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Research article

Anti-adipogenesis activities of *Zingiber cassumunar* Roxb. rhizome extracts on L929 cells evaluated by image-based analysis

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Abstract

Some species in family Zingiberaceae have been reported as having anti-adipogenesis activity, but to date *Zingiber cassumunar* has not been investigated. Therefore, this paper investigates of *Z. cassumunar* extracts on mouse pre-adipocytes, L929 cell line; the first time this cell has been used as a non-inductive in vitro model. Dry rhizome was extracted by dichloromethane, absolute ethanol, and hot water obtaining the crude extracts: ZCD, ZCE and ZCW respectively. A non-cytotoxic activity was shown on the 24 h exposed cells to all extracts, evaluated by using the MTT (methyl-thiazolyldiphenyl-tetrazolium bromide) assay. An anti-proliferation assay was carried out to each of the extracts for 72 h, to determine the non-toxic inhibition-concentration (IC_{20}). The quantity of lipid droplets in the cells, exposed to the IC_{20} or $IC_{20}/2$ of each extract, was assessed by using photomicrographs and image-based assay under the ImageJ computer software. Cellular lipid accumulation was significantly decreased in the exposed cells to each of the extract in a concentration-dependent manner, in comparison to the unexposed cells, by which ZCE was the most effective followed by ZCW and ZCD respectively. Expression of adipogenesis-related genes of the exposed cells to each extraction was evaluated through real-time PCR. All of the extracts down-regulated the activity of PPARγ (the key regulator of adipogenesis) while ZCE up-regulated Pref-1 (the pre-adipocyte regulatory gene inhibiting lipid accumulation). Four groups of genes were down-regulated by at least one or more of the extracts. These findings verified the anti-adipogenesis potential of *Z. cassumunar* in mammalian adipocyte.

Keywords: Adipogenesis, *Zingiber cassumunar*, Image-based analysis, L929

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INTRODUCTION

Fat tissue, containing lipid droplets (LDs), plays an important metabolic and signal function that regulates body homeostasis. LDs, in the form of triglycerides, are accumulated in mature adipocytes, most of which are defined as white adipose tissue (WAT). Adipogenesis and adipolysis occur equally through coupled biochemical pathways to maintain homeostasis. Disproportion of the pathways generally leads to metabolic abnormality which eventually causes obesity syndrome (Lefterova and Lazar 2009). Initiation of the syndrome is further related to incidence of several chronic diseases including hyperlipidemia, hypertension, atherosclerosis, fatty liver, diabetes mellitus, cancer and cardiovascular disease (Mukherjee, 2003). Thus the regulation of the coupled pathway is probably an essential route for obesity control. There are few FDA approved anti-obesity medications or weight loss drugs, including Orlistat (Xenical), lorcaserin (Belviq) and a combination of phentermine and topiramate (Qsymia®) (Lefterova and Lazar 2009). Although these drugs are effective, adverse side effects can occur including gastro-esophageal reflux diseases, depression, liver failure, increased heart rate, psychiatric and cognitive effects, and even death (Buyukhatipoglu, 2008; Shin and Gadde, 2013). Traditional natural medicines have progressively become an alternative option for healing many diseases, including obesity, (Hasani-Ranjbar et al., 2013). Also Natural medicines usually have fewer side effects (Najafian et al, 2014).

Cassumunar ginger (Plai or Phlai in Thai) or *Zingiber cassumunar* Roxb. (Family Zingiberaceae), a synonym of *Zingiber montanum* (J.König) Link ex A. Dietr (Sharma et al., 2011), is a perennial herb distributing mainly in India, Indochina and tropical Southeast Asia. Its rhizome has been applied to treat inflammation, muscle and joint problems, menstrual disorders, abscesses and skin diseases and for wound healing (Farnsworth and Bunyapraphatsara, 1992). Phytochemical investigations of *Z. cassumunar* (ZC) rhizomes have revealed the presence of phenylbutanoids, cyclohexene derivatives, naphthoquinones, vanillin, vanillic acid, veratric acid, terpenoids, β-sitosterol, and curcuminoid (Koparde and Magdum, 2017). Previous studies also reported that some plants in the family Zingiberaceae decreased blood glucose and lipids, which further helped in reducing body weight, by lessening lipid peroxidation without inhibiting pancreatic lipase (Mahmoud and Elnour, 2013).

Many strategies for obesity treatment have been evaluated by focusing on mechanisms of adipocyte differentiation, adipogenesis and adipolysis (or lipolysis) at cellular levels. Two classes of cell lines have been applied as precursors for adipocyte differentiation; pluripotent fibroblasts (10T1/2, Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts) and unipotent preadipocytes (3T3-L1, 3T3-F422A, 1246, Ob1771, TA1 and 30A5) (Ntambi and Young-Cheul, 2000). Both can be induced to mature adipocytes by using some chemical agents and maintaining them under appropriate culture conditions. However, the in vitro inductive adipogenesis usually takes a long time, e.g., up to 12 days on 3T3-L1 (Stoecker et al., 2017) and 14 days on human preadipocytes (Bombrun et al., 2017).

This research was the first trial that used the L929 mouse fibroblast cell line as a model for adipogenesis. L929 is a mouse pre-adipocyte that spontaneously differentiates in vitro to mature adipocytes, without requiring for any inducing agent (Jeney et al., 2000). Indeed, L929 has never been reported in any broadly applied for adipogenesis experiments, but the cells becomes more attractive to be used in a laboratory due to their abilities to accumulate lipid droplets within a few days. Also the spontaneous differentiation of the cells to mature adipocytes offer a benefit to avoid any unexpected interactions between the adipocyte inductive-agents and the herb extracts.

Consequently, this research examined *Z. cassumunar* on L929 cell lines and determined the non-toxic concentrations for downstream experiments. Lipid content in the cells was evaluated by conventional Oil Red O (ORO) staining, with image-based analysis verification. Adipogenic-related gene expression profiles were evaluated to get a better understanding how the extracts control through targeted gene regulation of adipocyte development.

MATERIALS and METHODS

Chemicals and reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and 100X antibiotics/antimycotics were purchased from GIBCO® (Grand Island, NY, USA). Isopropanol, dimethyl sulfoxide (DMSO), gelatin Type B from bovine skin and methylene blue (MB) were from Sigma-Aldrich (St. Louis, MO, USA). Oil Red O (ORO) was from Bio Basic (Amherst, NY, USA). Dichloromethane (CH_2Cl_2), absolute ethanol (CH_3CH_2OH) and formaldehyde (CH_2O) were from RCI Labscan (Samutsakorn,Thailand). Methyl-thiazolyldiphenyl-tetrazolium bromide (MTT; 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) was from Invitrogen® (Carlsbad, CA, USA).

Crude extracts of *Z. cassumunar* were prepared from the dry powder of the rhizome using three solvents under two standard methods. Soxhlet extraction was setup through dichloromethane and absolute ethanol to obtain ZCD and ZCE extracts, respectively. Water heat reflux was also performed to obtain a ZCW extract. The percentage yield of each extract was calculated relative to the rhizome dry weight.

Preparation of the extracts used in experiments

Each extract was dissolved in DMSO, as a stock solution, to reach a maximum concentration at 30 mg/ml (w/v) prior to preparation of working concentrations by mixing in the completed media. The working concentrations ranged from 7 to 200 µg/ml, depending on the requirement of each experiment.

Cell culture

The mouse fibroblast cell line, L929, was purchased from American Type Culture Collection (ATCC®, Manassas, VA, USA). Cells were cultured in 25 cm^2 culture flasks with the completed media (DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics / antimycotics). Cells were maintained under standard culture conditions at 37°C and 95% relative humidity in a 5% CO_2 atmosphere. The medium was changed every 2-3 days and sub-culturing was performed at 70% spatial confluency by trypsinization.

From our preliminary study, the seeding density of L929 at 50,000 cell/ml has given the best result of intracellular lipid accumulation within 5 days culture period. The cells in 6 and 7 days culture periods were observed with cell death and detachment from the culture surface (data not shown).

Pre-coating the 96-well culture plates

The surface of 96-well plates was pre-coated with 0.1% (w/v) gelatin solution, as suggested by ATCC® (PCS-999-027™). Briefly, the gelatin solution was prepared by dissolving gelatin type B from bovine skin in deionized water, followed by sterilization in autoclaving (121°C, 15 psi, 15 min). The solution was cooled to room temperature, before adding 60 µl of gelatin solution into each well and incubation for 30 min under standard culture conditions. After that, the solution was aspirated and discarded prior to adding serum free DMEM (100 µl/well). The coated plates were kept in a CO2 incubator for at least an hour, but not exceeding 3 days after coating. Whenever cell seeding was required, the media was discarded beforehand.

Couple staining of Oil Red O and methylene blue (MB)

Cells were seeded in gelatin-coated 96-well plates at a density of 50,000 cell/ml. The cultures were maintained under standard culture conditions for 5 days and the medium renewed every couple of days. The progression of L929 differentiation to adipocytes was primarily stained by ORO, as previously described by Kinkel et al. (2004). In short, the cells were gently rinsed with phosphate buffer saline twice, fixed by 10% (w/v) formaldehyde for an hour and then washed thoroughly with distilled water. Isopropanol (60% v/v) was added to wash and dehydrate the cells and discarded afterward. The cells were then completely air-dried at room temperature. ORO (1.4 g ORO, 400 ml isopropanol, 144 ml distilled water) was added to the cells, incubated for 15 min and afterward completely rinsed with distilled water 4 times. After that, MB counter staining was performed and the cultures were then incubated for 3 min before rinsing with distilled water 3 times as described by Todoric et al. (2011).

Cytotoxicity screening

L929 (50,000 cell/ml) were seeded in gelatin-coated 96-well plates and incubated for 24 h under standard culture conditions. The cultures were exposed to Z. cassumunar extracts (ZCE, ZCD and ZCW) at concentrations of 7, 13, 25, 50, 100 and 200 µg/ml and incubated for 24 h. A control was the cells received completed media in equivalent volume to the extracts. Cell viability was evaluated by MTT assay. Shortly, MTT (12 mM) was added into each well and incubated for 4 h at 37˚C. Afterwards, 100 µl of 10% (w/v) SDS-0.01 M HCl was added and the culture further incubated at 37˚C overnight. The amounts of viable cells were measured indirectly through the value of optical density at 570 nm (OD570) acquired under a microplate-reader (Rayto TR-2100C, Shenzhen, China). The half of maximal inhibitory concentration (IC50) of each crude extract was determined by the PriProbit program ver. 1.63 (Sakuma, 1998). The relative viable-cell was calculated by using mean of OD570 value of the exposed cells to each extract in percentage comparing to the control.

Anti-proliferation assay

This test was performed as described in the cytotoxic screening sub-topic with seeding cell density at 50,000 cell/ml. Cells were exposed to the extracts for up to 72 h before OD570 measurement. The PriProbit program methodically predicted an IC_{20} concentration which was further applied in the other experiments.

Evaluation of anti-adipogenesis efficacy from the extracts

Lipid content measurement by image-based analysis

Cells were seeded in the coated 96-well plates, at a density of 50,000 cell/ml, and incubated for 24 h under standard culture conditions. Cells were exposed to the extracts at concentrations of IC_{20} and half of IC_{20} ($IC_{20}/2$) for 72 h with media + extracts and refreshed every 48 h. During that time, assuming that the cells had differentiated to mature adipocytes and accumulated LDs in their cytoplasm under the extract influences. Thereafter, the ORO-stained mature adipocytes were counter stained with MB, as described above. The ORO-MB-stained cells from all treatments were randomly photomicrograph-taken under an Olympus CK40 equipped with CCD camera (Olympus, Japan), with approximately at least 200 cells per image. Images were stored as tagged image format files (*.TIFF). To detect and measure LDs area using the ImageJ software (https://imagej.nih.gov), the images were adjusted and processed, as described in Broeke et al. (2015). In brief, the images were normalized and calibrated before setting up a color threshold. A background subtraction was applied to reduce some background noises, resulting in vivid LDs, the areas of which were automatically detected by using the 'analyze particle' commands in the software. The LD areas from the treatments were further compared with the controls, resulting in percentage of relative LD areas.

Adipogenesis related gene expression

After the cells had been exposed to the extracts at concentrations of IC20 for 72 h, they were harvested for RNA extraction, using a NucleoSpin® RNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions.

The mRNA was reverse transcripted to cDNA, using ReverTra Ace® qPCR RT master mix (Toyobo Co., Osaka, Japan). Four groups of adipogenesis-related genes were examined: (1) adipocyte differentiation genes; C/EBPα, PPARγ, ADD-1, Pref-1, (2) glucose uptake genes; IRS-1, GLUT4, Adiponectin, (3) lipid metabolism genes; FAS, aP2 and (4) fatty acid oxidation genes; ATGL, HSL, PGC-1β. β-actin was used as a housekeeping gene or internal control. Primers (Table 1) were designed by using Primer 3, a primer design tool, followed by the primer specificity confirmation using nucleotide BLAST which both tools are available on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Target gene expression was quantitatively evaluated by real-time PCR (SensiFASTTM SYBER® No-ROX Kit (Bioline, London, UK)), performed in the EcoTM Real-Time PCR System (Illumina, Inc. SD, USA). The PCR mixtures were denatured at 94˚C for 3 min, followed by 35 thermal cycles: 40 sec at 94˚C, 30 sec at 60˚C and 30 sec at 72˚C. CT values were normalized by an internal and an external control or an experimental control. The expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001). Data were presented as relative gene expression by comparison of the treatments with the controls.

Table 1 Designed primers of adipogenesis related genes, using Primer 3 and BLAST available from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with accession number.

Gene	Accession number		Sequence (5'-3')
C/EBPα	NM_007678	fw	GGA TAC TCA AAA CTC GCT CC
		rv	CTA AGT CCC TCC CCT CTA AA
PPARγ	NM_011146	fw	TTT TCA AGG GTG CCA GTT TC
		rv	AAT CCT TGG CCC TCT GAG AT
ADD-1	NM_011480.3	fw	TGT TGG CAT CCT GCT ATC TG
		rv	AGG GAA AGC TTT GGG GTC TA
Pref-1	L12721.1	fw	CTA ACC CAT GCG AGA ACG AT
		rv	GCT TGC ACA GAC ACT CGA AG
IRS-1	NM_133249.2	fw	CAT GCA GAT AGG TTG TCC TC
		rv	CAG CAA GGA AGA GTG AGT AG
GLUT4	AB008453	fw	ACC TCT ACA TCA TCC GGA AC
		rv	TTG ATG CCT GAG AGC TGT TG
Adiponectin	U49915	fw	GCA ACT ACT CAT AGC CCA TA
		rv	CAT GTA AGA GTC GTG GAG AC
FAS	NM_007988.3	fw	TTG CTG GCA CTA CAG AAT GC
		rv	AAC AGC CTC AGA GCG ACA AT
aP2	NM_024406.2	fw	TCA CCT GGA AGA CAG CTC CT
		rv	AAT CCC CAT TTA CGC TGA TG
ATGL	AY894805	fw	TAT TGA GGT GTC CAA GGA GG
		rv	GTA CAC CGG GAT AAA TGT GC
HSL	NM_010719.5	fw	GAG GGA CAC ACA CAC ACC TG
		rv	CCC TTT CGC AGC AAC TTT AG
PGC-1β	NM_133249.2	fw	GGA AGA ACT TCA GAC GTG AG
		rv	CAC CTG GCA CTC TAC AAT CT
β-actin	NM_007393.4	fw	CCA CAG CTG AGA GGG AAA TC
		rv	AAG GAA GGC TGG AAA AGA GC

Statistical analysis

All data were represented as means ± standard errors (SE). Data were statistically evaluated by a one-way analysis of variance. Significant difference ($p \leq 0.05$) among the groups was analyzed by Duncan's multiple range tests. All experiments were repeated 3 times, each with 5 replicates of the designed concentration.

RESULTS

Crude extraction

Water was the most effective solvent. The percentage yields of ZCD, ZCE and ZCW were 4.8%, 1.7% and 14.7%, respectively.

Cytotoxicity and anti-proliferation effects of *Z. cassumunar*

After the cells had been exposed to the various concentrations of ZCD, ZCE and ZCW for 24 h, acute cytotoxicity was quantified as a percentage of the number of viable-cells relative to the controls (Figure 1A-1C). Cell-viability when exposed to ZCD (Figure 1A) and ZCE (Figure 1B) was concentration-dependent manner. In contrast, the effects of ZCW showed an astonishment of variability ranging from non-toxicity to slightly increases in cell-viability (Figure 1C). As the IC_{50} of each crude extract was >100 µg/ml in which classified by U.S. National Cancer Institute (NCI) as the non-acute-cytotoxic to the exposed cells (Iswantini et al., 2011). However, the most active extract, in according to IC50, was ZCD and followed by ZCE and ZCW.

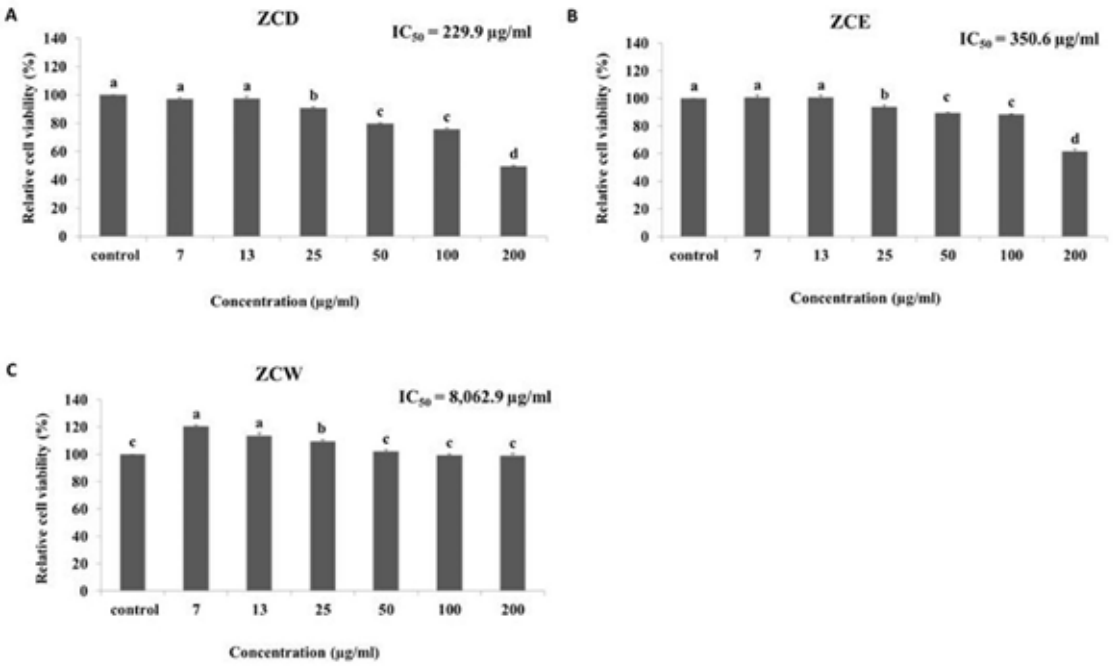

Figure 1 Cytotoxic effect after 24 h exposure of (A) ZCD, (B) ZCE and (C) ZCW at varied concentration. IC_{50} values, obtained from the PriProbit analysis, were shown on the top right of the graphs. Control was the cells received completed media in equivalent volume to the extracts. The small alphabets (a, b, c, d) indicate the statistical difference among the concentrations of each extracts ($p \leq 0.05$).

The anti-proliferation potential was also evaluated after the cells had been exposed to the extracts for 72 h. ZCD (Figure 2A) and ZCE (Figure 2B) tended to inhibit cell proliferation in the concentration-dependent manner in a similar pattern to that of cytotoxicity (Figure 1). In contrast, ZCW again exhibited no anti-proliferation effect (Figure 2C).

In this experiment the IC_{20} was calculated to determine the non-toxic concentrations on cellular proliferation, to be used in the anti-adipogenesis experiments. IC_{20} was individually obtained from the PriProbit analysis and is shown on each graph in figure 2 with ZCW as the most effective extract, followed by ZCE and ZCD. $IC_{20}/2$ was then calculated, which would also be used in the anti-adipogenesis efficacy evaluation.

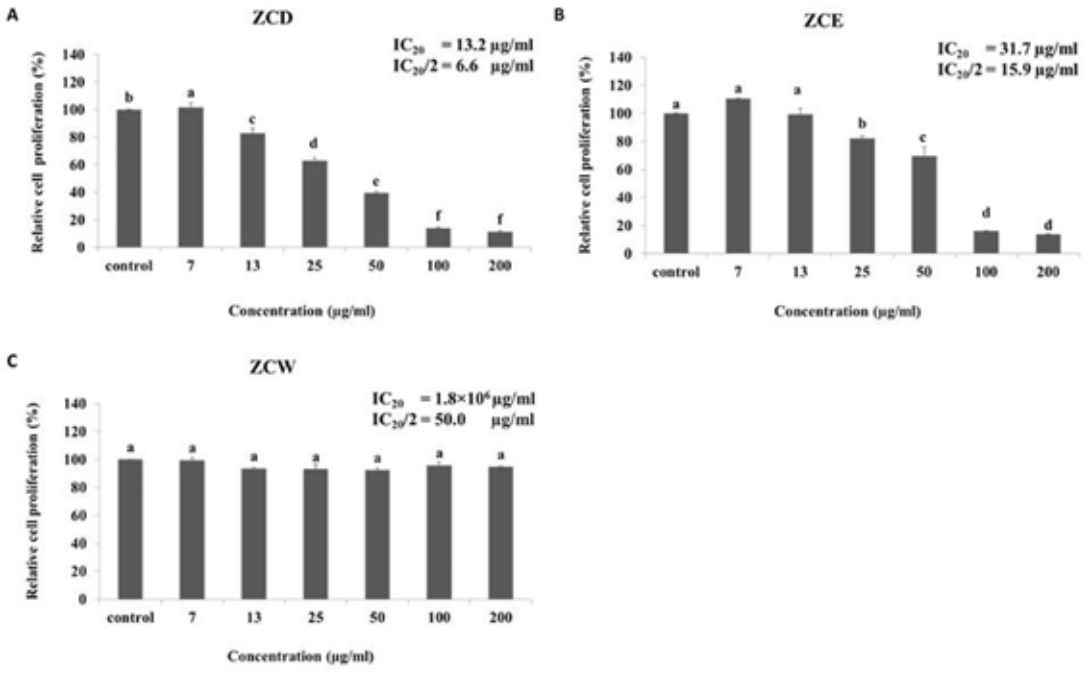

Figure 2 Anti-proliferation effect on the cells after 72 h of exposure to (A) ZCD, (B) ZCE and (C) ZCW at varied concentrations. IC_{20} and $IC_{20}/2$ values, obtained from the PriProbit analysis, were shown on the top right of the graphs. Control was the cells received completed media in equivalent volume to the extracts. The small alphabet (a, b, c, d) indicated the statistical difference among the concentrations of each extracts ($p \leq 0.05$). On (C) the *IC_{20} of ZCW, 1.8x106 µg/ml, was far too much to be used in the experiment and was speculated for the possible preparation at 100 µg/ml with *$IC_{20}/2$ at 50 µg/ml.

Evaluation of anti-adipogenesis efficacy of the *Z. cassumunar* extracts

Lipid content measurement by image-based analysis

Photomicrographs of the cells, before and after the Image J program processing, have been taken and presented in figure 3. Lesser LDs areas were observed in the exposed cells to the extracts in comparison to the unexposed control cells. The LDs areas were then quantified by the program and summarized in figure 4. All of the extracts significantly inhibited LDs accumulation. The most effective extract was ZCE, followed by ZCW and ZCD.

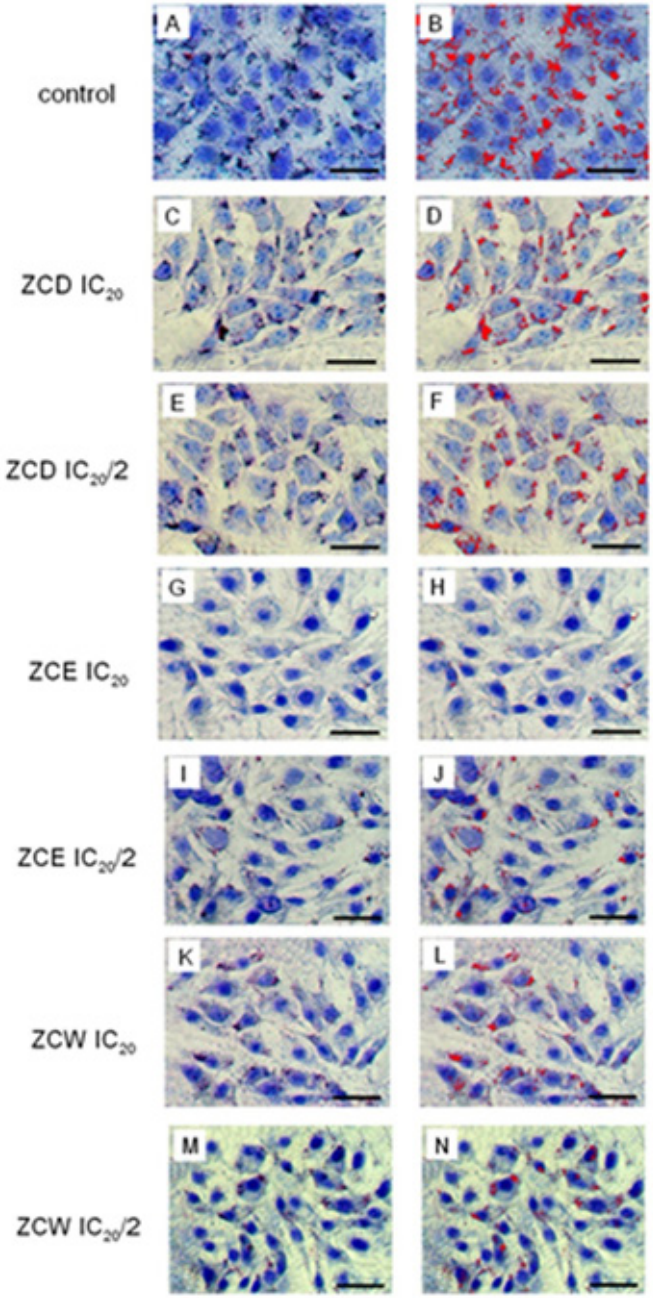

Figure 3 Samples of photomicrography taken before (A, C, E, G, I, K, M) and after (B, D, F, H, J, L, N) the processing under ImageJ computer program (Scale bar = 100 µm). Note that the nucleus was displayed in blue color of MB while LDs stained in darken red-dots with ORO (A, C, E, G, I, K, M) and appeared in vivid red cluster after the processing (B, D, F, H, J, L, N).

Adipogenesis related gene expression

Adipocyte differentiation genes; ZCD (Figure 5A) and ZCW (Figure 5C) significantly down-regulated the expression of all genes in this group, the lowest one was PPARγ. Whilst ZCE (Figure 5B) resulted in a different pattern of gene expression, with ADD-1 as the lowest down-regulated and Pref-1 up-regulated.

Glucose uptake genes; all extracts down-regulate these genes (Figure 5D, 5E, 5F). IRS-1 was expressed the least, followed by GLUT-4 and Adiponectin. ZCD and ZCW resulted in slightly greater down-regulation effect compared with ZCE.

Lipid metabolism genes; all extracts down-regulated FAS (Figure 5G, 5H, 5I), whilst expression of aP2 was similar in both the control cells and those exposed to ZCD.

Fatty acid oxidation genes; expression of these genes decreased after exposure to the extracts (Figure 5J, 5K, 5L). Expression was lowest for ATGL. ZCW was the most effective at decreasing ATGL expression.

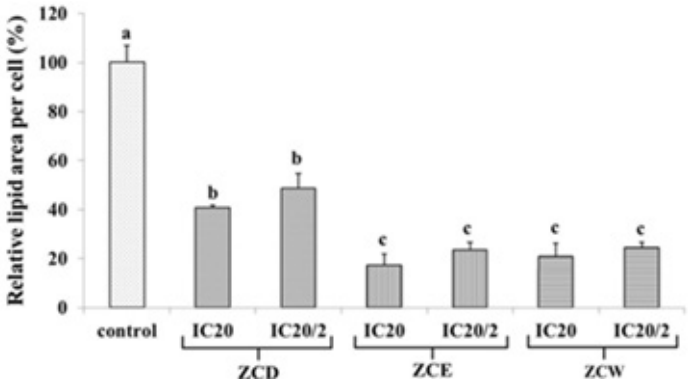

Figure 4 Relative percentage of LDs area derived from image-based assay. All extracts exhibited an inhibition potency of LDs accumulation in L929. The small alphabets (a, b, c) indicate the statistical difference among the treatments ($p \leq 0.05$).

DISCUSSION

The cytotoxicity and anti-proliferation assays of *Z. cassumunar* extracts were performed on L929. Percent yields of the extracts performed in this work were differed from those in previous records. Water as solvent resulted in the highest percent yield (Koparde and Magdum, 2017; Iswantini et al., 2011). Differences in polarity of solvents and extraction method achieved difference kinds of active ingredients from the rhizome. Dichloromethane, the lowest solvent polarity used in this work, had been reported to yield more effective compounds than ethanol and water extracts derived by Soxhlet extraction (Koparde and Magdum, 2017).

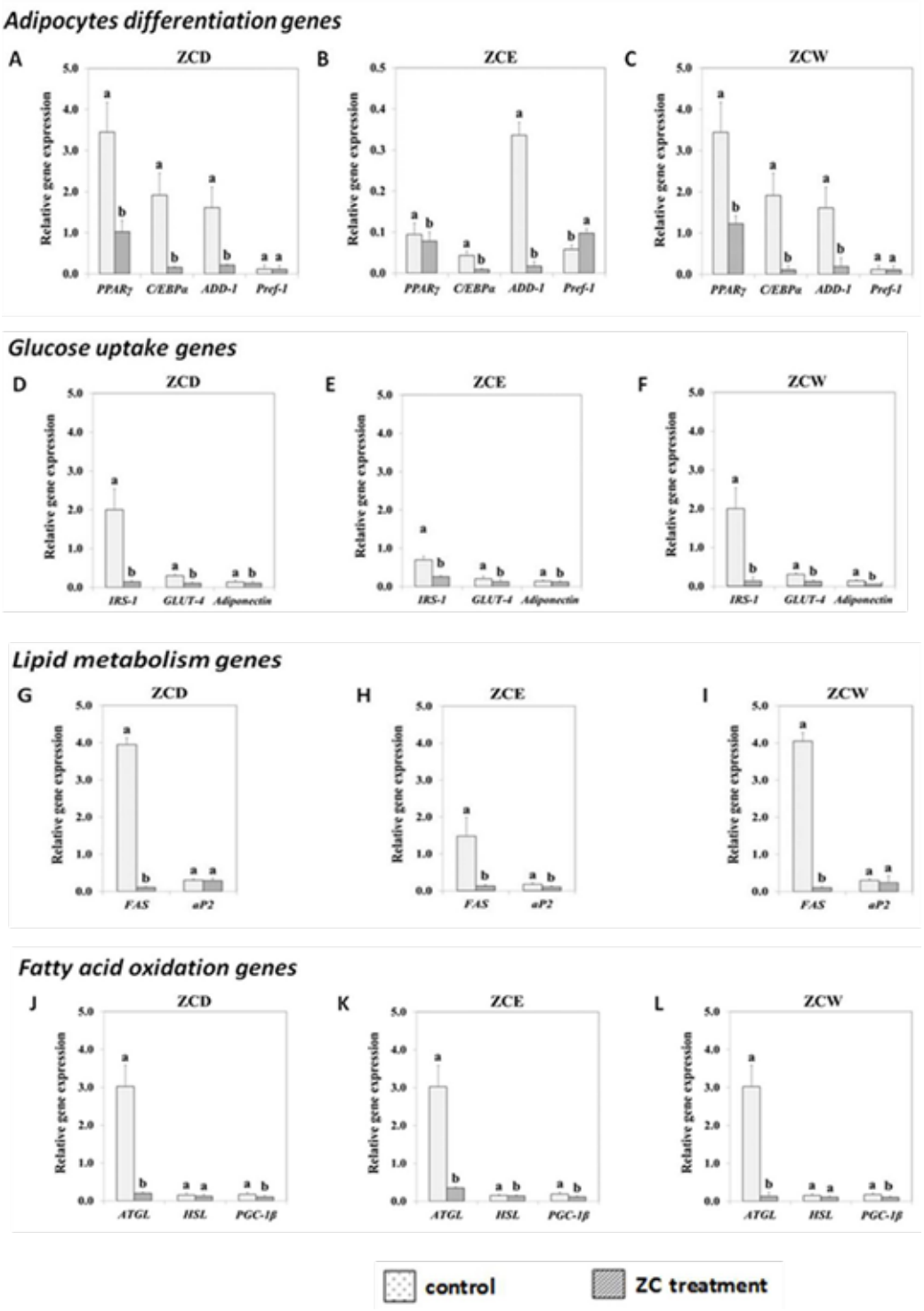

Figure 3 Relative gene expression of anti-adipogenic effects on L929 exposed to ZCD, ZCE and ZCW at IC20 concentration for 72 h. Expression level of the genes, in comparison to β-actin (given value as 1, but not shown in these graphs), presented in 4 groups; adipocyte differentiation genes (A, B, C), glucose uptake (D, E, F), lipid metabolism (G, H, I) and fatty acid oxidation (J, K, L). The small alphabets (a, b) indicate the significance of statistical difference with $p \leq 0.05$ on each of the gene between the controls and the exposed cells to ZC treatment.

Maceration in ethanol also collected more active compounds than in water (Iswantini et al., 2011). In other words, extraction of active ingredients from the rhizomes may probably comprise the most in dichloromethane followed by ethanol, and the least in water. However, all extracts (ZCD, ZCE and ZCW) exhibited non-cytotoxicity to the cells referred to Kalantari et al. (2017). At the IC_{50}, concentration of any crude extracts which >100 µg/ml was considered to contain any non-toxic substance to the cells (Iswantini et al., 2011). In other studies also revealed a non- to low- toxicity to other mouse fibroblast cell lines although different kinds of solvent were used (Sharma et al., 2007). In contrast, ZCD in this work, at the concentration of IC20, showed the highest activity to L929 followed by ZCE and ZCW. From previous report, ZCD may contain some compounds which would harm the cells including alkaloid, fat and oil (Koparde and Magdum, 2017). One of main constitute in non-polar soluble extracts from dichloromethane was in the form of oil (terpene) contains several phenylbutenoids which have been denoted as a cell proliferation inhibitor, apoptosis inducer and mitochondria disruptor in various cancer cell lines (Anasamy et al., 2013; Singh et al., 2015).

ZCD, ZCE and ZCW inhibited adipogenesis of L929 cells by significantly decreasing the expression of adipocyte differentiation genes: PPARγ, C/EBPβ and ADD-1. C/EBPα. PPARγ, the master regulator of adipogenesis, can induce their own expression and also activate a large number of downstream target genes, whose expression determines the adipocyte differentiation (Wang et al., 2006). ADD-1 is the coordinately activate the gene, responsible for maintaining the adipocyte phenotype. Its expression increases PPARγ transcriptional activity (Kim et al., 1998). Moreover, ZCE showed anti-adipogenic activity by increasing expression of Pref-1, the inhibitor of adipocyte differentiation, that was expressed in pre-adipocytes but was absent in adipocytes (Kim et al., 2007).

Expression of glucose uptake genes, IRS-1, Glut4 and Adiponectin was also evaluated. IRS-1 plays an important role in transmitting signals from insulin and insulin-like growth factor-1 receptors to the intracellular pathways and also to the glucose transporter, Glut4, to activate the uptake of glucose (Copps and White, 2012). Adiponectin is a protein hormone that is exclusively secreted from adipocytes into the bloodstream to modulate several metabolic processes, including glucose regulation and fatty acid oxidation (Díez and Iglesias, 2003; Chen et al., 2006). In this study, the three extracts significantly decreased expression of IRS-1 and Glut4. ZCD and ZCE also significantly down-regulated Adiponectin.

ATGL and HSL are key genes encoding the enzymes involved in intracellular degradation of triacylglycerols (Zimmermann et al., 2004). Both expressed predominantly in adipose tissue. ATGL regulates the initial hydrolysis of intracellular triglycerides into diacylglycerol. Then HSL continues breaking down to glycerol and free fatty acids (Morak et al., 2012). PGC-1β is a coactivator of PPARα in the transcriptional control of mitochondrial fatty acid oxidations that regulate the break-down of fatty acids into acetyl-Co-A and prevent insulin resistance by decreasing lipid storage (Serra et al., 2013). All kind of extracts inhibited adipocyte differentiation, by reducing expression of all genes in this group. ZCE significantly decreased ATGL, HSL and PGC-1β; ZCD significantly decreased both HSL and PGC-1β, while ZCW significantly decreased only ATGL.

To study activation of lipid metabolism genes by *Z. cassumunar* crude extracts, FAS and aP2 were focused. FAS is responsible for the synthesis of fatty acids from acetyl-CoA and malonyl-CoA resulting from the TCA cycle (Lodhi et al., 2012). Besides, aP2 is the pre-dominant fatty acid-binding protein, found in adipose tissue and plays an important role in the intracellular metabolism and the transport of fatty acids (Coe and Bernlohr, 1998). This protein responds to and regulates PPARγ and C/EBPα (Shan et al., 2013). All the extracts significantly reduced expression of FAS, while only ZCE reduced aP2.

Adipogenesis is a multi-step process, involving a cascade of gene expressions. As described earlier, all *Z. cassumunar* extracts (ZCD, ZCE and ZCW) reduced expression of adipogenic-related genes, adipocyte differentiation, glucose uptake, fatty acid oxidation and lipid metabolism. Such examinations have been extensively reported for rhizomes of other plant species in the same family (Zingiberaceae), such as ginger (*Zingiber officinale*), bitter ginger (*Zingiber zerumbet*) and turmeric (*Curcuma longa*) (Ejaz et al., 2009; Ahn and Oh, 2013; Tzeng and Liu, 2013). Two major bioactive compounds of ginger rhizome, 6-shogaol inhibited adipogenesis and increased glycerol release, but decreased intracellular lipid content, without any cytotoxic activity in 3T3-L1 cells (Suk et al., 2016). On the other hand, 6-gingerol inhibited adipocyte proliferation in concentration- and time-dependent manners, with more effective inhibition of adipocyte differentiation of 3T3-L1 (Seo, 2015). Furthermore, active compounds in bitter ginger rhizome (zerumbone and 6-gingerol) decreased expression of adipogenic-related genes in 3T3-L1 cells (Tzeng and Liu, 2013; Tzeng et al., 2014). Moreover, the active compound in turmeric rhizome, curcumin, also decreased expression of adipogenic-related genes (Ejaz et al., 2009).

Although, L929 cells have been used as an in vitro model for several fields of research, for example; monoclonal antibody production (Harada et al., 2014), biocompatibility (Serrano et al., 2004) and chemical compound testing models (Eljezi et al., 2017), but investigation of anti-adipogenic activity by image-base analysis, has never been reported. The ImageJ software was used effectively on the 3T3-L1 cell model, stained with ORO, on the purposes to study LDs accumulation (Kirchner et al., 2010; Li et al., 2011). We proposed in this study the successful of using L929 culture system to study the activity of herbal ingredients to inhibit adipogenesis. Significant decreases of LDs in cells exposed to *Z. cassumunar* extractions could be clearly monitored.

Spontaneous adipocyte differentiation in pre-adipocytes of L929 mouse fibroblasts was verified in this work through the photomicrographs of LD accumulation and ORO staining. This study also applied gelatin-coated surfaces for culture instead of glass as mentioned previously (Jeney et al., 2000). In contrast, most of the recent reports on the in vitro model for anti-adipogenesis study generally focus on the 3T3-L1, mouse pre-adipocytes cell line, with the inevitable of using the inductive chemical reagents (Kwon et al., 2017; Hwang et al., 2017; Lauvai et al., 2017). However, one has to keep in mind that L929 was cultured in the completed media, containing 10% FBS, which was approximately composed of 10 μU/ml of insulin (range 6-14 μU/ml) (Gstraunthaler, 2003). As insulin is the key hormone involved in mammalian fibroblast adipogenesis (Romao et al., 2011), most of the cells in the L929 population should be very sensitive to respond to such a small amount of insulin and initialize accumulation of LDs. More evidence is required to decide which of the two cell lines serves as the better model for studying of lipid metabolism.

CONCLUSION

Rhizomes of *Zingiber cassumunar* Roxb. have been shown to have anti-adipogenesis effects using the in vitro model of L929, mouse pre-adipocytes cell line. Non-cytotoxic activity was evaluated on the extracts using 3 types of solvent including dichloromethane, absolute ethanol and warm water. Lipid droplet accumulation was inhibited by the extracts in a concentration-dependent manner. This anti-adipogenesis activity was verified by down-regulation of pertinent genes, via the key regulator of adipogenesis, PPARγ. This study showed that L929 has high potential as an alternative for the in vitro model for the study of adipogenesis compared with the other cell lines.

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