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Lipogenesis inhibition and adipogenesis regulation via PPARγ pathway in 3T3-L1 cells by *Zingiber cassumunar* Roxb. rhizome extracts

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ABSTRACT

Zingiber cassumunar (ZC) is used by tribal people in northern Thailand in traditional remedies for anti-obesity and in food recipes. Extracts from this plant have been studied for several pharmacological effects including anti-obesity, but with no clear evidence on cellular mechanism of activity. This study aim to investigate the lipolytic and anti-adipogenic activity of crude extracts from ZC on *in vitro* cultures of the mouse adipocyte cell-model, 3T3-L1. Dry rhizome powder was extracted with absolute ethanol and boiled-water. On the exposed pre-adipocytes to the extracts, cytotoxicity was not detected by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Lipid content and glycerol release were assessed using Oil Red-O and a commercial Adipolysis Assay kits respectively. The extracts exhibited no significant lipolytic activity on the exposed mature-adipocytes, in serial dilutions ranging from 1 to 800 μg/ml. However, anti-lipogenic activity was presented. All extracts significantly reduced the lipid content of exposed differentiating-adipocytes. This anti-lipogenic activity was confirmed by the expression of selected genes, determined by using real-time PCR techniques, in four groups namely: adipocyte differentiation genes, glucose uptake genes, lipid metabolism genes and fatty acid oxidation genes. ^{1}H NMR spectrum of the extracts exhibited the prominent olefinic protons of phenylbutanoids, the group of compounds previously proved with several bioactivities. This study provided evidences of mechanisms that apparently verify the traditional use of ZC to prevent obesity.

1. Introduction

The rhizome, young leaves and flowers of *Zingiber cassumunar* Roxb. (ZC) (Zingiberaceae) have long been used as a flavorings in Thai cuisine and as fresh ingredients in northern Thai salads. Synonyms of the scientific name include *Zingiber montanum* (J. Koenig) Link ex A. Dietr. (ZM) and *Z. purpureum* Roscoe. [1]. The Thai name of this monocotyledonous plant is Phlai or Plai [2,3] and the international common name is cassumunar ginger [4]. ZC has been investigated for several pharmacological properties, including antioxidant [5], anti-inflammation [6], anti-allergic [7], anti-proliferative [8], antiulcer [9] and insecticidal [10]. Anti-adipogenic activity of closely related species has been mentioned in several publications (e.g. *Z. officinale* Roscoe (ginger) [11,12], *Z. zerumbet* Smith (bitter ginger) [13] and *Z. mioga* Roscoe (mioga ginger) [14]), while for ZC only one reports have been published on the *in vitro* inhibitory effect on pancreatic lipase activity [15]. In northern Thailand, ZC is an ingredient in several pharmacological remedies to treat obesity among tribal people [16]. Consequently, this study investigated the potential and cellular mechanism of anti-adipogenic and lipolytic activity of crude extracts from ZC rhizomes on an *in vitro* culture system using the mouse adipocyte cell-model, 3T3-L1.

2. Methods and materials

2.1. Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and 100x antibiotics/antimycotic were purchased from GIBCO

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(Grand Island, NY, USA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), dimethyl sulfoxide (DMSO), isopropanol, sodium dodecyl sulfate (SDS) and gelatin Type B from bovine skin from Sigma-Aldrich (St. Louis, MO, USA). Oil Red-O (ORO) from Bio Basic (Amherst, NY, USA). Absolute ethanol, hydrochloric acid and formaldehyde from RCI Labscan (Samutsakorn, Thailand) and 3-(4,5-dimethylthiazol-2-yl)-2, 5diphenyltetrazolium bromide (MTT) from Invitrogen (Carlsbad, CA, USA).

2.2. Plant materials

2.2.1. Collection sites and type specimens

Fresh rhizomes of ZC were purchased from a city market (originally transported from suburban vegetable-garden) in Chiang Mai, Thailand. Ten pieces were sampled randomly from the pile and grown in a greenhouse at the Department of Biology, Faculty of Science, Chiang Mai University, to prepare herbarium specimens. Voucher specimens were deposited in the Herbarium Collection Section at the Human and Animal Cell Technology Research Unit, Faculty of Science, Chiang Mai University, Thailand with a voucher type specimens No. HC 255902-Z09.

2.2.2. ZC extraction

The rhizomes were extracted with 2 types of solvents: absolute ethanol (ZCE) and distilled water (ZCW).

A. Absolute ethanol: The rhizomes were thinly sliced and oven-dried at 50 °C for 2 weeks, with dry-weight checking for stabilization. The dried material was pulverized into powder (500 g) and soaked in 1.5 L of the absolute ethanol at ambient temperature (25–30 °C) for 3 days.

B. An aqueous extraction: Boil 60 g of the pulverized powdered ZC rhizome in 1 L of distilled water, under heat-reflux (100 °C) for 60 min, before cooling to room temperature.

At the end of each extraction, the mixture was taken to gauze filtration. The filtered solutions from each solvent were then re-filtered through Whatman™ no.1 filter paper, using a vacuum pressure pump. Each of the final filtered solutions was concentrated with a rotary vacuum evaporator (Rotavapor R-114, Buchi, Switzerland) to obtain a dark-brown, sticky, crude extract. Crude extracts were stored in brown bottles in darkness at 4 °C. The yield of each extraction process was calculated as a percent of the starting weight of the pulverized powder.

2.2.3. Serial dilutions of crude extracts

Stock solutions (10 mg/ml) were prepared by dissolved each crude extract in DMSO. Serial dilutions, ranging from 0.25 to 1000 μg/ml were prepared by dissolving the stock solution in the completed media.

2.3. Cell culture

2.3.1. Pre-Adipocytes culture

The mouse embryonic pre-adipocyte fibroblast, 3T3-L1 cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in 25 or 75 cm^2 culture flasks with the completed media (DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics/antimycotic) under standard culture conditions (37 °C in a 95% humidified atmosphere, with 5% CO_2). The media was changed every 2–3 days and sub-culturing was performed at 70% spatial confluency. In this paper, "pre-adipocyte fibroblast" is abbreviated to "PAs", to avoid confusion with "mature adipocyte", "MAs". Also, cells differentiating from PAs into MAs (with or without chemical induction) are abbreviated as "DAs" (differentiating adipocytes).

2.3.2. Induction of adipogenesis

The 96-well plates were pre-coated with 0.1% (w/v) gelatin and modified as described by ATCC® (PCS-999-027™). Briefly, 0.1% gelatin solution was prepared by dissolving gelatin type B, from bovine skin, in deionized water and sterilized under a pressure-cooker (121 °C, 15 psi, 15 min). Add 60 μl of the solution into each well and incubated for 30 min in standard culture conditions. The solution was aspirated before adding 100 μl/well of serum free DMEM and incubated for at least 1 h to equilibrate the attach surface. The pre-coated plate was used within 3 days after preparation. The medium was discarded prior to the cell seeding.

Adipogenesis was induced in PAs (to finally result in MAs) using methods modified from those of Ambati, et al. [17]. Briefly, 3000 PA cells/well were plated in pre-coated 96-well plates and maintained for 2 days to achieve confluency. After confluency (day-0 of induction), cells were exposed for 48 h to sequential inductive-media, including initial media (mixtures of completed media, 0.5 μM IBMX, 1 μM DEX and 167 nM insulin). The cultures were then replaced every 2 days with insulin media (a mixture of completed media and 167 nM insulin), until day-14 to obtain MAs.

However, in this study, spontaneous adipogenesis of PAs, without any induction, occurred sparsely in a few cells at 14 days of culture, after the initial cell-seeding. Such cells are abbreviated as "SA".

2.4. Cellular quantitative assay

2.4.1. MTT assay

Numbers of viable-cells were counted by MTT assay. Any cell culture experiment, brought to the MTT assay, have been performed in a 96 well-plate. After each cell treatment, MTT stock solution (12 mM) was added into each well and incubated for 4 hrs, under standard culture conditions. Subsequently, formazan crystal products were dissolved in 100 μl of 10% (w/v) SDS-0.01 M HCl, incubated overnight and then the absorbance was read at 570 nm, using a microplate-reader (Rayto TR-2100C, Shenzhen, China). Relative viable-cell number was calculated in an untreated group control, as a reference.

2.4.2. Oil Red-O colorimetric assay

The lipid content of MAs was quantified by ORO staining, as previously described by Kinkel, et al. [18]. Briefly, cells in a pre-coated 96-well plate, were rinsed with phosphate buffer saline twice, fixed for 1 h in 10% (w/v) formaldehyde and then washed thoroughly with distilled water. Isopropanol (60% v/v) was added to wash and dehydrate the wells and discarded afterward. Then the cells were air-dried and stained for 15 min with ORO solution (1.4 g ORO + 400 ml isopropanol + 144 ml distilled water) and completely rinsed 4 times with distilled water. In order to determine lipid content, isopropanol was used to elute the ORO stain. Finally, absorbance was monitored at 492 nm with a microplate-reader. Relative lipid content was calculated by comparison with untreated controls.

2.5. Cytotoxic assay of pre-adipocyte

PAs were seeded in 96-well plates at 5000 cells/well and incubated for 24 h, under standard culture conditions. Then the cultures were exposed to the crude extract solution at serial 10-dilutions (see above) and incubated for a further 72 h. Viable-cell numbers were detected by colorimetric MTT assay (described above). The inhibition concentration (IC) of the crude extract was evaluated by using the PriProbit Program ver. 1.63 [19]. The IC of 50% of the cell population in cultures (IC_{50}) was used to assess the activity of cytotoxic assay. Non-toxic concentrations at IC_{10} to IC_{20}, with values rounded to whole numbers, were obtained for use in the adipogenic assay.

2.6. Adipogenic assay

2.6.1. Lipolytic assay

A sub-stock solution at IC_{10}-IC_{20} (see above), of each extract, was freshly re-prepared by dissolving the designated concentration of the stock solution in the completed media. A set of serial 5-dilutions was then prepared from the IC_{10}-IC_{20} sub-stock solution. The concentration of each crude extract in the 5-dilutions therefore differed among the extracts.

PAs were cultured in pre-coated 96-well plates and induced to differentiate into MAs, by the methods described above. The resultant MA cells were then exposed to the 5-dilutions of each extract on the last day of adipogenesis (i.e. day-14) for 3 days, under standard culture conditions. The lipid content of the MAs was then detected by ORO colorimetric assay. The number of viable exposed MA cells was counted by colorimetric MTT assay on another similarly-treated plate, set up alongside. This 3-day treatment was set up to focus on the dose-dependent effect of each extract on the cells.

From the results of the 3-day treatment, the most effective lipolytic concentration of each extract was selected. This single concentration (S-Conc.) was re-prepared and applied for 6 days, to evaluate the time-dependent effect of the extracts. The procedure of exposure was similar to that of the 3-day experiments.

2.6.2. Glycerol release assay

Glycerol release was assessed using the Adipolysis Assay Kit (Millipore, Billerica, MA, USA). The methodology was adapted from that provided in the company's manual. Briefly, the induced MAs were exposed for 5 days to mixture-1 of each extract (DMEM + 10%FBS + S-Conc.). Subsequently, mixture-1 was replaced with mixture-2 of each extract (Kit's Incubation Solution + 2% BSA + S-Conc.) and incubated for 24 h. Thereafter, the medium and the cells were collected separately. The quantity of glycerol released was determined with the Kit's colorimetric assay protocol, i.e. 25 μl of the medium was transferred into a new 96-well plate and 200 μl of the Free Glycerol Assay Reagent was added to each well. Isoproterenol, at 10 μM (provided with the Kit) was used as a positive control to determine lipolysis. After 15 min's incubation at ambient temperature, the absorbance at 540 nm was determined using a microplate-reader. Lipid content of the cells was determined by the ORO colorimetric assay described above.

2.6.3. Anti-lipogenic assay

An anti-lipogenic assay was performed, as described above, except that the IC_{10}-IC_{20} of each extract was mixed with the initial media and with the insulin media before the cells were exposed to the extracts. Four cell-groups of PAs were set up: 1) the induction control group, exposed to the set of induction media, which became MAs, but not treated with any extract (MA/nt, 0 μg/ml); 2) the group with neither induction nor extract, which became SAs under complete media only (SA/nt, 0 μg/ml) and 3–4) the three treatment groups which were exposed to IC_{10}-IC_{20} of each extract during their differentiation, DA/ZCE and DA/ZCW. Lipid content determination and the count of viable-cells were performed after the 14 days, as described above.

2.7. Adipogenetic evaluation

In this study, we focused on 4 groups of genes i.e. those involved in the following processes: 1) adipocyte differentiation (*C/EBPα* (CCAAT/enhancer binding protein alpha), *PPARγ* (Peroxisome proliferator-activated receptor gamma), *ADD-1* (Adipocyte determination and differentiation-dependent factor 1) and *Pref-1* (Pre-adipocyte factor 1)); 2) glucose uptake (*IRS-1* (Insulin receptor substrate 1), *GLUT4* (Glucose transporter type 4) and *Adiponectin*); 3) lipid metabolism (*FAS* (Fatty acid synthase) and *aP2* (Adipocyte protein 2)) and 4) fatty acid oxidation (*ATGL* (Adipose triglyceride lipase), *HSL* (Hormone sensitive lipase) and *PGC-1β* (PPARγ coactivator 1 beta)). The relative mRNA expression of real-time PCR products was evaluated. ZCE and ZCW extracts were chosen in this work, since other solvents pose human health hazards and are not used in traditional medicine.

Total RNA was isolated from cells, obtained after the anti-lipogenic assay, using a NucleoSpin® RNA isolation kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The mRNA was synthesized to cDNA, using ReverTra Ace® qPCR RT master mix (Toyobo Co., Osaka, Japan). Quantitative real-time PCR was conducted with a SensiFAST™ SYBER® No-ROX Kit (Bioline, London, UK) in an Eco™ Real-Time PCR System (Illumina, Inc. SD, USA). The following primers were used: *C/EBPα* forward: 5′-GGA TAC TCA AAA CTC GCT CC-3′, reverse: 5′-CTA AGT CCC TCC CCT CTA AA-3′; *PPARγ* forward: 5′-TTT TCA AGG GTG CCA GTT TC-3′, reverse: 5′-AAT CCT TGG CCC TCT GAG AT-3′; *ADD-1* forward: 5′-TGT TGG CAT CCT GCT ATC TG-3′, reverse: 5′-AGG GAA AGC TTT GGG GTC TA-3′; *Pref-1* forward: 5′-CTA ACC CAT GCG AGA ACG AT-3′, reverse: 5′-GCT TGC ACA GAC ACT CGA AG-3′; *IRS-1* forward: 5′-CAT GCA GAT AGG TTG TCC TC-3′, reverse: 5′-CAG CAA GGA AGA GTG AGT AG-3′; *GLUT4* forward: 5′-ACC TCT ACA TCA TCC GGA AC-3′, reverse: 5′-TTG ATG CCT GAG AGC TGT TG-3′; *Adiponectin* forward: 5′-GCA ACT ACT CAT AGC CCA TA-3′, reverse: 5′-CAT GTA AGA GTC GTG GAG AC-3′; *FAS* forward: 5′-TTG CTG GCA CTA CAG AAT GC-3′, reverse: 5′-AAC AGC CTC AGA GCG ACA AT-3′; *aP2* forward: 5′-TCA CCT GGA AGA CAG CTC CT-3′, reverse: 5′-AAT CCC CAT TTA CGC TGA TG-3′; *ATGL* forward: 5′-TAT TGA GGT GTC CAA GGA GG-3′, reverse: 5′-GTA CAC CGG GAT AAA TGT GC-3′; *HSL* forward: 5′-GAG GGA CAC ACA CAC ACC TG-3′, reverse 5′-CCC TTT CGC AGC AAC TTT AG-3′; *PGC-1β* forward: 5′-GGA AGA ACT TCA GAC GTG AG-3′, reverse: 5′-CAC CTG GCA CTC TAC AAT CT-3′ and *β-actin* forward: 5′-CCA CAG CTG AGA GGG AAA TC-3′, reverse 5′-AAG GAA GGC TGG AAA AGA GC-3′. Initial denaturation of PCR mixtures was carried out at 94 °C for 3 min, followed by 40 thermal cycles: 40 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. The amounts of each gene were determined, relative to the housekeeping gene, *β-actin*, normalized as an internal control, using the $2_T^{-\Delta\Delta C}$ method [20].

2.8. ^1H NMR spectroscopy

To evaluate the chemical composition of crude extracts, 10 mg of each, ZCE and ZCW, was dissolved in 0.7 ml of deuterated methanol (CD_3OD) and in 0.7 ml of deuterium oxide (D_2O) respectively. Each solution was then transferred into an NMR tube. ^1H NMR spectra of the extracts were recorded on a Bruker Avance 300 spectrometer (^1H at 300 MHz).

2.9. Statistical analyses

Results are presented as the mean ± standard error of the mean. Data were statistically evaluated by a One-way Analysis of Variance. Determination of significant differences ($p \leq 0.05$) between means was followed by Duncan's multiple range test. Individual treatments was performed in triplicates, each with 5 wells of replication.

3. Results

3.1. ZC extraction

The higher percentage yield was obtained with boiled water (ZCW, 14.7%), followed by the ethanol (ZCE, 1.7%).

3.2. Cytotoxic assay of pre-adipocytes

Considering the mean IC_{50} of the 2 types of extract (Table 1), ZCE appeared to be the more toxic to PA cells than ZCW with significant difference among the mean values.

3.3. Lipolytic assay of mature adipocytes

The extracts did not break down lipids. Neither dose-dependent nor time-dependent effects of the crude ZC extracts on lipolytic activity of MA cells were detected. At the concentration ranges used in the study, the lipolytic activity of the 2 extracts on MAs did not differ significantly over 3 day's exposure (Fig. 1). The most concentrated solution of each extract was chosen to be the S-Conc, i.e. 25 and 800 μg/ml for ZCE and ZCW, respectively. Exposure of MAs for 6 days to the S-Conc also had no effect on lipid content and glycerol release (see Supplement file, Fig. e-Sup 1).

Table 1
Mean values ± standard error of ICs (μg/ml) presenting the cytotoxic activity of the 2 crude extracts, ZCE and ZCW on Pas.

	ZCE	ZCW
IC_{10}	11.8 ± 3.2	560.4 ± 3.5
IC_{20}	25.8 ± 1.9	816.1 ± 2.1
IC_{50}	166.3 ± 2.4	1,674.8 ± 3.4

3.4. Anti-lipogenic assay

The extracts inhibited lipid accumulation. Although, the 2 ZC extracts (at non-toxic concentrations, IC_{10}-IC_{20}) did not inhibit differentiation of fat cells (adipogenesis), they did significantly reduce their fat content by significantly inhibiting lipogenesis in DA cells, compared with MA/nt (Fig. 2). ZCW and ZCE reduced lipid content in DA cells by 29 and 62% respectively, compared with the non-treated induced MA cells. Interestingly, the cells that underwent spontaneous adipogenesis (without induction), the non-treated SA/nt cells, contained the lowest lipid content, just 7% of that found in non-treated MA cells. Furthermore, 2 sub-populations of SA/nt were detected, spontaneous sensitive adipocyte (SSA) containing intracellular lipid droplets and spontaneous-insensitive adipocyte (SIA) with no visible lipid droplet. These sub-populations of cells with such biological properties have not previously been recorded among 3T3-L1 cultures. Nonetheless, how the extracts differently reacted to these cells was unclear in this experiment.

3.5. Adipogenetic evaluation

ZCE and ZCW significantly suppressed the expression of the adipocyte differentiation genes: *C/EBPα*, *PPARγ* and *ADD-1* (Fig. 3A). We also detected the expression of *Pref-1* gene and found that SA/nt cells have higher levels of gene expression than MA/nt cells.

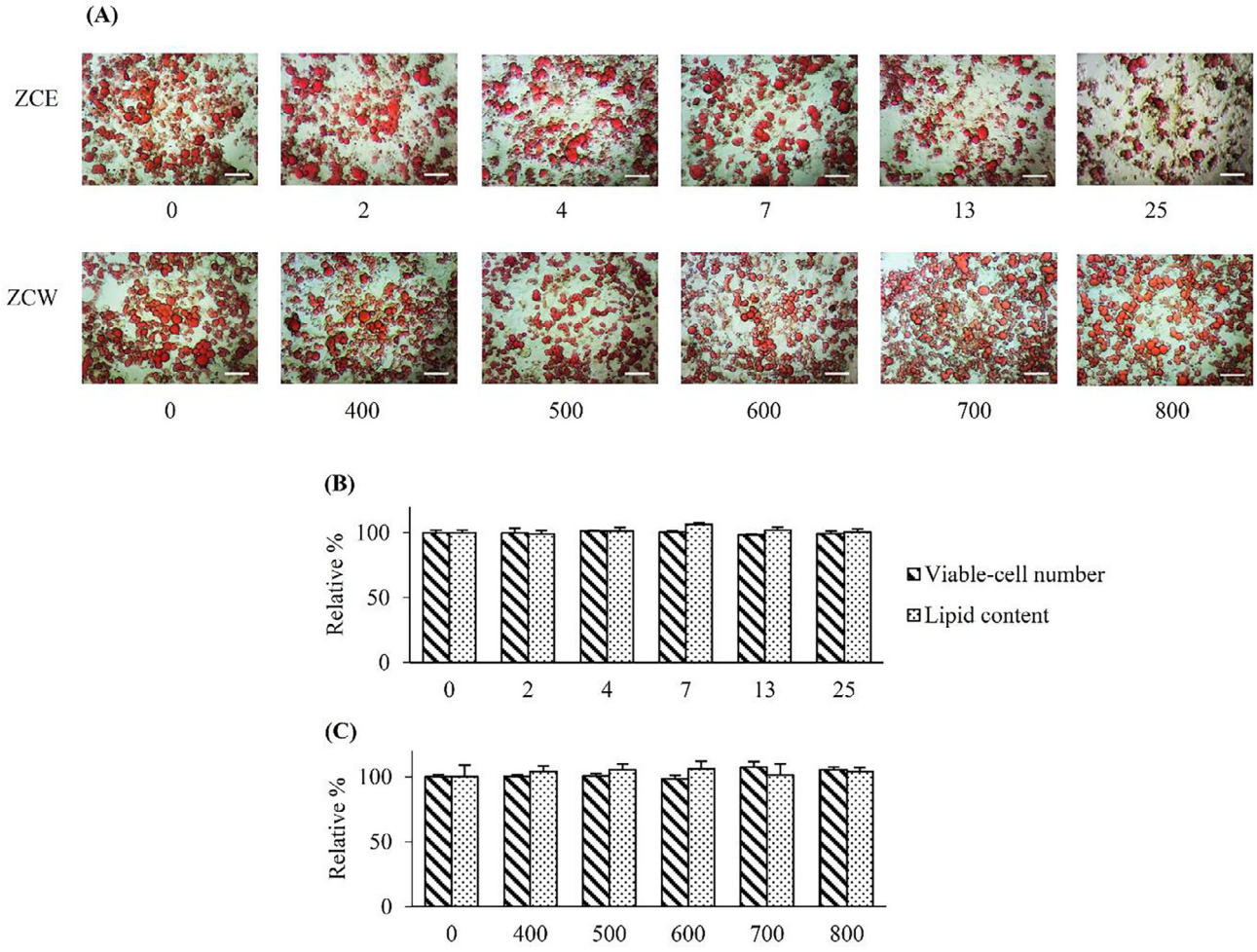

Fig. 1. Lipolytic activity of the crude extracts on MAs after 3 days of treatment. (A) – Image of lipid-droplets, stained with ORO, scale bar = 50 μm. Horizontal values indicated the 5-dilutions concentrations of the crude extracts in μg/ml. Relative percentage of lipid content by ORO colorimetric assay and relative percentage number of viable-cells, in comparison to those of the control group at 0 μg/ml, were presented on (B) – ZCE and (C) – ZCW in comparison to that of the media control (0 μg/ml). Differences among the means were not significant ($p \leq 0.05$).

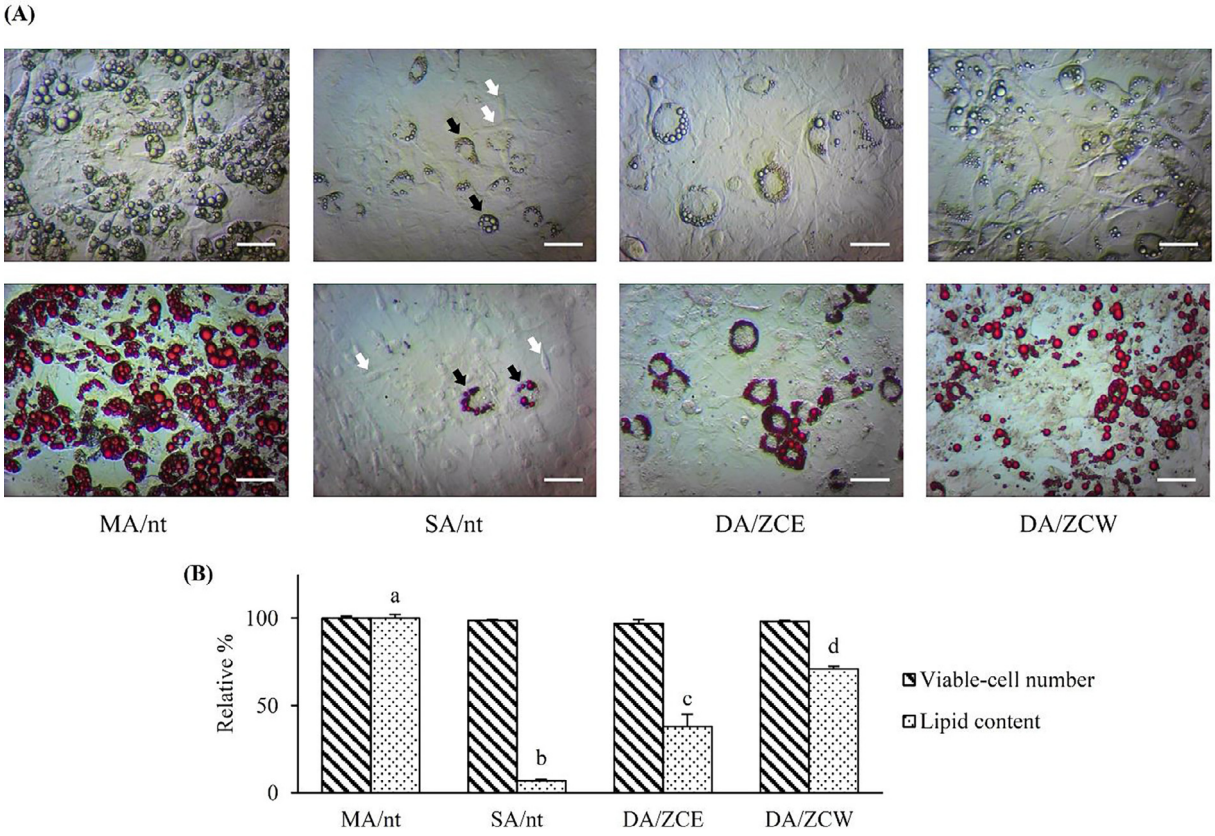

Fig. 2. Anti-adipogenic assay of the crude extracts on MAs after induction for 14-days. (A) – the fresh cells (upper row) and the cells with ORO staining (lower row) (scale bar = 50 μm). In SA/nt, black and white arrows indicated SSA and SIA cells respectively (see text). (B) – Relative number of viable-cells and the lipid content in compared with MA/nt. The exposed groups of DA/ZCE and DA/ZCW at 25 and 800 μg/ml respectively. The significant difference ($p \leq 0.05$) of the means was shown as a, b, c and d on top of the histograms.

However, *Pref-1* expression was drastically reduced in the MA/nt cells and in DA/ZCE and DA/ZCW, compared with SA/nt cells. Regarding the glucose uptake genes (Fig. 3B), expression of both *GLUT4* and *Adiponectin* was significantly reduced, but not *IRS-1*. In view of the lipid metabolism genes, expression of *FAS* and *aP2* was significantly reduced in DA/ZCE and DA/ZCW compared with MA/nt (Fig. 3C). Considering the genes that regulate fatty acid oxidation, expression of *ATGL* and *HSL* was significantly down regulated in DA/ZCE and DA/ZCW, compared with MA/nt (Fig. 3D). However, we observed no significant change in the level of *PGC-1β* expression in all experimental groups.

3.6. NMR spectroscopy

ZCE was analyzed by ^{1}H NMR spectrum (Fig. 4). Signals of the anomeric protons of glucose were clearly observable at 5.4 ppm (α-form) and at 4.5 ppm (β-form), while protons attached to hydroxyl groups of glucose had the resonances at 3.13–4.18 ppm. There were many signals of aromatic protons at 6.7–7.1 ppm indicating that the majority of the metabolites in the ethanol extract are natural products with aromatic rings. Signals of olefinic protons at 6.0–6.5 ppm were observed in the ^{1}H NMR spectrum and these signals were most likely to be of phenylbutanoids [21,22].

^{1}H NMR spectrum of ZCW was also obtained and analyzed (Fig. 5). Glucose was presented in the water extract because of the appearance of its anomeric protons at 5.3 ppm (α-form) and at 4.5 ppm (β-form), as well as protons attached to hydroxyl groups at 3.10–4.16 ppm. Two signals of aromatic protons at 6.85 ppm and 6.98 ppm and two signals of olefinic protons at 6.08 ppm and 6.32 ppm were observed in the spectrum. These signals are characteristics for phenylbutanoids [21,22].

4. Discussion

With the synonym of scientific name to ZC, the review of ZM have been considered in parallel. Percentage yield of extractions from different works was found with result variations depended on the methodology of extraction and solvent [7,22]. In this work, the lesser-polar fractions, ZCE, exhibited with lesser % yield than the higher-polar fraction, ZCW.

Using the generally acceptable IC_{50} of between 20 and 40 μg/ml for crude extracts from herbal materials seems to be accepted in some cases as 'high-toxic' to either cancer cells [24,25] or normal cells [26], ZC appeared to show no cytotoxic activity (IC_{50} > 40 - μg/ml). Methanol extracts of ZC were previously reported to be cytotoxic to 3T3-L1 with only 24% of cells surviving (i.e. IC_{76}) at a concentration of 200 μg/ml [27]. However, that report focused anti-oxidant activity, rather than anti-adipogenic activity. Essential oils of ZC are not cytotoxic to human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines at concentrations close to 100 μg/ml [8].

Considering adipogenic effects of active compound from herbs that are closely related to ZC, gingerol (a phenolic compound from *Z. officinale*) enhances glucose uptake by increasing cell surface GLUT4 on L6 rat skeletal muscle cells *in vitro* [28]. The same compound from *Z. zerumbet* prevents adipocyte proliferation in a dose- and time-dependent manner and prominently inhibits adipogenic differentiation of 3T3-L1 cells [13]. Shogaol, another type of

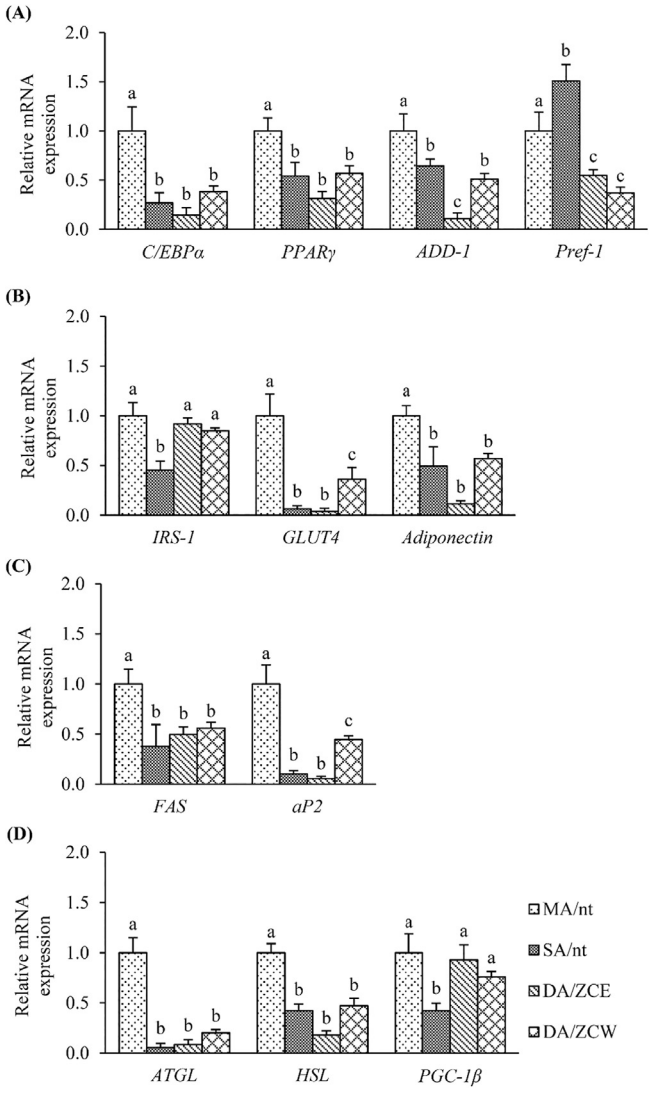

Fig. 3. Expression of adipogenic-related genes on the 4 cell-groups (abbreviation, see text). (A) –adipocyte differentiation genes, (B) – glucose uptake genes, (C) – lipid metabolism genes and (D) – fatty acid oxidation genes. The significant ($p \leq 0.05$) of mRNA expression of each gene is shown in relation to MA/nt (control) cells.

phenolic compound and gingerol from *Z. officinale* possess lipolytic and anti-adipogenesis activity [12].

ZC yields a diverse range of polyphenols, steroids and terpenes [23,29,30]. Pure compounds of different types from ZC and *Z. officinale* have been identified elsewhere [31–34]. Previously, several phenylbutanoids were isolated from the rhizomes of ZC [21,22,35,36]. They were found to have anti-inflammatory activity by the inhibition of lipopolysaccharide (LPS)-induced nitric oxide production [22] and inhibition of cyclooxygenase-2 [35], anti-cancer activity [36], and inhibition of invasion of cancer cells [21]. Recently, the phenylbutanoid, *E*-4-(3′,4′-dimethoxyphenyl)but-3-en-1-ol, from ZC showed anti-inflammatory activity in Wistar rats [37]. Isolated chemical compounds from ZC/ZM have never been tested for anti-adipogenesis or lipolysis. Since we now proved anti-lipogenesis activity of ZC crude extract, we recommend that similar trials are now performed on the main groups of chemical constituents or on individual compounds.

The effects of the extracts on gene regulation are shown in the Graphical Abstract. After induction of adipogenesis in PAs, *C/EBPα* and *PPARγ*, are up-regulated [38]. *C/EBPα* then activates the expression of other adipocyte genes and thus stimulates differentiation. *PPARγ* regulates fatty acid storage, glucose metabolism and initiates lipid accumulation. *ADD-1* plays an important role in fat cell gene expression and differentiation and enhances the regulatory pathway of *PPARγ* [39]. Our study showed that ZCE and ZCW suppress expression of *C/EBPα*, *PPARγ* and *ADD-1*, *i.e.* the extracts suppress adipogenesis (Fig. 3A). *Pref-1* is a transmembrane protein that is highly expressed in PAs and it can be used as a marker for PA characteristics. Down-regulation of this gene is related to the end of the PA stage and is probably required for adipocyte differentiation [40]. Therefore, the reduction in *Pref-1* expression indicates that the PA stage is ending and the MA has been triggered. The high expression of *Pref-1* in SA/nt cells showed that most of the cells in that culture maintained their status as PAs, after 14 days of cultivation (Fig. 3A).

Regarding the glucose-uptake genes (*IRS-1*, *GLUT4* and *Adiponectin*), differences in the level of *IRS-1* gene expression in DA/ZCE and DA/ZCW were not significant, compared with the MA/nt group (Fig. 3B). The extracts, therefore, did not interfere with insulin signal transduction. Our observation that *GLUT4* was reduced, after the exposure to the extracts, strongly suggests that adipogenic suppression at least performed through down regulation of the insulin-regulated glucose transporter. Reduction in GLUT4 on the membrane led to a decrease in glucose transport from the extracellular environment into the cells. This may explain the smaller sizes of lipid droplets in DA/ZCE and DA/ZCW indicating lower lipid content (Fig. 2A). The mRNA expression level of *Adiponectin* was significantly decreased in DA/ZCE and DA/ZCW (Fig. 3B). Adiponectin is an adipokine, secreted predominantly by adipose cells, which regulates both metabolic and vascular homeostasis *in vivo*. Adiponectin also increases insulin's ability to maximally stimulate glucose uptake through increasing expression of the *GLUT4* gene and increasing the recruitment of GLUT4 protein to the plasma membrane [41]. Suppression of *Adiponectin* by the extracts supports the idea that glucose transport through *GLUT4* is reduced. This may also be a major target pathway of the extracts for their mechanisms of action to prevent the accumulation of lipid content in the cells.

Focusing on the lipid metabolism genes, *FAS* regulates the synthesis of fatty acids from acetyl-CoA and malonyl-CoA via the tricarboxylic acid cycle [42]. Therefore, reduction of *FAS*, by ZCE and ZCW extracts (Fig. 3C), could also explain how the extracts suppress lipid accumulation. *aP2* is a fatty acid binding protein, which reacts as a key factor in intracellular fatty acid transport and lipid metabolism. This gene is activated in response to *PPARγ* regulation [43]. In our study the extracts reduced the mRNA level of *aP2*, suggesting that intracellular fatty acid transport and lipid metabolism are reduced due to low lipid accumulation.

Considering fatty acid oxidation genes, ATGL is a patatin-derived lipase, with differentiation-dependent up-regulation during adipogenesis of 3T3-L1 cultures [44]. ATGL selectively hydrolyses triglycerides into diacylglycerol, where HSL (together with another enzyme, monoacylglycerol lipase) continues breaking down to glycerol and free fatty acids (FFA) [45]. FFAs are then taken into mitochondria to facilitate fatty acid oxidation. The extracts down regulated expression of *ATGL* and *HSL* genes (Fig. 3D). These results suggest that in addition to the suppression of the early responsive genes activation for initiation of the adipocyte differentiation, glucose uptake and synthesis-and-transport of fatty acid, the extracts can also suppress the processes of lipid break down. This phenomenon is not surprising, since cells have smaller lipid content due to inhibition of glucose uptake and lipid synthesis and thus there is no need for cells to induce the production of lipases to break down lipid. Normally, up-regulation of *PGC-1β* increases fatty acid oxidation in the mitochondrial pathway [46,47]. However, in this study when we measured the level of *PGC-1β*, there was no change in all experimental groups. PGC-1β

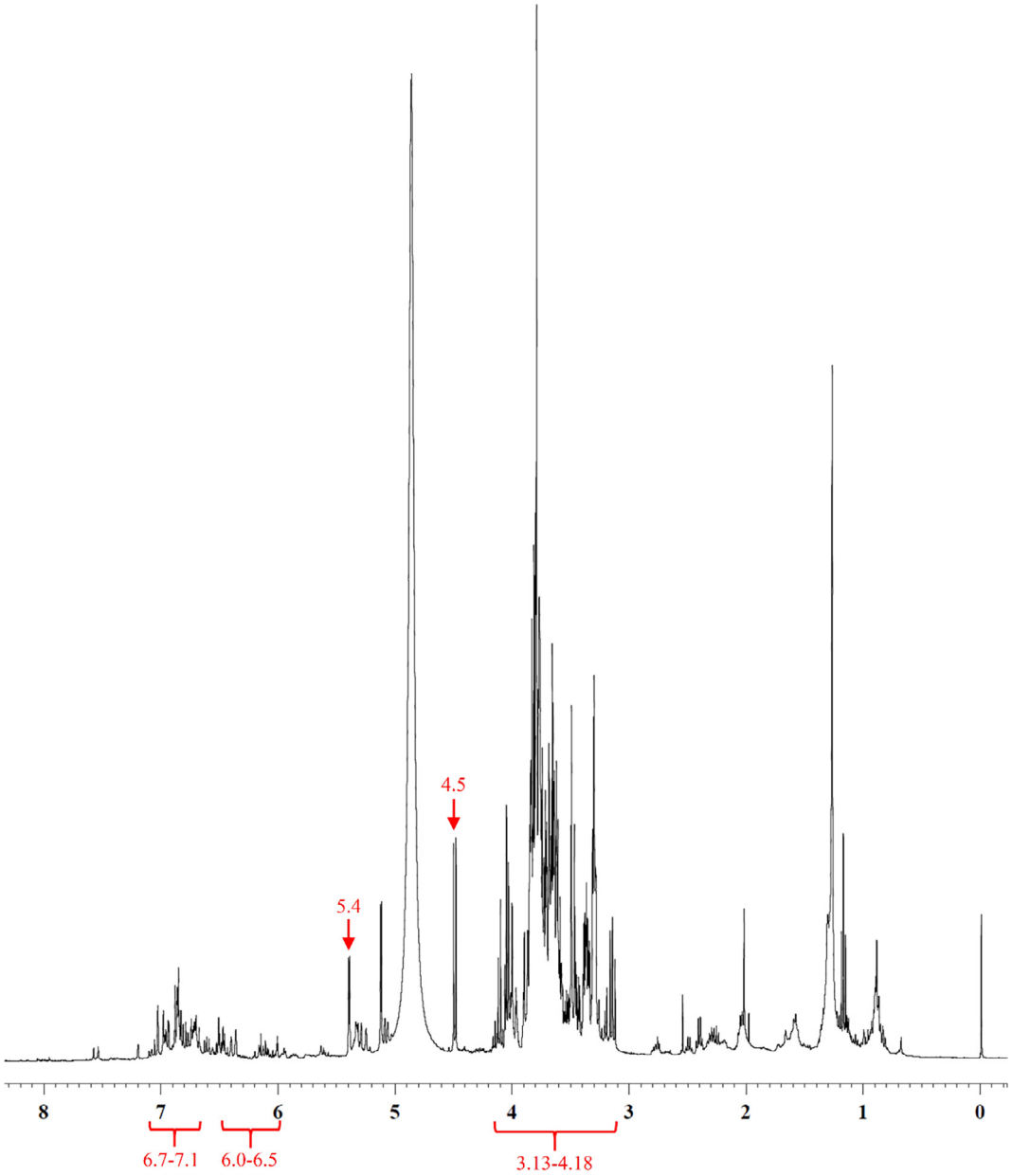

Fig. 4. ^{1}H NMR spectrum (CD_3OD) of the ethanol crude extract of ZCE. Anomeric protons peaks of glucose were shown at 5.4 and 4.5 ppm, with the resonances of hydroxyl groups' protons at 3.13–4.18 ppm. Aromatic protons of metabolite majorities appeared at 6.7–7.1 ppm. Olefinic protons of phenylbutanoid compound were at 6.0–6.5 ppm.

works as a co-activator of PPARγ in the transcriptional control of mitochondrial oxidative metabolism [48]. Therefore, ZCE and ZCW extracts did not suppress adipogenesis via down regulation of *PGC-1β*.

Admitting ZC in this study showed the supported evidences of anti-obese activity, according to the Thai tribe traditional used, but precaution must be highlighted with over-dose consumption. Rats fed with single dose of ZC extracts at 5000 mg/kg.bw showed a remarkable decrease of body weights, but significant weight increase was detected in certain internal organs; spleen and epididymis in male, while lung and kidney in female [2].

5. Conclusion

Rhizome extracts of ZC have no effect on lipolysis, the breakdown of fats in mature adipocytes, and also has no effect on adipogenesis, the differentiation of mature adipocyte from the preadipocyte. However, both ethanolic and aqueous extracts of ZC similarly reduced lipogenesis, the synthesis of lipids within the differentiated adipocytes, at the gene level, through inhibition of genes involved in adipocyte lipogenesis, glucose transport and fatty acid synthesis. Phenylbutanoid compounds in the extracts were believed to play an important role in such the activities. Whilst ZC is certainly not a cure for obesity, it may have applications in preventing the onset of obesity or preventing obese patients from becoming more obese. But before such recommendations can be considered, animal and human trials should be carried out to test the efficacy of the crude aqueous extract. If successful, further research, to determine which of the vast range of compounds present in the ZC extracts are responsible for inhibiting lipogenesis might yield more refined dietary supplements of medicines capable of controlling the onset or exacerbation of obesity.

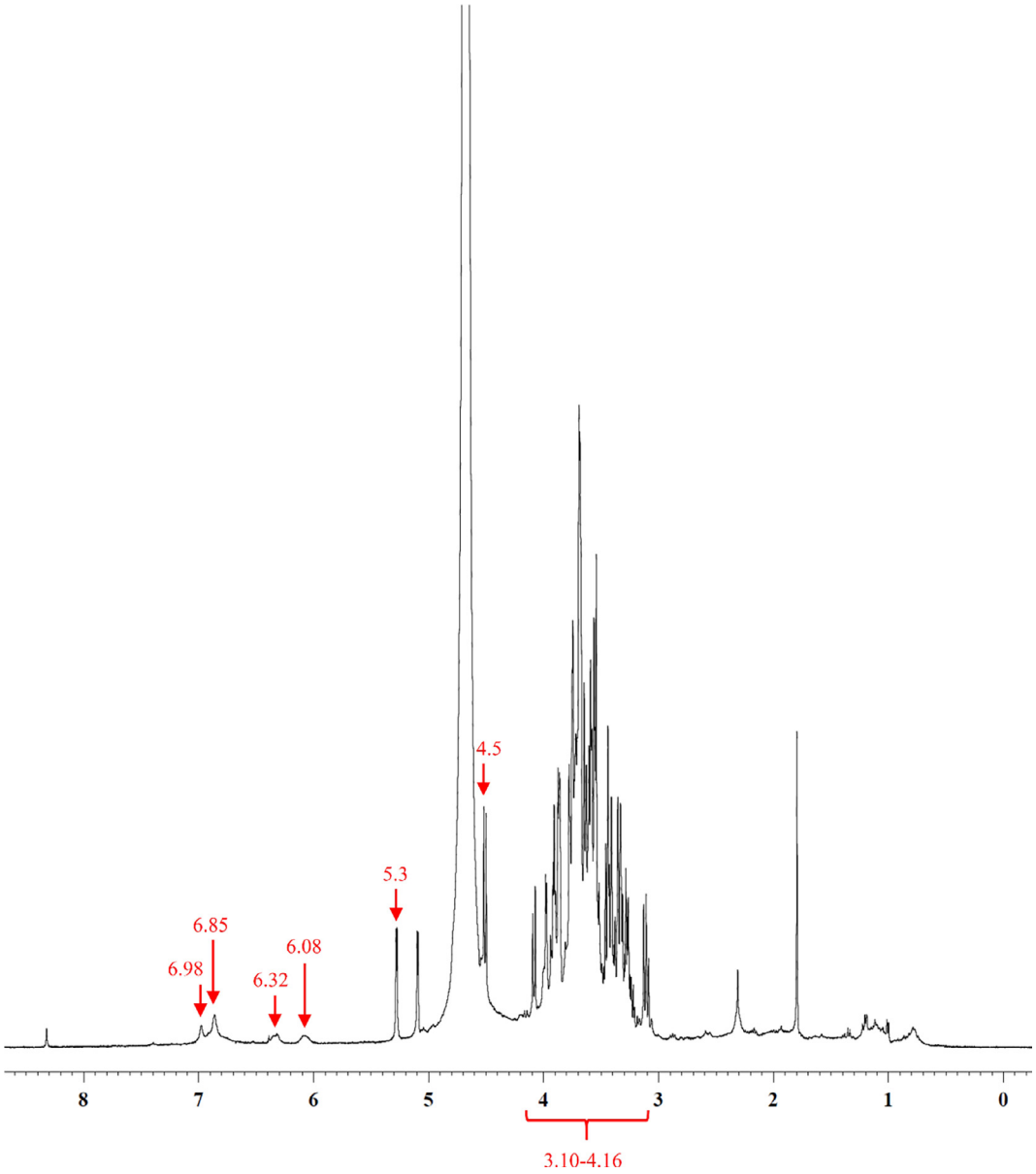

Fig. 5. ^{1}H NMR spectrum (D_2O) of ZCW. Anomeric protons peaks of glucose were shown at 5.3 and 4.5 ppm for α-form and β-form respectively, with the resonances of hydroxyl group protons at 3.10–4.16 ppm. Aromatic protons of metabolite majorities appeared at 6.85 and 6.98 ppm. Olefinic protons of phenylbutanoid compound were at 6.08 and 6.32 ppm.

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Conflict of interest statement

We declare that we have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbas.2018.09.001.

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