Jurnal Teknologi, 44(F) Jun 2006: 53–68

A PROANTHOCYANIDIN FROM *CINNAMOMUM ZEYLANICUM* STIMULATES PHOSPHORYLATION OF INSULIN RECEPTOR IN 3T3-L1 ADIPOCYTES

MUHAMMAD TAHER[1]*, FADZILAH ADIBAH ABDUL MAJID[2] & MOHAMAD ROJI SARMIDI[3]

Abstract. Non-insulin-dependent diabetes mellitus is due to in large part to insulin resistance, a state where the target cells are no longer responding to ordinary levels of circulating insulin. A drug that promotes the initiation of insulin receptor (IR) signaling by enhancing IR phosphorylation should be useful in the treatment of Type 2 diabetes. To investigate a compound that is able to phosphorylate IR, a proanthocyanidin, cinnamtannin B1, has been isolated from the stem bark of *Cinnamomum zeylanicum* Blume (Lauraceae). The structure of the compound was established by spectroscopic methods. Cinnamtannin B1 (0.11 mM) activates the phosphorylation of insulin receptor β-subunit on 3T3-L1 adipocytes. Like insulin, cinnamtannin B1-stimulated phosphorylation of insulin receptor was inhibited by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase (PI3K) and cytochalasin B, an inhibitor of the glucose transporter (GLUT4); otherwise the phosphorylation was enhanced by vanadate, a tyrosine phosphatase inhibitor. These results suggest that the activity of cinnamtannin B1 is specially mediated by phosphorylation of IR through activation of the PI3K.

Keywords: *Cinnamomum zeylanicum*, cinnamtannin B1, phosphorylation, insulin receptor, adipocyte

Abstrak. Sebahagian besar penyakit kencing manis yang tidak bergantung kepada insulin adalah berpunca daripada hambatan terhadap insulin. Keadaan ini berlaku apabila sel sasaran gagal bertindak balas dengan insulin pada paras biasa. Ubat yang mampu memulakan isyarat penerimaan insulin dengan menaikkan fosforilasi penerima insulin adalah sangat berguna untuk merawat kencing manis Jenis 2. Dalam usaha untuk mencari suatu sebatian yang mampu untuk memfosforilasikan penerima insulin, sejenis proantosianidin, cinnamtannin B1, telah diasingkan daripada kulit pokok kayu manis (*Cinnamomum zeylanicum*). Struktur sebatian dibina dengan menggunakan kaedah spektroskopi. Cinnamtannin B1 didapati mampu menggalakkan fosforilasi β-subunit penerima insulin pada 3T3-L1 adiposit. Seperti insulin, keupayaan cinnamtannin B1 untuk menggalakkan fosforilasi penerima insulin disekat oleh wortmannin (pembantut khusus bagi fosfatidilinositol 3-kinase) dan cytochalasin B (pembantut yang menjejaskan prestasi pengangkut glukosa). Sebaliknya, fosforilasi penerima insulin ditingkatkan oleh vanadate, iaitu pembantut enzim tirosin fosfatase. Keputusan uji kaji menunjukkan bahawa aktiviti cinnamtannin B1 dalam meningkatkan pengambilan glukosa dalam sel berlaku apabila cinnamtannin B1 melekat pada penerima insulin dan seterusnya memfosforilasikannya melalui pengaktifan PI3-kinase.

Kata kunci: *Cinnamomum zeylanicum*, cinnamtannin B1, fosforilasi, penerima insulin, adiposit

[1,2&3] Department of Bioprocess, Faculty of Chemical and Natural Resources Engineering, Universiti Teknologi Malaysia, 81310 UTM, Skudai, Johor, Malaysia

* Corresponding author: Tel: +607-5535884, Fax: +607-5581463, Email: muhammad_taher@yahoo.com

1.0 INTRODUCTION

Insulin plays a key role in regulating a wide range of cellular processes [1]. Insulin mediates a wide spectrum of biological responses including stimulation of glucose uptake, glycogen, lipid and protein synthesis, antilipolysis, activation of transmembrane of specific genes, and modulation of cellular growth and differentiation [2]. Insulin resistance is an important feature of Type 2 diabetes, which is due to a reduce ability of insulin in activating its signaling pathways [3]. Insulin binding activates the tyrosine kinase, leading to autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit and tyrosine phosphorylation of the IRS-protein [4].

Phosphorylation of protein has been postulated to be an important regulatory mechanism in the action of insulin and other hormones. Immunoprecipitation of the labeled receptor demonstrated that there are two major subunits of insulin receptor phosphorylated with molecular weight of 135 000 and 95 000 that correspond to the receptor of alpha and beta subunit of the insulin receptor [5]. The cellular response to insulin is mediated through the insulin receptor, which is a tetrameric protein consisting of two identical extracellular α-subunits that bind insulin as well as two identical transmembrane β-subunits that have intracellular tyrosine kinase activity [6]. When insulin binds to the α-subunit of the receptor, the β-subunit tyrosine kinase is activated, resulting in autophosphorylation of β-subunit tyrosine residues [7].

The genus *Cinnamomum* (cinnamon) comprises 250 species which are distributed in Asia and Australia. Many species of cinnamon yield volatile oils on distillation. The most important cinnamon oils in world trade are those from *C. zeylanicum*, *C. cassia*, and *C. camphora*. Cinnamon offers a variety of oils with different aroma characteristics and composition which benefited to the flavor industry. The root bark oil was reported to have camphor as the main constituent, but does not seem to have commercial value, unlike leaf and stem bark oils. Major compounds present in stem bark oil and root bark oil are cinnamaldehyde (75%) and camphor (56%), respectively. Cinnamon bark oil possesses the delicate aroma of the spice with a sweet and pungent taste [8].

Cinnamomum zeylanicum (in Malay called *kayu manis*) has mildly astringent and aromatic properties and is used in European medicine. In the list of Johor medicines, *kayu manis* has a place for the treatment of colic and diarrhea [9].

Nonaka *et al.* [10] pioneered the study on proanthocyanidins from cinnamon. Among several plant materials of the genus *Cinnamomum* examined, only the bark of *Cinnamomum zeylanicum* contained a major phenolic metabolite of doubly linked proanthocyanidins (A-Type). While in other materials, such as the bark of *C. burmanii* and *C. cassia* and the root bark of *C. camphora*, the major phenolic metabolites consisted of linearly linked proanthocyanidins.

Proanthocyanidin is an important class of secondary plant metabolites. It is found mostly in woody plants which consist of flavan-3-ol units singly or doubly through interflavanoid linkages [11]. Proanthocyanidins possess doubly-linked structures (the dimer belonging to this class are designated as A-type) that are rarely found in nature and limitedly to members of the families Apocynaceae [12], Hippocastanaceae [13], Ericaceae [14], Lauraceae [10], Polypodiaceae [15], Rosaceae [16], Rubiaceae [17], and Sapindaceae [18].

The increasing interest in proanthocyanidin derivatives is based on their biological activity [19]. The prominent biological and pharmacological effects reported for proanthocyanidin can be classified into antimicrobial [14] and antiviral activities [20], enzyme inhibition, antioxidative effects [21], antimutagenic and antitumoral properties [22], and some more specific interactions e.g. with cardiovascular systems and inflammation processes [23].

This paper describes the isolation and the biological activity of cinnamtannin B1 in stimulating phosphorylation of insulin receptor β-subunit on 3T3-L1 adipocytes. The insulin receptor phosphorylation plays an important role in glucose uptake cascade. The impairing of glucose uptake mostly occurs in patients with Type 2 diabetes. A drug that promotes the initiation insulin receptor signaling by stimulation of phosphorylation is useful in the treatment of Type 2 diabetes.

2.0 MATERIALS AND METHODS

2.1 Chemicals

All cell culture grade chemicals were purchased from Sigma-Aldrich and Gibco BRL. Analytical grade chemicals were purchased from Merck-Schuchardt (Mallinckrodt). Silica gel 60 F_{254} for thin layer chromatography and silica gel for column chromatography were purchased from Merck (Darmstadt, Germany). Sephadex-LH 20 was purchased from Pharmacia (Uppsala, Sweden). Molecular weight markers for proteins (broad range) were purchased from Promega (Madison, WI). Antiphosphotyrosine (4G10) (HRP conjugate), anti-insulin receptor β-subunit, phosphotyrosine control (EGF-stimulated A431 cell lysate), and secondary antibody (goat anti-rabbit IgG, HRP conjugate) were purchased from Upstate Biotechnology (Lake Placid, NY).

Ultraviolet spectra was recorded on the UV-100PC Shimadzu in methanol. Infrared (IR) spectra was run using KBr disc on FT-IR Perkin Elmer 1600. FAB-MS was recorded on a VG (Micromass) 70-SE mass spectrometer. ^{1}H NMR (300 MHz), ^{13}C (100 MHz), and ^{1}H-^{1}H COSY NMR spectrum were recorded on a Bruker Avance 300 MHz spectrometer.

2.2 Plant Materials

Cinnamomum zeylanicum Blume was collected from West Sumatra province, Indonesia in July 2001. A voucher specimen (MT-09) is deposited at the Herbarium of Universitas Andalas, Padang, Indonesia.

2.3 Isolation and Purification

Dried and powdered stem bark of *C. zeylanicum* Blume (750 g) was extracted by soxhlet extractor for 18 hours with *n*-hexane, and acetone successively. The acetone (50 g) extract was fractionated by vacuum liquid chromatography on silica gel 60 (230-400 mesh, Merck) and eluted with EtOAc and EtOAc: MeOH (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) and methanol. Each fraction was analyzed by TLC (Silica gel 60 F_{254}, Merck). The combined fraction of EtOAc: MeOH (8:2) was evaporated by rotary evaporator and separated by column chromatography on silica gel 70-230 mesh and eluted with *n*-hexane: EtOAc (1:1), EtOAc, acetone, and MeOH. The fractions were collected in a test tube (25 mL) and the same R*f* was combined to give fractions 1-5, 6-20, and 21-44. The combined fraction of 6 - 20 was separated using silica gel column chromatography and eluted with EtOAc and EtOAc: MeOH (8:2) afforded fraction of 4-10, 11-19, and 20-29. Fraction of 4-10 was washed with acetone to yield an off-white amorphous solid. The isolated compound was then purified using Sephadex LH-20 and eluted with methanol to produce Compound 1 (0.6403 g, 0.085%) (Figure 1).

2.4 Acetylation of Compound 1

Compound 1 (60 mg) was acetylated with Ac_2O/pyridine (1:1). The mixture was stirred at room temperature for 24 hours. The mixture was poured into ice cold distilled water and extracted with ethyl acetate. The ethyl acetate extract was evaporated and applied to column chromatography on Sephadex LH-20 and eluted with hexane: ethyl acetate (1:1) to give acetate derivative as pale brown amorphous solid (58 mg) (Compound 2) (Figure 1).

2.5 Cell Culture

The 3T3-L1 adipocytes cells were cultured and maintained as previously described by Student *et al*. [24]. Cell culture was propagated at 37°C in a humidified atmosphere of 5% CO_2 in DMEM containing 10% fetal bovine serum, 1% penicillin (10 000 U/mL), and 1% streptomycin (10 000 µg/mL). Induction of differentiation was done one to two days post confluence. Cells were maintained in differentiation DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin and proliferation cocktail (0.25 mM dexamethasone, 0.5 mM 1-isobutyl-3-methylxanthine (IBMX), and 1 µg/mL insulin) for four days and the medium was

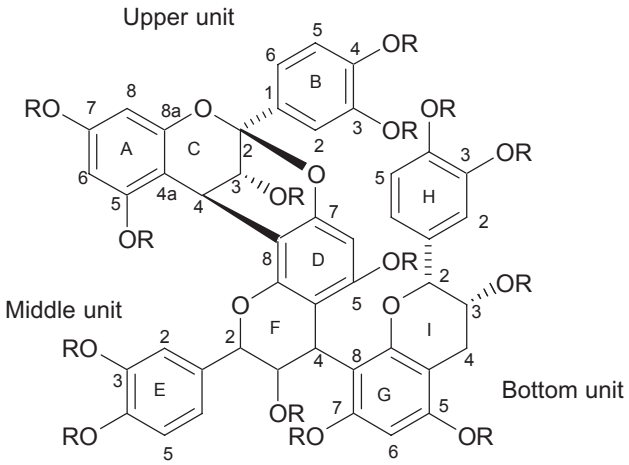

Figure 1 **R=H** is a cinnamtannin B1 (Compound 1), **R=acetate** is an acetate derivative (Compound 2)

changed every two days. At day 4, the dexamethasone and IBMX were removed with insulin remaining on the cells for an additional two days. Differentiation was allowed to continue in DMEM supplemented with 10% fetal bovine serum. The sample was tested at day 9 - 14 post-induction.

2.6 Immunoprecipitation

Adipocytes were cultured and differentiated in 25 cm^2 T-flask. Prior to the treatment, cells were serum starved in DMEM for three hours, washed two times, and equilibrated for 15 minutes with KRPH buffer (5 mM Na_2HPO_4, 20 mM HEPES, pH 7.4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 136 mM NaCl, and 4.7 mM KCl). After treatment, cells were washed three times with ice-cold PBS pH 7.4 and lysed with 1 mL of ice cold immunoprecipitation buffer (10 mM phosphate, pH 7.2, 0.14 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl-fluoride, 50 mM NaF, 1.25 mM Na_3MoO_4, 1.25 mM Na_3 VO_4, 12.5 mM $Na_4P_2O_7$,10 µg/mL pepstatin A, 10 µg/mL aprotinin, and 10 µg/mL leupeptin). Cell lysates were clarified by centrifugation at 14 000 rpm. 4 µg precipitating antibody (anti-insulin receptor β-subunit) was added to 300 µL cell lysates (1 mg protein) and incubated with shaking overnight at 4°C. 50 µL of Protein A/G Plus Sepharose (50% slurry in RIPA buffer; 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and 1 mM PMSF) was added and incubated for one hour with shaking at 4°C. Beads were washed three to four times with immunoprecipitation buffer. 30 µL of sodium dodecyl sulfate (SDS)—sample buffer (1 ml glycerol, 0.5 mL β-mercapthoethanol, 3 mL 10% SDS, 1.25 mL Tris-HCl pH 6.8 and 1 mg bromophenol blue) was added, and the beads were boiled for five minutes at 100°C. Proteins were

separated on a 7.5% SDS-polyacrylamide gel electrophoresis (Bio-Rad). The separated protein in SDS PAGE was transferred to nitrocelullose membrane (Hybond C extra, Amersham) using transblot cell (Bio-Rad). Blots were blocked in TBST (10 mM Tris, pH 7.4, 0.9% NaCl, and 0.1% Tween 20) containing 3% milk at room temperature for 30 minutes. Primary antibody was incubated on the blots at a 1:1000 dilution in TBST for 1½ hour at room temperature. Blots were washed several times with TBST, and the secondary antibody was added (1:5000 dilution) for 1½ hour at room temperature. Blots were again washed several times. Immunoreactive proteins were detected with chemiluminescense using ECL Western Blotting Analyses System (Amersham, Little Chalfont, Buckinghamshire, UK). The cells were pretreated with wortmannin (500 nM), cytochalasin B (40 mM), or sodium orthovanadate (5 mM) to examine the specificity of the signaling pathways.

2.7 Enzyme Linked Immunosorbent Assay

3T3-L1 preadipocytes were seeded in 100 µL medium at density of 2×10^3 cells per well in 96 well plates. 2 days post confluent the cells were induced into adipocytes as described in formerly Cell Culture section. After 11 days of induction, the cells were treated with the samples (100 nM insulin and 0.11 mM cinnamtannin B1) for 30 minutes. After treatment, the cells were fixed by adding 100 µl methanol/acetic acid (50: 50) to each well for 10 minutes. The solution was removed by inverting the plate and washed three times with PBS. The plate was tapped by upside down to remove excess PBS. Non-specific antibody was blocked with 200 µl blocking buffer (3% skim milk in PBS) per well for 1 hour at 37°C. The cells were gently washed with PBS and the plate was tapped to remove excess PBS. Antiphosphotyrosine antibody (5 µL) was diluted in 5 mL freshly prepared blocking buffer and added 50 µl to each well excluded a blank. The plate was incubated overnight at 4°C. The cells were washed three times with PBS-Tween 0.05%. HRP conjugate secondary antibody in blocking buffer (1:5000) was added to each well and incubated for 1 hour at room temperature. The complex antigen antibody was washed three times with a washing solution by tapping the plate. 100 µl of mixed ABTS reagent (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate and peroxidase solution B) (KPL, Gaithersburg, MD) was added to each well and incubated for 30 minutes at room temperature and the reaction was stopped by adding 50 µL 4% SDS. The plate was read with ELISA plate reader (Bio-Rad) at 410 nm.

2.8 Statistical Analysis

Data was expressed as the means ± standard error of the means (SEM). Statistical analysis was performed using Sigma plot 8.0. Differences among the results were considered to be statistically significant when P value was < 0.05.

3.0 RESULTS

3.1 Isolation of Proanthocyanidin

The acetone extract of cinnamon consists of essential oil and proanthocyanidins where its TLC pattern showed that the major components were polyphenol that gave positive reaction with ferric chloride. Purification of the acetone extract was carried out by chromatographic methods using silica gel and Sephadex LH-20 to yield Compound 1.

Compound 1 responded positively to the $FeCl_3$ with dark green colour and with anisaldehyde sulphuric acid with dark purple colour. Retention factor (R_f) was 0.26 in EtOAc-HCOOH-HOAc-H_2O (140:2:1:59) (upper phase), m.p 205-207°C (decomposed). a_D^{24} + 70° (c = 0.5, MeOH) (JASCO DIP-370 digital polarimeter). UV (MeOH) λ_{max} (log ε) : 278 (4.09). IR (KBr) ν_{max}: 3400, 2932, 1615, 1525, 1440, 1383, 1284, 1146, 1115, 1065, 1012, 968, 816 cm^{-1}. ^1H and ^{13}C NMR: see Table 1. Positive ion FAB MS (VG (Micromass) 70-SE magnetic sector instrument) *m/z* (rel. int.): 865 $[M+H]^+$ (13), 804 (2), 766 (9), 715 (2), 613 (2), 460 (12), 397 (1), 307 (84), 219 (11), 154 (100).

The acetate derivative (Compound 2) resulted in a negative reaction to iron (III) chloride and dark purple with anisaldehyde-sulphuric acid reagent. R_f. 0.78 in EtOAc-HCOOH-HOAc-H_2O (140:2:1:59) (upper phase), and 0.54 in toluene:acetone (2:1). IR (KBr) ν_{max}: 2938, 1772, 1602, 1508, 1431, 1372, 1208, 1119, 1044, 1014, 901, 840, 588. Positive ion FAB-MS *m/z* (rel. int): 1452 $[M+H]^-$ (13), 1348 (19), 1073 (1), 851 (2), 630 (6), 455 (100), 329 (64), 179 (98).

3.2 Insulin Receptor Phosphorylation

In an attempt to discover a small molecule that can stimulate phosphorylation like insulin, cell-based assay using 3T3-L1 adipocytes was carried out. After inducing the 3T3-L1 preadipocytes with the proliferation cocktail, the mature adipocytes was tested with insulin and/or cinnamtannin B1. Cell lysates were immunoprecipitated with anti-insulin receptor β-subunit, separated by SDS-Page and transfered to nitrocellulose membrane. The blot was immunoblotted with antiphosphotyrosine antibody (Figure 2(a)). Phosphorylation activity of cinnamtannin B1 was inhibited by wortmannin and cytochalasin B, on other hand, enhanced by vanadate (Figure 2(b)).

3.3 ELISA for Phosphorylation

To confirm the phosphorylation effect of cinnamtannin B1 on 3T3-L1 adipocytes, the direct ELISA to intact cell was carried out to detect phosphorylation of insulin receptor β-subunit. Figure 3 shows that cinnamtannin B1 strongly exhibits effect in phosphorylation of insulin receptor. The phosphorylation effect of cinnamtannin B1

Table 1 ^{13}C and ^{1}H NMR data for Compound 1 (300 MHz, in CD_3OD)

Ring	Position	^{13}C NMR	^{1}H NMR[a]
C	2	98.5	-
	3	68.7	3.28, *d* (3.4)
	4	27.5	4.14, *d* (3.4)
F	2	77.2	5.69, *br.s*
	3	71.1	4.14, *d* (3.2)
	4	36.9	4.52, *br.s*
I	2	81.9	4.38, *br.s*
	3	65.8	3.85, *br t*
	4	29.2	2.82, *m*
A	4a	103.4	-
	5	155.2	-
	6	96.9	5.96, *d* (2.0)
	7	156.4	-
	8	95.1	6.00, *d* (2.0)
	8a	153.0	-
D	4a	104.9	-
	5	154.1	-
	6	95.1	5.83, *s*
	7	149.4	-
	8	104.9	-
	8a	150.2	-
G	4a	100.2	-
	5	143.4	-
	6	94.7	6.10, *s*
	7	154.0	-
	8	107.8	-
	8a	154.1	-
B	1	130.9	-
	2	114.3	7.02, *d*
	3	144.2	-
	4	145.1	-
	5	115.1	6.83, *d* (7.2)
	6	118.6	6.84, *dd* (7.2, 1.8)
E	1	130.2	-
	2	115.3	7.31, *d* (2.0)
	3	144.8	-
	4	145.0	-
	5	114.6	6.81, *d* (6.0)
	6	119.7	7.18, *br*
H	C-1	131.6	-
	C-2	114.0	6.80, *br*
	C-3	144.0	-
	C-4	144.3	-
	C-5	114.8	6.75, *d* (8.0)
	C-6	118.4	6.60, *d* (8.0)
OH	-	-	7.94, *s*

[a]δ values in part per million and coupling constant (J) values are given in parentheses (*br*: broad, *d*: doublet, *m*: multiplet, *s*: singlet, *t*: triplet)

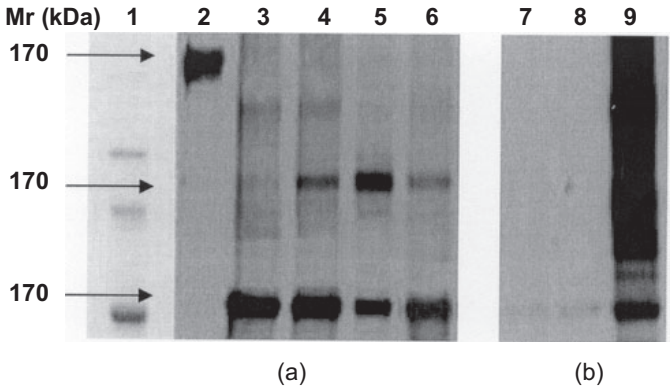

Figure 2 Effect of cinnamtannin B1 on phosphorylation of insulin receptor β-subunit. Differentiated 3T3-L1 adipocytes in culture were serum starved in DMEM for 3 hours before treatment and equilibrated in KRPH buffer for 15 min. (a) The cells were treated with 100 nM insulin (lane 4), 0.11 mM cinnamtannin B1 (lane 5), and mixture of 0.11 mM cinnamtannin B1 and 100 nM insulin (lane 6). Lane 1, 2, and 3 show protein maker, EGF control, and untreated, respectively. (b) 15 minutes prior treatment, the cells were treated with wortmannin (500 nM), cytochalasin B (40 mM), and sodium orthovanadate (5 mM). Cells were lysed and immunoprecipitated with antibody against antiphosphotyrosine

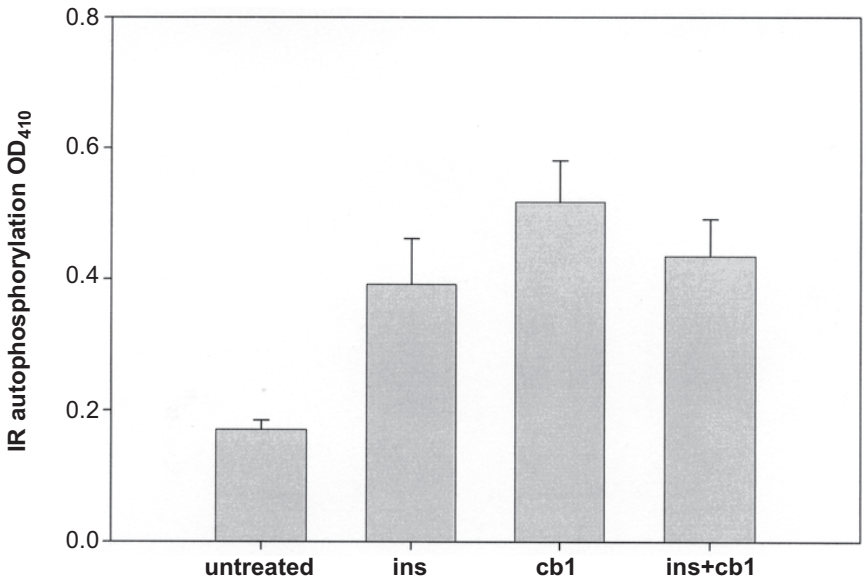

Figure 3 ELISA for phosphorylation of insulin receptor in intact cells. At day 11 after induction, the cells were serum starved for 3 hours in DMEM and treated with the samples (100 nM insulin and 0.11 mM cinnamtannin B1) for 30 minutes. After treatment, the cells were fixed by adding 100 µL methanol/acetic acid (50:50) to each well for 10 minutes. Non-specific antigen was blocked with skim milk and probed with antiphosphotyrosine antibody

on insulin receptor β-subunit was observed by increasing intensity of reaction mixture of ABTS reagent with HRP conjugated secondary antibody that bound to primary antibody.

4.0 DISCUSSION

4.1 Structure Characterization of Proanthocyanidin

Compound 1 was obtained as an off-white amorphous powder and gave orange-red colour with anisaldehyde-sulfuric acid reagent and reacted positively to ferric chloride that is characteristic of proanthocyanidins. Ultraviolet spectrum of Compound 1 revealed absorption maxima at 278 nm, all of which suggested that it had one or more flavan-3-ol moieties present in the molecule [13]. In addition, its infrared spectrum showed hydroxyl functionality as a broad band at 3400 cm^{-1} and aromatic ring absorption was observed at 1615 cm^{-1}.

HPLC analysis of Compound **1** showed the sample to be predominantly a single compound, suggesting that the multiple proton signals observed were likely a result of conformational isomerism due to steric hindrance to rotation about the interflavonoid bond [13].

The ^{1}H NMR spectrum of Compound 1 revealed signal at δ 3.28 (d) and 4.14 (d, J = 3.4 Hz) corresponding to an A-type unit from the AB coupling system [12, 25]. The correlations of those assignments were clearly observed in ^{1}H-^{1}H correlated spectroscopy (COSY) spectrum. The ^{13}C NMR spectrum of Compound 1 showed two signals at δ 77.2 and 81.9 attributable to flavan C-2 carbon of middle and terminal units respectively. The appearance of a ketal carbon signal at δ 98.5 indicated that Compound 1 possesses an A-type unit in the molecule [17]. Its DEPT spectrum showed the sum of protonated carbon is 21 carbons, which consist of 20 methine carbons (C-H) and a methylene carbon (CH_2). The interflavonoid linkage between the middle and bottom unit was assumed to be the C-4 (F-ring) and C-8 (G-ring) positions due to the chemical shifts of the signals assignable to the C-H at δ 36.9 and 107.4 respectively (in the DEPT spectrum).

The mass spectra was analyzed using FAB technique which is generally applicable for very polar compounds (sugars polysaccharides and polyphenol), labile polar compounds, and high molecular weight biological compounds (polypeptides and poly nucleotides) [26]. The fast atom bombardment mass spectrum (FAB-MS) of Compound 1 exhibited an $[M+H]^{+}$ ion peak at m/z 865, corresponding to a triflavonoid structure. The examination of plant materials reveals that in the bark of *C. zeylanicum*, the major phenolic metabolites consist of a series of proanthocyanidins with doubly linked (A-type) unit, while in the barks of *C. burmanii* and *C. cassia* and the root bark of *C. camphora,* the main phenolic metabolites consist of linearly linked proanthocyanidins [10]. On the basis of spectral data and comparison with several literatures, it was concluded that Compound 1 was cinnamtannin B1 that previously

has been isolated by Nonaka *et al.* [10] from the same species. The compound was also found in *Aesculus hippocastanum* [13], *Parameria laevigata* [12], and *Pavetta owariensis* [17]. The IUPAC name of the cinnamtannin B1 is epicatechin-(4β→8, 2β→*O*→7)-epicatechin-(4α→8)-epicatechin with the empirical formula of $C_{45}H_{36}O_{18}$ [27].

Acetylation of cinnamtannin B1 with anhydride acetic acid/pyridine at room temperature followed by column chromatography on Sephadex LH-20 resulted in an acetate derivative (Compound 2). In its infrared spectrum, the complete acetylation was shown by no broad band for hydroxyl groups in the region 3100-3700 cm^{-1}. The presence of C=O acetate was observed at 1772 cm^{-1}. Its FAB-MS showed the [M+H] ion at *m/z* 1452 corresponding to $C_{73}H_{64}O_{32}$ indicated that all 14 protons attached in the hydroxyl groups were replaced by acetate. Other ions were observed at *m/z* 1348, 1073, 851, 630, 455, 329, and 179.

4.2 Cinnamtannin B1 Stimulated Phosphorylation

Non-insulin-dependent diabetes mellitus is due to insulin resistance, a state when the target cells no longer respond to ordinary levels of circulating insulin. A drug that promotes the initiation IR signaling by enhancing IR autophosphorylation should be useful in treatment Type 2 diabetes.

The insulin receptor is a heterodimer composed of two identical insulin-binding subunits (the α-subunit, 135 kDa) covalently attached by disulfide bonds to two identical kinase subunits (the β-subunit, 95 kDa). It is known that insulin stimulates not only the extent of autophosphorylation of the α-subunit but also the catalytic activity toward artificial substrates [5, 28].

The receptor could provide the initial steps in a cascade leading eventually to activation of the glucose transport system. These steps would include insulin binding, autophosphorylation of the receptor, and receptor mediated phosphorylation of an endogenous substrate. Cinnamtannin B1 strongly induced tyrosine phosphorylation of insulin receptor.

One of the main signaling pathways that become activated following recruitment to the IRS proteins is phosphatidylinositol 3-kinase cascade, which mediates many of the major metabolic effects of insulin stimulation. PI3K is composed of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. The regulatory subunit binds phosphorylated tyrosine residues on the IRS proteins via its SH2 domain activating the catalytic subunit by dissociation [29]. All cell lysates express phosphorylation between 50-55 kDa, an isoform of p85 that have been suggested to play a role in specificity of signaling or differential localization within the cell [30].

It should be noted that the effect of cinnamtannin B1 (0.11 mM) with the absence of insulin was significantly greater than insulin alone (100 nM). While the effect of combination of insulin and cinnamtannin was not given a high intensity dot blot.

Phosphorylation was quantitatively assayed using ELISA, suggesting that cinnamtannin-induced signal transduction leading to phosphorylation is similar mechanism to that induced by insulin. Together treatment of insulin and cinnamtannin B1 may cause a competition to activation of PI3K. These data suggested that insulin and cinnamtannin B1 could give a similar effect (mimicker). Previously, some researchers [31 - 34] have reported the *in-vitro* and *in-vivo* activity of *Cinnamomum* as antihyperglycemic agent.

In order to determine whether the activity of cinnamtannin B1 is affected by several chemicals, the 3T3-L1 adipocyte was treated with wortmannin (500 nM), cytochalasin B (40 mM), or sodium orthovanadate (5 mM) for 15 minutes prior to addition of cinnamtannin B1. Wortmannin has previously been shown to inhibit insulin dependent 2-deoxy-D- [1-^{3}H] glucose uptake in 3T3-L1 adipocytes by inhibiting PI3K [35]. Differentiated cells were washed and glucose-starved as described in Section 2.0. Prior to addition of sample, the cells were pretreated with wortmannin at final concentration of 500 nM. As shown in Figure 1, the activity of cinnamtannin B1 was fully inhibited by wortmannin.

The cytochalasin B is known to block glucose uptake by binding to the membrane [36]. Pretreatment of the cells with cytochalasin B inhibits all phosphorylation sites. It is found that the cytochalasin B inhibited all insulin signaling causing loss in cell communication.

The stimulation of phosphorylation was demonstrated using sodium orthovanadate, an inhibitor of protein phosphatase. Vanadate inhibits protein phosphotyrosine phosphatases and also activates insulin receptor β-subunit phosphorylation; the possibility considered was that vanadate-stimulated glucose uptake by 3T3-Ll adipocytes [37]. Pretreatment of the cells with vanadate did not influence the phosphorylation activity of cinnamtannin B1.

Tyrosine phosphorylation of β-subunit of the insulin receptor is one of the earliest cellular responses to insulin binding [38]. It is well established that insulin signaling, including activation of insulin receptor tyrosine kinase activity, is impaired in most patients with Type 2 diabetes [39]. Phosphorylation of IR leads to activation of the PI3K. Stimulation of glucose transport and disposal, glycogen synthesis, and inhibition of lipolysis are mediated by PI3K activity [40].

5.0 CONCLUSION

The isolation of bioactive compound from *C. zeylanicum* produced a compound called cinnamtannin B1. Cinnamtannin B1 may have insulin-like activity similar to insulin by binding to the α-subunit of the receptor in the cell membrane and then tyrosine residues of β-subunit undergo autophosphorylation. Autophosphorylation subsequently activates PI3K, GLUT4 translocation, and glucose uptake. In addition, the experimental of results with cinnamtannin B1 indicate that the phosphorylation of IR β-subunit is an important molecular target for treatment of diabetes.

ACKNOWLEDGEMENTS

We thank Drs. Rusdi Tamin for identifying plant material from Herbarium of Universitas Andalas, Padang, Indonesia, where the plant specimen is deposited. The authors wish to thank Intensified Research in Priority Area (IRPA), Ministry of Science, Environment and Technology Malaysia for the grant of 72139, 74054, and 75091 (IRGS) for financial supports.

REFERENCES

[1] Shepherd, P. R., D. J. Withers, and K. Siddle. 1998. Phosphoinositide 3-kinase: The Key Switch Mechanism in Insulin Signaling. *Biochemical Journal*. 333(Pt 3): 471-490.

[2] Taha, C., and A. Klip. 1999. The Insulin Signaling Pathway. *Journal of Membrane Biology*. 169(1): 1-12.

[3] Goldfine, I. D. 1999. Unravelling the Ridle of Insulin Resistance. *Journal of Laboratory and Clinical Medicine.* 134(2): 100-102.

[4] White, M. F. 1997. The Insulin Signalling System and the IRS Protein. *Diabetologia*. 40: S2-S17.

[5] Kasuga, M., Y. Zick, D. L. Blith, F. A. Karlsson, H. U. Haring, and C. R. Kahn. 1982. Insulin Stimulation of Phosphorylation of the β-subunit of the Insulin Receptor. Formation of Both Phosphoserine and Phosphotyrosine. *Journal of Biological Chemistry.* 257(17): 9891-9894.

[6] Goldfine, I. D. 1987. The Insulin Receptor: Molecular Biology and Transmembrane Signaling. *Endocrinology Review*. 8(3): 235-255.

[7] White, F. M., and C. R. Kahn. 1994. Minireview: The Insulin Signaling System. *Journal of Biochemistry.* 269(1): 1-4.

[8] Jayaprakasha, G. K., L. J. Rao, and K. K. Sakariah. 2003. Volatile Constituents from *Cinnamomum zeylanicum* Fruit. *Journal of Agricultural and Food Chemistry*. 51(15): 4344-4348.

[9] Burkill, I. H. 1966. *A Dictionary of the Economic Products of the Malay Peninsular*. 2nd Ed. Kuala Lumpur: Ministry of Agriculture and Cooperatives.

[10] Nonaka, G., S. Morimoto, and I. Nishioka. 1983. Tannins and Related Compounds. Part 13. Isolation and Structures of Trimeric, Tetrameric and Pentameric Proanthocyanidins from Cinnamon. *Journal of the Chemical Society, Perkin Transaction I*. 2139-2145.

[11] Morimoto, S., G. I. Nonaka, and I. Nishioka. 1985. Tannins and Related Compounds. XXXV. Proanthocyanidins with a Doubly Linked Unit from the Root Bark of *Cinnamomum sieboldii* Meisner. Chemical and Pharmaceutical Bulletin. 33(10): 4338-4345.

[12] Kamiya, K., C. Watanabe, H. Endang, M. Umar, and T. Satake. 2003. Studies on Constituents of *Parameria laevigata* MOLDENKE. Chemical and Pharmaceutical Bulletin. 49(5): 551-557.

[13] Morimoto, S., G. I. Nonaka, and I. Nishioka. 1987. Tannins and Related Compounds. LIX. Aesculitannins, Novel Proanthocyanidins with Doubly-Bonded Structures from *Aesculus hippocasnatum* L. Chemical and Pharmaceutical Bulletin. 35: 4717-4729.

[14] Foo, L. Y., Y. Lu, A. B. Howell, and N. Vorsa. 2000. A-Type Proanthocyanidin Trimers from Cranberry that Inhibit Adherence of Uropathogenic P-Fimbriated *Escherichia coli*. *Journal of Natural Products*. 63(9): 1225-1228.

[15] Baek, N. I., E. J. Kennely, L. B. S. Kardono, S. Tsauri, K. Padmawinata, D. D. Soejarto, and A. D. Kinghorn. 1994. Flavonoids and a Proanthocyanidin from Rhizomes of *Selligue feei*. *Phytochemistry*. 36(2): 513-518.

[16] Bilia, A. R., I. Morelli, M. Hamburger, and K. Hostettman. 1996. Flavans and A-Type Proanthocyanidins from *Prunus prostata*. *Phytochemistry*. 43(4): 887-892.

[17] Balde, A., L. A. Pieters, V. Wray, H. Kolodziej, D. A. Vanden Berghe, M. Claeys, and A. J. Vlietinck. 1991. Dimeric and Trimeric Proanthocyanidins Possesing a Doubly Linked Structure from *Pavetta owariensis*. *Phytochemistry*. 30(12): 4129-4135.

[18] Roux, E. L, T. Doco, P. Sarni-Manchado,Y. Lozano, and V. Cheynier. 1998. A-Type Proanthocyanidins from Pericarp of *Litchi chinensis*. *Phytochemistry*. 48(7): 1251-1258.

[19] De Bruyne, T., L. Pieters, H. Deelstra, and A. Vlietinck. 1999. Condensed Vegetable Tannins: Biodiversity in Structure and Biological Activities. *Biochemical Systematics and Ecology*. 27(4): 445-459.

[20] Ferrea, G., A. Canessa, F. Sampietro, M. Cruciani, G. Romussi, and D. Bassetti. 1993. In Vitro Activity of a *Combretum micranthum* Extract Against Herpes Simplex Virus Types 1 and 2. *Antiviral Research*. 21(4): 317-325.

[21] Park, Y. C., G. Rimbach, C. Saliou, G. Valacchi, and L. Packe. 2000. Activity of Monomeric, Dimeric, and Trimeric Flavonoids on NO Production, TNF-α Secretion, and NF-KB-Dependent Gene Expression in RAW 264.7 Macrophages. *FEBS Letter*. 465(2-3): 93-97.

[22] Cos, P., G. Calomme, J. P. Hu, K. V. Cimanga, B. Poel, L. Pieters, and A. J. Vlietinck. 1998. Structure-activity Relationship and Classification of Flavonoids as Inhibitors of Xanthine Oxidase and Superoxide Scavengers. *Journal of Natural Products*. 61(1): 71-76.

[23] Garbacki, N., L. Angenot, C. Bassleer, J. Damas, and M. Tits. 2002. Effects of Prodelphinidins Isolated from *Ribes nigrum* on Chondrocyte Metabolism and COX Activity. *Naunyn-Schmiedeberg's Archives of Pharmacology*. 365(6): 434–441.

[24] Student, A. K., R. Y. Hsu, and M. D. Lane.1980. Induction of Fatty Acid Synthase Synthesis in Differentiating 3T3-L1 Preadipocytes. *Journal of Biological Chemistry*. 255(10): 4745-4750.

[25] Santos-Buelga, C., H. Kolodziej, and D. Treutter. 1995. Proanthocyanidin Trimers Possessing A-Doubly Linked Structure from *Aesculus Hippocastanum*. *Phytochemistry*. 38(2): 499-504.

[26] Bloor, S. J., and L. J. Porter. 1993. Mass Spectrometry. In *Bioactive Natural Products: Detection Isolation and Structural Determination*. Edited by S. M. Colegate, and R. J. Molyneux. Boca Raton: CRC Press.113.

[27] Kolodziej, H., D. Ferreira, and G. Lemiere. 1993. On the Nomenclature of Oligoflavonoids with an A-Type Unit. *Journal of Natural Products*. 56(7): 1199-1200.

[28] Kohanski, R. A., S. C. Frost, and M. D. Lane.1986. Insulin-dependent Phosphorylation of the Insulin Receptor-protein Kinase and Activation of Glucose Transport in 3T3-L1 Adipocytes. *Journal of Biological Chemistry*. 261(26): 12272-12281.

[29] Frost, S. C., R. A. Kohanski, and M. D. Lane. 1987. Effect of Phenylarsine Oxide on Insulin-dependent Protein Phosphorylation and Glucose Transport in 3T3-L1 Adipocytes. *Journal of Biological Chemistry*. 262(20): 9872-9876.

[30] Inukai, K., M. Funaki, T. Ogihara, H. Katagiri, A. Kanda, M. Anai, Y. Fukushima, T. Hosaka, M. Suzuki, B. C. Shin, K. Takata, Y. Yazaki, M. Kikuchi, Y. Oka, and T. Asano. 1997. p85α Gene Generates Three Isoforms of Regulatory Subunit for Phosphatidylinositol 3-kinase (PI 3 kinase), p50α, p55α, and p85α, with Different PI 3-kinase Activity Elevating Responses to Insulin. *Journal of Biological Chemistry*. 272(12): 7873-7882.

[31] Imparl-Radosevich, J., S. Deas, M. M. Polansky, D. A. Baedke, T. S. Ingebritsen, R. A. Anderson, and D. J. Graves. 1998. Regulation of PTP-1 and Insulin Receptor Kinase by Fractions from Cinnamon: Implications for Cinnamon Regulation of Insulin Signalling. *Hormones Research*. 50(3): 177-182.

[32] Jarvill-Taylor, K. J., R. Anderson, and D. J. Graves. 2001. A Hydroxychalcone Derived from Cinnamon Functions as a Mimetic for Insulin in 3T3-L1 Adipocytes. *Journal of the American College of Nutrition*. 20(4): 327-336.

[33] Khan, A., M. Safdar, M. M. Ali Khan, K. N. Khattak, and R. A. Anderson. 2003. Cinnamon Improves Glucose and Lipids of People with Type 2 Diabetes. *Diabetes Care*. 26(12): 3215-3218.

[34] Anderson, R. A., C. L. Broadhurst, M. M. Polansky, W. F. Schmidt, A. Khan, V. P. Flanagan, N. W. Schoene, and D. J. Graves. 2004. Isolation and Characterization of Polyphenol Type-A Polymers from Cinnamon with Insulin-like Biological Activity. *Journal of Agricultural and Food Chemistry*. 52(1): 65-70.

[35] Arcaro, A., and M. P. Wymann. 1993. Wortmannin is a Potent Phosphatidylinositol 3-kinase Inhibitor: the Role of Phosphatidylinositol 3, 4, 5-trisphosphate in Neutrophil Responses. *Biochemical Journal*. 296(Pt 2): 297-301.

[36] Jung, C. Y., and A. L. Rampal. 1977. Cytochalasin B Binding Sites and Glucose Transport Carrier in Human Erythrocyte Ghosts. *Journal of Biological Chemistry*. 252(15): 5456-5463.

[37] Huyer, G., S. Liu, J. Kelly, J. Moffat, P. Payette, B. Kennedy, G. Tsaprailis, M. J. Gresser, and C. Ramachandran. 1997. Mechanism of Inhibition of Protein-tyrosine Phosphatases by Vanadate and Pervanadate. *Journal of Biological Chemistry*. 272(2): 843-851.

[38] White, M., E. W. Stegman, T. J. Dull, A. Ullrich, and C. R. Kahn. 1987. Characterization of Endogenous Substrate of the Insulin Receptor in Cultured Cells. *Journal of Biological Chemistry*. 262(20): 9769-9777.

[39] Thies, R. S., J. M. Molina, T. P. Ciaraldi, G. R. Freiden, and J. M. Olefsky. 1990. Insulin-receptor Autophspohorylation and Endogenous Substrate Phosphorylation in Human Adipocytes from Control, Obese, and NIDDM Subjects. *Diabetes*. 39: 250-259.

[40] Saltiel, A. R., and C. R. Kahn. 2001. Insulin Signalling and the Regulation of Glucose and Lipid Metabolism. *Nature*. 414: 799-806.