a wide range of biological properties such as antioxidant, anticancer, antimicrobial, anti-inflammatory and immunosuppressive activities (Saleem *et al*., 2005). Cubebin was firstly obtained from the fruit of *Piper cubeba*, which has been used since centuries ago. It was reported that cubebin exhibited bioactivities as anti-inflammatory (Souza *et al*., 2004), antiprotozoal (Esperandim *et al*., 2013), cytotoxic and antimutagenicity (Niwa *et al*., 2013). Cubebin has been used for starting material in synthesizing dibenzylbutyrolactone lignan derivatives (da Silva *et al*., 2005) and unusual features of lignans (Batterbee *et al*, 1969). As reported previously, the stembark of *Knema patentinervia* contained a sesquiterpene (Taher, 2013). Therefore, this paper reports the presence of a new naturally occurring lignan from the stem bark of *K. patentinervia* together with its glucose uptake activity in 3T3-L1 adipocytes cells.

MATERIALS AND METHODS

General experimental procedures

The melting point was measured on a Buchi B545 melting point apparatus and is uncorrected. The ultraviolet (UV) spectrum was recorded using a Secomam Uvi light XT2 spectrophotometer with methanol as the solvent. The infrared (IR) spectrum was obtained from a Perkin Elmer infrared spectrophotometer. The NMR spectra were recorded on a Bruker Avance 400MHz in $CDCl_3$. Chemical shifts were reported in ppm and the coupling constants were given in Hz. The ESI-MS spectra were recorded on a Thermo LTQ-FT mass spectrometer (mass accuracy= RMS 2 ppm with external calibration) in the Mark Wainwright Analytical Centre at The University of New South Wales, Sydney, Australia. All chemicals and organic solvents used for extraction, fractionation and purification were purchased from Merck, Germany (analytical grade). Thin layer chromatography (TLC) of compounds was conducted on thin layer aluminium plates of 0.2mm of Merck-pre-coated silica gel F_{254}. Merck silica gel 230-400 and 70-230 mesh were used for vacuum liquid chromatography (VLC) and column chromatograph (CC), respectively.

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Cell culture materials

High glucose Dulbecco's modified eagle medium (DMEM), foetal bovine serum (FBS) and penicillin-streptomycin (10,000 units) were obtained from Gibco (Invitrogen Co.). 2-Deoxy-D-[^{3}H]-glucose was obtained from Perkin Elmer (Switzerland). Sodium orthovanadate, metformin, 3-isobutyl-1-methyl-xanthine (IBMX), dexamethasone and insulin were purchased from Sigma (St. Louis, MO).

Plant material

The stem bark of *K. patentinervia* was collected from Taman Pertanian Kuantan in March 2010. The plant was identified and confirmed by Dr. Shamsul Khamis (Institute of Bioscience, Universiti Putra Malaysia). A voucher specimen (MT17) of this plant was deposited at the Herbarium of the Faculty of Pharmacy, International Islamic University Malaysia, Kuantan, Pahang, Malaysia.

Cell lines

Mouse 3T3-L1 fibroblast (CL-173) was obtained from the American Type Culture Collection (ATCC) and grown in complete medium containing DMEM, foetal bovine serum and penicillin-streptomycin.

Extraction process

Dried and powdered *K. patentinervia* (758g) was extracted with hexane (3L), ethyl acetate (3L) and methanol (3L), successively. Each extract was evaporated under reduced pressure to obtain *n*-hexane (1.2g, 0.15%), EtOAc (29.9g, 3.9%) and MeOH (81g, 10.6%) extracts. A portion of the EtOAc extract (25g) was fractionated by VLC on silica gel with hexane, DCM: hexane (1:1), DCM, DCM: acetone (1:1) and acetone. The hexane: DCM (1:1) fraction (0.9g) was chromatographed by CC with hexane, hexane: DCM (9:1 →1:9) and DCM to yield 22 fractions. Fractions 1-8 were combined to give compound 1 (162 mg).

Isocubebinic ether 1. White amorphous powder, mp 113-114ºC, UV λ_{max} nm MeOH: 235, 287. IR (KBr) υ_{max} cm^{-1}: 1636, 1488, 1240, 1038, 925, 809, 640. ESI-MS m/z: 338, 204, 203, 136, 135 (100), 79, 77. ^{1}H NMR and ^{13}C NMR (table 1).

Cell culture

The 3T3-L1 preadipocytes were cultured and maintained as previously described by Susanti *et al*. (2012). After counting, cells were seeded in 25 ml flasks at a density of 2.0 x 10^4 cells/ml for 3T3-L1 cells. A total volume of 10 ml of pre-warmed growth medium was added to the cells. 3T3-L1 preadipocytes were grown in DMEM medium supplemented in a 37˚C incubator with 5% CO_2 in DMEM containing 10% foetal bovine serum, 1% penicillin (10.000U/ml) and 1% streptomycin (10.000µg/ml).

Adipocyte differentiation

3T3-L1 preadipocytes were grown in a complete medium containing DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37ºC under a humidified 5% CO_2 incubator. The cells were then seeded into 12-well plates at a density of 2.0 x 10^{4}cells/ml. For differentiation, two days post-confluence (defined as day-0), cells were stimulated to differentiate with an adipogenic cocktail containing MDI (0.5mM IBMX, 0.25mM dexamethasone, 1µg/ml insulin) for two days. To examine the effect of compounds on adipocyte differentiation, confluence 3T3-L1 preadipocytes were treated with various concentrations of test compounds (10 µM, 20µM and 50µM) at day-0 or with insulin (100nM) in the presence of the adipogenic cocktail. On day 2, differentiating media was replaced with a complete medium containing 1µg/ml insulin and incubated for another 2 days (day-4). Thereafter, the cells were maintained in the complete medium for an additional 4 days (day-8) with the medium changed once every 2 days (Nidhina *et al.*, 2011).

Deoxy-[^{3}H]-D-Glucose uptake assay

Glucose uptake activity was analysed by measuring the uptake of radiolabelled glucose from the culture medium by adipocytes. Briefly, the differentiated adipocytes, which were grown in 12-well plates, were washed twice with serum-free DMEM and incubated for 3h at 37ºC with 1 ml of fresh serum-free DMEM. The cells were washed three times with Krebs-Ringer HEPES (KRPH) buffer (118mM NaCl, 5mM KCl, 1.3mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4 and 30mM HEPES, pH 7.4) and incubated with 0.9 ml of KRPH buffer for 30 min at 37ºC. Test compounds including the control were added, and the cells were incubated at 37ºC for a further 60 min. Glucose uptake was initiated by the addition of 0.1ml of KRPH buffer containing 2-deoxy-D-[^{3}H] glucose (0.037 MBq; Perkin Elmer) and glucose (0.001mM). After 60 min, the cells were washed three times with ice-cold PBSA to stop the glucose uptake. The cells were then lysed through incubation for 20 min at 37ºC with 0.7ml of 1% Triton X-100. The radioactivity levels in the cell lysates were determined using a Tri-Carb 2700TR liquid scintillation counter Packard Instrument Co. Samples from each lysate were counted and measured in triplicate.

STATISTICAL ANALYSIS

Results were presented as means ± standard error of three experiments. Data were analysed by ANOVA using SPPS version 19. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Compound 1 (fig.1) was found a white amorphous powder. Its molecular formula was deduced as $C_{20}H_{18}O_5$ on the basis of an ion at m/z 338 which appeared in ESI-

MS data. It is in agreement with isocubebinic ether, a synthetic product of (-)-cubebin which gave cleavage to m/z 203 and 135 (Batterbee *et al*., 1969).

The UV spectrum of compound 1 showed absorbance maxima at 235 and 287 nm exactly typical of cubebin derivatives (Batterbee *et al*. 1969). The infrared spectrum of the compound exhibited bands at 1636 and 1488 (aromatic rings) and 925cm^{-1} (methylenedioxy) (Batterbee *et al*. 1969; Sing *et al*, 1989). The ^{1}H and ^{13}C NMR data are given in Table 1.

Adipocytes which were treated with compound 1 significantly stimulated the glucose uptake dose-dependently with 2.14 fold, 2.93 fold and 3.30 fold increases at doses 10, 20 and 50µg/ml, respectively (fig. 2), as compared to basal conditions. Metformin and sodium orthovanadate were used as positive controls.

DISCUSSION

The NMR spectrum showed *ortho*-coupled aromatic signals at δ 6.78 (1H, d, *J* 8.0 Hz) and 6.69 (1H, dd, *J* 8.0,

Table 1: NMR data of compound 1 in $CDCl_3$

C Number	δ_H (multiplicity, J/Hz)[a]	δ_C (ppm)[b]	HMBC
2	4.45 (1H, s)	79.7	C-4, C-6, C-5, C-6’
3	2.31 (1H, t, 8.0)	48.4	C-5, C-6
4	2.44 (1H, m)	38.0	
5’	3.70 (1H, d, 8.0)	71.2	C-6
5’	4.16 (1H, ddd, 8.0, 5.6, 2.0)		C-3, C-6
7’	2.73 (1H, dd, 14.0, 8.0)	36.7	C-2, C-4, C-2”, C-6
7’	2.60 (1H, dd, 14.0, 8.0)		C-3, C-4
6’	2.76 (1H, dd, 16.0, 2.0)	38.2	
6b	3.05 (1H, ddd, 16.0, 2.0, 2.0)		
1’	-	133.4	
2’	6.59 (1H, s)	108.2	C-2
3’	-	147.4	
4’	-	145.5	
5’	6.61 (1H, s)	109.3	C-1’, C-3’
6’	-	127.7	
4’,5’-OCH_2O	5.90 (2H, m)	100.8	C-3’, C4’
1”	-	133.9	
2”	6.73 (1H, d, 1.6)	109.3	C-6”
3”	-	147.7	
4”	-	145.9	
5”	6.78 (1H, d, 8.0)	107.8	C-1”, C-3”
6”	6.69 (1H, dd, 8.0, 1.6)	121.9	C-4”
4”,5”-OCH_2O	5.96 (2H, m)	100.8	C-3”, C-4”

[a]recorded at 400 MHz; [b]recorded at 100 MHz.

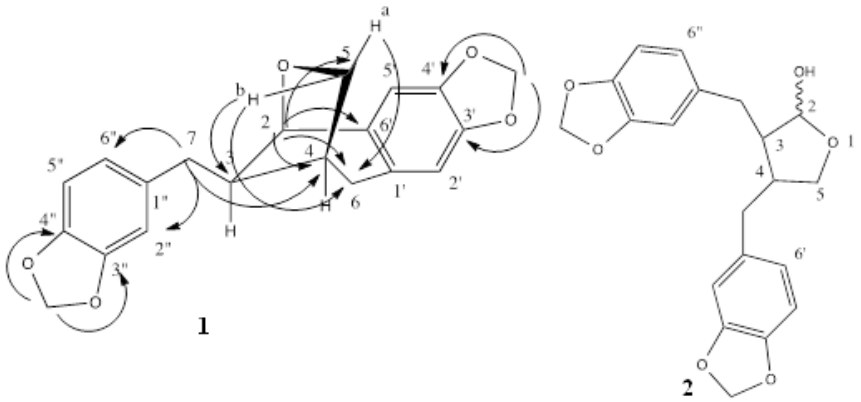

Fig. 1: Key HMBC correlations of compound 1 and structure of (-)-cubebin (2)

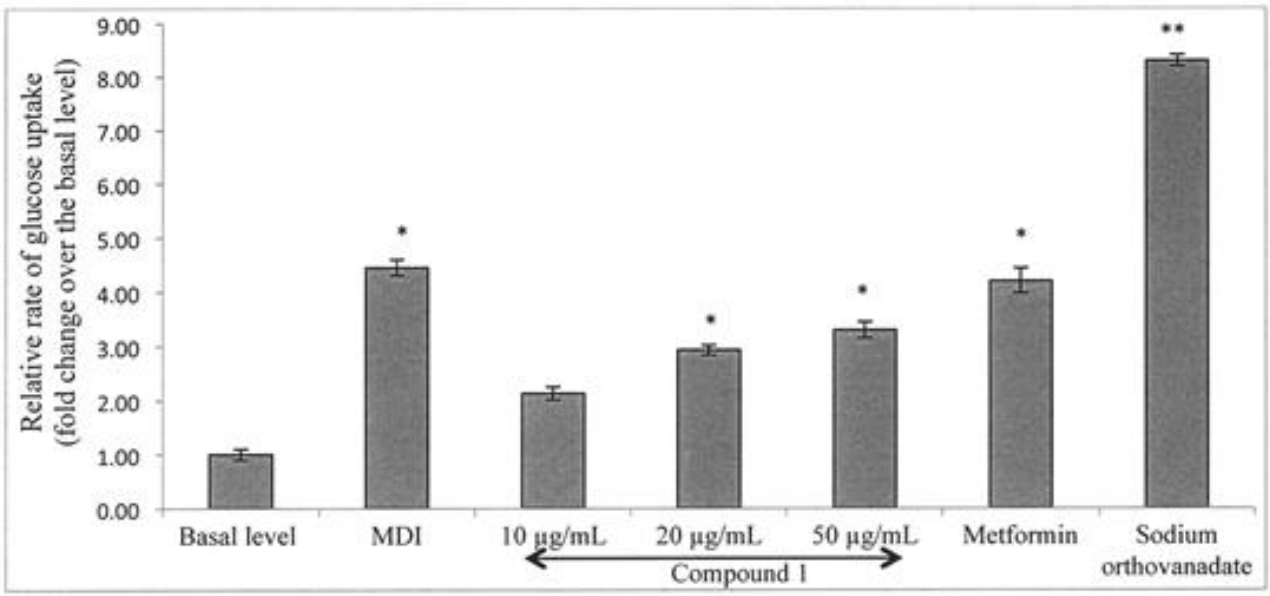

Fig. 2: The effects of compound 1 on glucose uptake in 3T3-L1 adipocytes. Adipocyte cells in 12-well plates were incubated for 60 min with compound 1 extracts at dose 10, 20 and 50µg/ml or with MDI, metformin (1mM) and sodium orthovanadate (5mM) as a positive control, or with normal glucose (basal cell), then assayed for 2-deoxy-D-[^{3}H] glucose uptake. Levels of radioactivity in the cell lysates were determined using a liquid scintillation counter. Data are means ± SD, (n = 3). *p<0.05, **p<0.01 compared with the control group (basal).

2.0 Hz) with *meta*-coupling to a signal at δ 6.73 (1H, d, *J* 1.2 Hz), indicative of a 1,3,4-trisubstituted benzene. Another two singlet aromatic proton signals at δ 6.59 (1H, s) and 6.61 (1H, s) indicated a 1,2,4,5-tetrasubstituted benzene fragment. Two characteristic signals of methylenedioxy groups appeared as a non-identical signal at d 5.90 (2H, m) and 5.96 (2H, m). In (-)-cubebin (2), these signals appeared as an overlapping signal for four protons (Batterbee *et al*., 1969). These correlated with two overlapping carbon signals at δ 100.86 in an HMQC experiment. These ^{1}H NMR spectroscopic signal patterns accounted for 14 of the total 20 carbons and were consistent with the combination of two safrole-like fragments possibly linked through a 3,4-type lignan coupling, but possessing additional bond formation at one of the adjacent aromatic ring positions. The remaining nine aliphatic C–H protons were assigned to three CH_2 and three CH groups with the help of ^{13}C NMR DEPT and ^{1}H–^{13}C HMQC experiments (table 1). A signal for one proton adjacent to oxygen was clearly downfield at δ 4.45 (1H, s, H-2) and no coupling could be observed due to dihedral angle of H(2)-H(3) *ca*. 80^o (Batterbee *et al*. 1969); it makes unusual features of the structure compared to that of (-)-cubebin. The H-5 methylene protons which are non-equivalent appeared as two signals at δ 3.70 and δ 4.16 indicated for H-5a and H-5b, respectively with coupling constants $J_{5a\text{-}5b}$ (8 Hz)-this is in agreement with Batterbee *et al*. (1969). A large shift of the H-5 methylene was due to a relatively rigid cyclic molecule like the cyclic ether. There is a coupling between H-5b and H-4 as indicated in COSY spectrum. The HMBC spectrum showed the correlations between δ_H 4.45 (H-2) with δ_C 38.0 (C-4), δ_C 71.2 (C-5), δ_C 38.2 (C-6) and δ_C 127.7 (C-6') and supported by correlations of δ_H 3.70 (H-5a) and δ_H 4.16 (5b) with δ_C 48.4 (C-3) and δ_C 38.2 (C-6) confirmed the ether cyclisation. The correlations of proton at δ_H 2.73 (H-7a) with carbons at δ_C 109.3 (C-2") and δ_C 121.9 (C-6") indicated the substitution was at δ_C 133.9 (C1"). The position of methylene groups at H-6 which is adjacent to one methylenedioxy group was confirmed by a coupling of H-6a and aromatic carbon C-2' in HMBC spectrum. The other methylene protons appeared at δ 2.60 and 2.73 were assigned for H-7b and H-7a, respectively. The multiple coupling to the proton at C-4 is consistent with the structure and configuration. On the basis of these data, the structure of the lignan is proposed as isocubebinic ether (1). This substance has not been reported as a natural product, but has been proposed as a synthetic product derived from (–)-cubebin via acetic acid and sulfuric acid reactions (Batterbee *et al*. 1969).

Lignans are a class of phenylpropanoids found in edible plant sources (Smeds *et al*., 2007). A review by Saleem *et al*. (2005) reported that lignans have anticancer, antioxidant, antimicrobial, anti-inflammatory and immunosuppressive activities. Kong *et al* (2014) reported that phillyrin, a natural lignan may have the potential to ameliorate insulin resistance in obese adipose tissue. In relation to that, we evaluated the effect of compound 1 on glucose uptake in 3T3-L1 adipocytes. It was found that compound 1 significantly (p<0.05) improved glucose

uptake glucose uptake in 3T3-L1 adipocytes dose dependent manner, which was compared to control group (basal). Further study is recommended to evaluate antidiabetic effect of the compound in animal model.

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