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FULL PAPER

Chemical profiling of *Zanthoxylum acanthopodium* essential oil and its antidiabetic activity

[1,*]Yanti and [2]Limas, R.W.

[1]*Food Technology Study Program, Faculty of Biotechnology, Atma Jaya Catholic University, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia*
[2]*Biology Study Program, Faculty of Biotechnology, Atma Jaya Catholic University, Jalan Jenderal Sudirman 51, Jakarta 12930, Indonesia*

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Abstract

Inflammation plays an important role in the development of type 2 diabetes mellitus (T2DM), including obesity-related insulin resistance. Biomarkers of inflammation, such as C-reactive protein (CRP), tumor necrosis factor alpha (TNF-α), interleukin-6, and monocyte chemoattractant protein-1 (MCP-1) are present at increased concentrations in insulin resistant and obese individuals. In this study, we investigated the antidiabetic activity of *Zanthoxylum acanthopodium* essential oil (ZAEO) on attenuation of proteins related to diabetes in hepatocytes *in vitro*. ZAEO was extracted from *Z. acanthopodium* fruits using hexane and identified by using pyrolysis gas chromatography-mass spectrometry (py-GC/MS). ZAEO was tested for its cytotoxicity against human Chang liver cells as an *in vitro* cultured hepatocytes model. Effect of ZAEO (1-50 µg/mL) on protein expression related to T2DM, including CRP, TNF-α, and MCP-1 was quantified by enzyme-linked immunosorbent assay (ELISA) assay. Chromatogram profile demonstrated that ZAEO mainly consisted of carveol (~47.7%). MTT profile showed that safest dose of ZAEO against hepatocytes was reached up to 10 µg/mL. ELISA data showed that among three proteins, ZAEO exerted significant inhibitory effect against MCP-1 protein expression secreted by hepatocytes. At lowest dose (1 µg/mL), ZAEO attenuated MCP-1 expression up to 30%, respectively. These findings suggest that ZAEO may down-regulate inflammation related to T2DM through inhibiting MCP-1 expression that leads to the increase of insulin-stimulated glucose uptake.

1. Introduction

The fruit of *Zanthoxylum acanthopodium* or lemon pepper is locally known as andaliman and belongs to endemic spicy plant in Tapanuli region, North Sumatera province (Indonesia). Ripen *Z. acanthopodium* fruit has reddish-purple colour and the pericarp has strong lemon aroma due to its geranyl acetate and limonene contents (Rakić *et al*., 2009). *Z. acanthopodium* fruit has been reported to possess antioxidant, antimicrobial, and immunostimulant activities. Ethanolic extract of *Z. acanthopodium* fruits exerted anti-inflammatory potential by ameliorating several pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and matrix metalloproteinase (MMP)-9 in macrophages treated with lipopolysaccharide (Yanti *et al.,* 2011). In terms of management for inflammation, it is thought that active constituents of *Z. acanthopodium* fruit may have potential protective and therapeutic effects for inflammation-related disorders.

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that can be occurred genetically or cause of an unhealthy diet and physical inactivity habit. Chronic inflammation has an important role in developing T2DM in particular obesity-related insulin resistance (Wellen and Hotamisligil, 2005). Inflammation mediators, such as TNF-α, IL-6, and C-reactive protein (CRP) are present at increased concentrations in insulin resistant and obese individuals (Qatanani and Lazar, 2007).TNF-α induces the development of insulin resistance *in vitro* and *in vivo* (Ryden *et al*., 2002; Ruan and Lodish, 2003). TNF-α also activates Jun N terminal kinase (JNK)-1, a serine/ threonine protein kinase that leads to serine phosphorylation of IRS-1 and impairs insulin action. TNF-α mediates inhibitor of nuclear factor (NF)-κB kinase-β (IKKβ) that impacts insulin signaling by directly phosphorylating serine residues on IRS-1 and

*Corresponding author.
Email: *yanti@atmajaya.ac.id*

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phosphorylating inhibitor of NF-κB. Activation of NF-κB leads to the production of inflammatory mediators including TNF-α and IL-6 (Qatanani and Lazar, 2007).

Accumulation of TNF-α stimulates preadipocytes and endothelial cells to produce monocyte chemotactic protein (MCP)-1 (Greevenbroek *et al.,* 2013). MCP-1 is known to decrease the insulin-stimulated glucose uptake rate, and over-expressed MCP-1 is reported to cause insulin resistance (Sartipy and Loskutoff, 2003). MCP-1 has a role as primary macrophage attractant and its presence elevates macrophage infiltration in adipose tissue (Greevenbroek *et al.,* 2013). Among two types of macrophages located within adipose tissue, i.e. M1 macrophages (considered as pro-inflammatory macrophages) and M2 macrophages (considered as anti-inflammatory macrophages), M1 phenotype is predominantly dominated in adipose tissue in obesity (Greevenbroek *et al.,* 2013). CRP is produced primarily in the liver under the stimulation of TNF-α and IL-6. It has a major role in developing T2DM. CRP significantly inhibits cells proliferation and increases cells apoptosis on pancreatic β-cells (Badawi *et al*., 2010).

The major current therapeutic agents to treat T2DM patients were sulfonylureas, metformin, and insulin-sensitizing glitazones. Those agents are targeted to improve metabolic control and lead to normalization of circulating inflammation markers through interaction with the innate immunity-related pathways (Badawi *et al.,* 2010). In this study, we investigated whether *Z. acanthopodium* fruit particularly the essential oil fraction possessed antidiabetic activity which can suppress the development of T2DM using *in vitro* cultured hepatocytes model.

2. Materials and methods

2.1 Plant materials

Z. acanthopodium fruits were collected from traditional markets at Central Tapanuli, North Sumatera (Indonesia). The fruits were identified by Herbarium Bogoriense, Bogor Botanical Garden, Bogor, Indonesia and stored at Faculty of Biotechnology, Atma Jaya Catholic University, Jakarta, Indonesia (voucher specimen No. LY25). *Z. acanthopodium* fruits (pericarp and seed) were dried using freeze dryer and ground to obtain the powder. The powder had 9.6% w/w of moisture content and approximately 60 mesh particle size.

2.2 Extraction and identification of essential oil from Z. acanthopodium

Dried *Z. acanthopodium* powder was extracted using hexane with a ratio of 1:5, then stirred and incubated for overnight. The solution was separated from *Z. acanthopodium* residue. A solution containing essential oil and hexane was evaporated to obtain *Z. acanthopodium* essential oil (ZAEO). ZAEO was further identified for its chemical compounds by using pyrolysis gas chromatography-mass spectrometry (py-GC/MS, Thermo Scientific). A 0.5 g of ZAEO was injected to the capillary column (Phase Rtx-5MS, Restek) with a film thickness of 0.10 mm, 0.25 mm ID, and 60 m length. Pyrolysis temperature was set to 280°C using helium as a carrier gas.

2.3 Cell growth, morphology, and viability

Cultured human Chang liver cells (ATCC CCL-13) were grown to confluence in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL of streptomycin. Cells were incubated at 37°C in 5% CO_2 until confluence. Cells were seeded at a concentration of 5×10^5 cells/mL at 96-well microplate for 24 hrs. Cells were washed by Dulbelcco's phosphate-buffered saline (DPBS) and re-incubated using serum-free DMEM added with samples in various concentrations from 1-100 µg/mL, serum-free DMEM for positive control, and DPBS for negative control. Cells were incubated for 48 hrs at 37°C in 5% CO_2. Cells morphology was analyzed with an inverted microscope with 10×100 magnification. After that, cells were further used for viability test.

Cells viability were evaluated with the 3—(4, 5—dimethyl—2-thiazolyl)—2, 5—diphenyl-2H-tetrazolium bromide (MTT) colorimetric assay (Yanti *et al*., 2011). Cells were added with MTT reagent, followed by incubation for 4hr at 37°C. MTT reagent was discarded and the remaining cells were dissolved with 70% ethanol. The purple formazan absorbance was measured at 565 nm with macroplate reader. This assay was done in triplicate. Viability percentage is counted by (absorbance average for each sample) / (absorbance average for positive control) × 100%.

2.4 Sample treatment

ZAEO was dissolved in 100% DMSO to obtain the stock solution of 10^5 µg/mL, followed by further dilution in 25% DMSO into serial concentrations of 10^4, 10^3, and 10^2 µg/mL. Cells were seeded at a concentration of 10^5 cells/mL in 12-well plates and cultured for 24hr in DMEM-FBS. Cells were washed with DPBS and re-incubated using serum-free DMEM added with ZAEO at various concentrations (1-50 µg/mL), serum-free DMEM (as positive control), and DPBS (as negative control). Cells were incubated for 48hr at 37°C in 5% CO_2. After that, media were collected for further experiments.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Effect of ZAEO at various concentrations (1-50 µg/mL) on protein expression of CRP, TNF-α, and MCP-1 were tested by using Quantikine ELISA kits (R&D Systems, USA). Each specific antibody of CRP, TNF-α, and MCP-1 protein was pre-coated onto 96-well microplate. Cell media which contain these targeted proteins was added to microplate in order to bind the expressed proteins to their antibody. The microplate was then washed in order to dispose other substances to unbind the antibody. After that, enzyme-labeled antibody which will bind to protein was added to detect the expression of the proteins. The microplate was then washed again to dispose other substances unbind to protein, followed by the addition of substrate to measure protein expression. Color develops in the presence of each enzyme which binds to the expressed protein. The reaction was stopped and the intensity of the color was measured. The optical density of each well was assessed with ELISA reader at 450 nm.

2.6 Statistical analysis

All data were presented as the mean±standard deviation (SD). Statistical analysis was performed using the statistical package for the social science software (SPSS) program. Student's *t*-test was used to assess the differences between control and sample treatment. Statistical significance was considered at $P<0.05$.

3. Results

3.1 Identification of chemical compounds in ZAEO

Chromatogram profile showed that there were 32 of chemical compounds found in ZAEO (Figure 1). Most compounds were grouped in essential oils with a total concentration of 69.03%, respectively (Table 1). The major compounds in ZAEO were carveol (47.70%), myrtenyl acetate (12.55%), phytol (2.81%), and citronellyl acetate (2.27%). Meanwhile, other minor compounds (<1%) in ZAEO were β-myrcene, limonene, α-ocimene, geraniol, geranyl acetate, geranyl isovalerate, nerol, neryl propionate, and myrtanol.

3.2 Effect of ZAEO on cell morphology and viability

Cultured human Chang liver cells were used in this study to represent the in vitro hepatocytes model for prevention and treatment of T2DM. Figure 2 showed that ZAEO with concentration up to 10 mg/mL did not affect cells morphology and cytotoxicity. At higher concentration (>10 µg/mL), ZAEO caused the change of

Table 1. Identification of chemical compounds in *Z. acanthopodium* essential oil

Peak	Retention time	Compounds	Concentration (%)	Group
1	2.646	Propanethiol	7.49	Thiol
2	11.826	β-Myrcene	0.68	Monoterpene
3	12.511	Limonene	0.18	Monoterpene
4	12.710	α-Ocimene	0.17	Monoterpene
5	15.254	Geraniol	0.44	Monoterpene
6	16.419	Geranyl acetate	0.36	Monoterpene
7	20.957	Palmitic acid	2.96	Fatty acid
8	21.033	1-(3,3-Dimethylbuthyn-1-yl)-2,2-dimethylcyclopropene	1.51	Alkane
9	21.240	1-(3,3-Dimethylbuthyn-1-yl)-2,2-dimethylcyclopropene	1.81	Alkane
10	21.493	Cyclohexanone	0.49	Ketone
11	21.662	5,9-Dimethyl-2-(1-methylethyl)-1-cyclodecanone	0.73	Ketone
12	21.813	10,13-Octadecadienoic acid	0.74	Fatty acid ester
13	21.959	Phytol	1.23	Diterpene
14	22.286	Linoleic acid	11.34	Fatty acid
15	22.652	Phytol	1.58	Diterpene
16	22.765	Neoherculin	1.56	Carboximidic acid
17	22.917	1-Chlorooctadecane	0.27	Alkane
18	23.050	Farnesol	0.26	Sesquiterpene
19	25.404	Farnesol	0.26	Sesquiterpene
20	25.557	Farnesol	0.29	Sesquiterpene
21	25.777	Geranyl isovalerate	0.34	Monoterpene
22	28.085	Citronellyl acetate	0.85	Monoterpene
23	28.515	Nerol	0.45	Monoterpene
24	28.901	Carveol	16.97	Monoterpene
25	30.166	Neryl propionate	0.28	Monoterpene
26	30.705	Neryl propionate	0.37	Monoterpene
27	31.748	Citronellyl acetate	1.42	Monoterpene
28	32.018	Myrtanol	0.44	Monoterpene
29	33.050	Carveol	30.72	Monoterpene
30	33.318	Myrtanyl acetate	12.55	Monoterpene
31	40.017	Clionasterol	0.51	Sterol
32	43.720	Sesamin	0.74	Lignan

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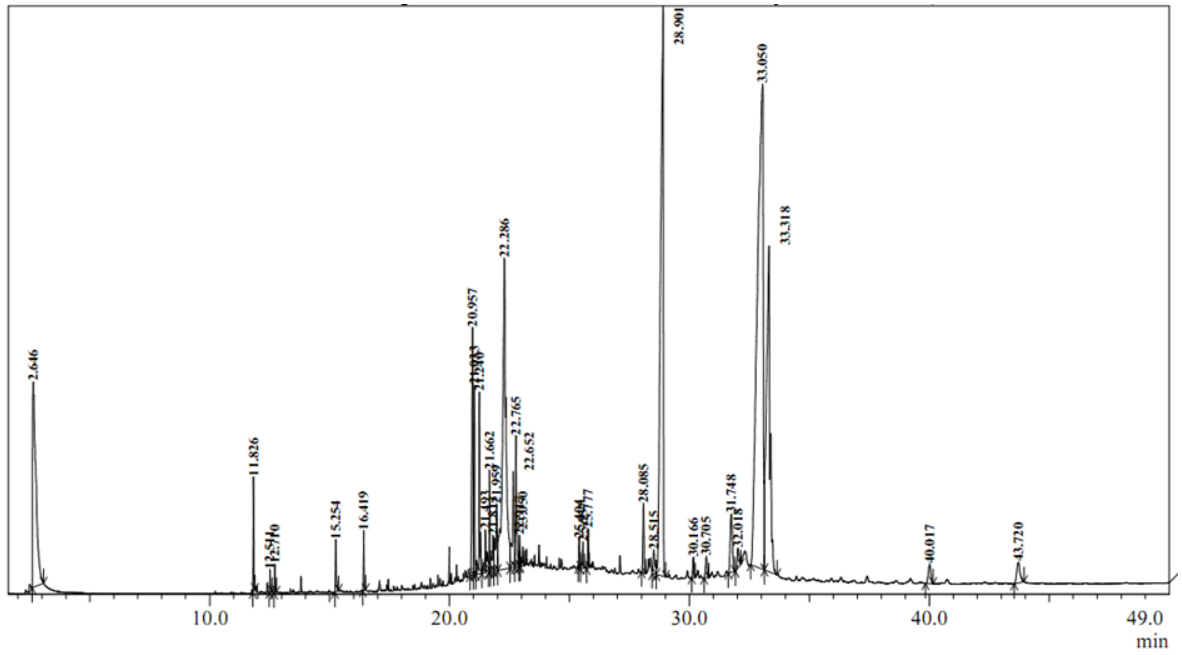
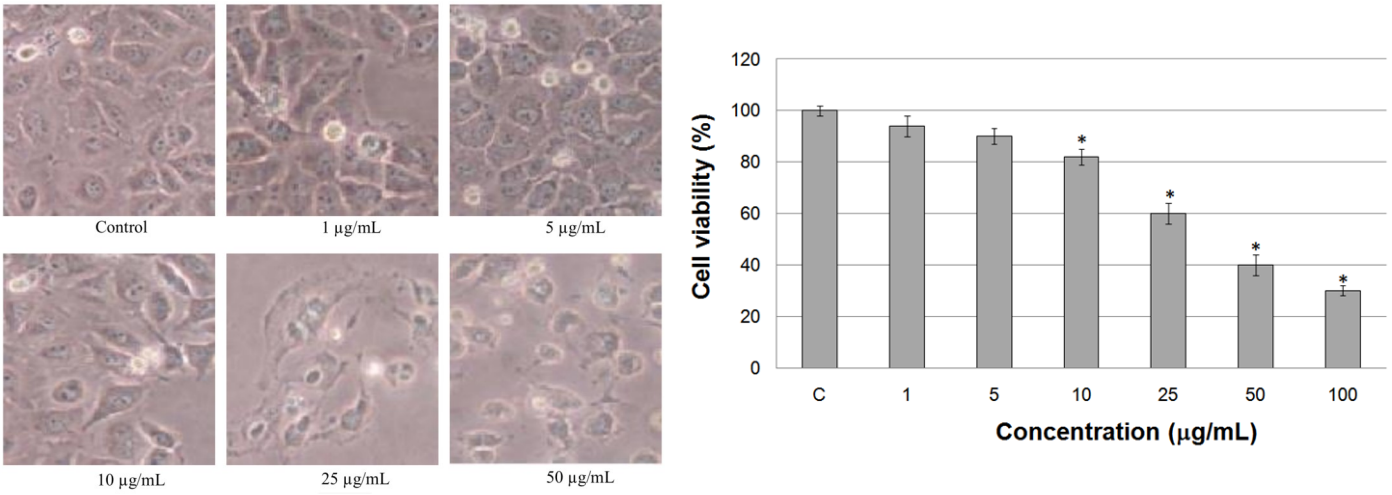


Figure 1. Chromatogram profile of *Z. acanthopodium* essential oil by py-GC/MS.



(A) (B)

Figure 2. Cell morphology (A) and cytotoxicity (B) of Chang liver cells after treated with ZAEO at various concentrations (1-100 mg/mL). *P<0.05 against control (C).

cell morphology and the significant decrease of cell viability. Thus, the safest concentration of ZAEO was used for further test.

3.3 Effects of ZAEO on secretion of CRP, TNF-α, and MCP-1

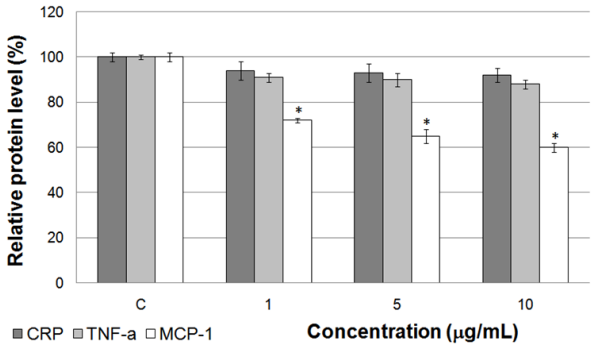


Figure 3. Effect of ZAEO at various concentrations (1-10 mg/mL) on protein expression of CRP, TNF-α, and MCP-1 secreted in cultured media of hepatocytes by ELISA. *P<0.05 against control (C).

Protein expression of CRP, TNF-α, and MCP-1 after treatment with ZAEO at various concentrations (1, 5, and 10 mg/mL) were assayed by ELISA (Figure 3). At lowest concentration (1 µg/mL), ZAEO was found to significantly inhibit MCP-1 expression secreted by hepatocytes up to 30%. However, ZAEO up to 10 mg/mL slightly reduced TNF-α and CRP expression in hepatocytes.

4. Discussion

Insulin resistance as the major factor of T2DM is developed as the impact of obesity-induced inflammation and the lack of fatty acid utilization (Greevenbroek *et al.,* 2013). In the previous study, we demonstrated that ethanolic extract of *Z. acanthopodium* exerted potential anti-inflammatory activity through inhibiting several pro-inflammatory mediators in macrophages treated with lipopolysaccharide (Yanti *et al*., 2011). Here, we

investigated whether ZAEO had the action on reducing obesity-induced inflammatory factor to interfere with the development T2DM.

Chemical profiling of ZAEO demonstrated that ZAEO mainly contained carveol constituent (Figure 1). Carveol belongs to the family of monocyclic monoterpenes with a molecular weight of 152 g/mol and has a function as a flavoring agent. Carveol is mostly found in oil of grapefruit, mandarin orange, blackcurrant berries, celery, dill, and caraway seed. Several reports demonstrated that carveol exerted pharmacological effects, including antimicrobial, antioncogenic, and anti-inflammatory activities (e Sá *et al*., 2013; Lopez-Romero *et al.,* 2015). In line with our study, ZAEO was also reported to have geranyl acetate and limonene constituents that possessed anti-inflammatory properties (Hirota *et al.,* 2010; Goncalves *et al.,* 2012). We suggest these activities could down-regulate the development of T2DM which is affected by several inflammatory factors, including CRP, TNF-α, and MCP-1.

In this study, human Chang liver cells were used as the *in vitro* hepatocytes system for T2DM and object of ZAEO treatment. The liver is centrally placed in relation to insulin secretion and nutrient intake since both insulin and nutrients must pass through the liver (Staehr *et al.,* 2004). Therefore, Chang liver cells were selected because they express liver function markers. It also could be used as a source for liver support in bioartificial livers in *in vivo* experiment (Yang *et al.,* 2013). MTT profile showed that ZAEO at concentration of 10 mg/mL had no cytotoxicity effect in Chang liver cells (Figure 2).

Inhibition of signaling downstream of the insulin receptor by inflammation mechanism leads to insulin resistance (Wellen and Hotamisligil, 2005). ELISA profile showed the protein expression of several inflammatory factors (CRP, TNF-α, and MCP-1) were decreased compared to the untreated cells after treatment with ZAEO (Figure 3). Among these proteins, ZAEO at 1 mg/mL possessed significant inhibition on MCP-1 protein expression in Chang liver cells. These results indicate that ZAEO may attenuate inflammation related to T2DM through inhibiting MCP-1 expression that leads to the increase of insulin-stimulated glucose uptake. Interestingly, carveol as a major essential oil isolated from several plants extracts, such as *Anethum graveolens* leaves and fruits*, Mentha spicata* leaves, and *Cymbopogon citrates* leaves had also been reported for its potential antidiabetic activity *in vitro* and *in vivo* (Bharti *et al*., 2013; Goodarzi *et al.*, 2016; Bayani *et al*., 2017).

It is known that MCP-1 elevates macrophage infiltration in adipocytes (Greevenbroek *et al.,* 2013). Inflammatory factors were secreted from white adipose tissue (WAT) in obesity. Recent data indicate that obese WAT is infiltrated by macrophages, which may be a major source of locally-produced pro-inflammatory cytokines (Bastard *et al*., 2006). Down-regulation of MCP-1 potentially could increase insulin sensitivity due to eliminating such inflammatory factors were produced by macrophage that infiltrated inside adipocytes. Meanwhile, slightly down-regulation of CRP may affect the reduction of a little pancreatic β-cells apoptosis (Badawi *et al.,* 2010). Down-regulation of TNF-α also prevents disruption of insulin signaling by restraining the activities of JNK1, IKKβ, and p38 MAPK signaling pathways (Hoareau *et al.,* 2010). These results suggest that essential oil extracted from *Z. acanthopodium* contained carveol as the major constituent and it has a potential antidiabetic effect via downregulation of MCP-1 expression in hepatocytes *in vitro*.

Conflict of Interest

The authors declared there is no conflict interest. The authors alone are responsible for the content of the paper.

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