Curcuminoid cider fermented from *Curcuma xanthorrhiza* curcuminoids attenuates gene expression related to obesity-induced inflammation in hypercholesterolaemic rats

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Abstract

Obesity is an important risk factor in the development of atherosclerosis, and often associated with hypercholesterolemia. Curcuminoid cider, a fermented beverage made from curcuminoid fraction isolated from *Curcuma xanthorrhiza*, has been reported to have high contents in organic acids and curcuminoids. Herein, we investigated whether curcuminoid cider affected the expression of genes related to obesity-induced inflammation (peroxisome proliferator-activated receptors γ (PPARγ), CCAT/enhancer binding protein α (C/EBPα), fatty acid-binding protein 4 (FABP4)) and genes generating pro-inflammatory cytokines (interleukin (IL) 1β (IL1β), tumour necrosis factor α (TNFα) and chemokine) in hypercholesterolaemic rats by conducting quantitative real time-PCR (qRT-PCR). Twenty-four male Sprague-Dawley rats were divided into six groups: normal group diet, high cholesterol diet (HCD), HCD+ 1% v/v curcuminoid cider, HCD + 2% v/v curcuminoid cider, HCD + 100 mg/kg bw curcuminoid fraction, and HCD + 300 mg/kg bw curcuminoid fraction for four weeks. Rats' lung and liver were collected for total RNA extraction, followed by quantitative analysis to determine gene expression related to obesity and pro-inflammatory cytokines. Results showed that curcuminoid cider at 1 and 2% v/v significantly reduced >80% gene expression of obesity-induced inflammation, including PPARγ, C/EBPα, FABP4, IL1β, TNFα and chemokine in hypercholesterolaemic rats. Meanwhile, curcuminoid fraction at 100 and 300 mg/kg was effective to attenuate up to 80% PPARγ gene expression correlated to obesity. These results indicate that curcuminoid cider may exert antiobesity potential at molecular level through suppressing various genes related to obesity and pro-inflammatory cytokines in hypercholesterolaemic rats. It may be used as a potential candidate in functional beverage for management of obesity-induced inflammation.

Introduction

Imbalance between energy intake and expenditure causes obesity which is the major health challenge globally. Obesity contributes to metabolic syndromes such as hypertension, atherosclerosis, dyslipidaemia and insulin resistance (Grundy *et al.*, 2004). Obesity is also associated with chronic low-grade inflammation due to the increased levels of several cytokines, such as tumour necrosis factor α (TNFα), interleukin (IL) 6, IL1β and chemokine (C-C motif) ligand 2 (CCL2), and acute phase proteins associated with inflammation (Tucakovic *et al.*, 2015). Furthermore, TNFα can indirectly modify the sensitivity of insulin via down-regulating the expression of adipogenic gene, such as peroxisome proliferator-activated receptor (PPAR)-γ (Aggarwal, 2010; McArdle *et al.*, 2013).

Curcuma xanthorrhiza, also known as Javanese turmeric or *temoe lawak*, is a member of the ginger family (Zingiberaceae), and one of the Curcuma species that belongs to a native Indonesian plant. *C. xanthorrhiza* is rich in secondary metabolites in particular terpenoids and phenols (Halim *et al.*, 2012). The main constituents isolated from *C. xanthorrhiza*

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rhizomes are curcuminoids (diarylheptanoids) and sesquiterpenes (β-curcumene, ar-curcumene, xanthorrizol and camphor) (Van Galen and Kroes, 2014). Curcuminoids, which is a group of phenolic substances including curcumin, demethoxycurcumin and bisdemethoxycurcumin, are known as the main constituents of C. longa and C. xanthorriza. Previous studies demonstrated that among the popular Indonesian Zingiberaceae rhizomes, *C. xanthorrhiza* and its active constituents exerted potential effect as antiatherosclerotic candidate via inhibiting matrix metalloproteinase (MMP)-2, -9 and IL-6 expression in various vascular culture cells *in vitro* (Yanti, 2011; 2014; 2015).

Curcumin, a non-toxic yellow pigment with molecular weight of 368, has been investigated most extensively as a treatment for obesity-related metabolic diseases (Aggarwal, 2010). At the molecular level, curcumin is found to modulate a wide range of signalling molecules by up-regulation or down-regulation of gene expression involved in energy metabolism, lipid accumulation and lipogenesis. Curcumin also protects from liver injury, prevents LDL-C oxidation, increases adiponectin production, decreases hepatic NF-κB activity and inflammatory markers of liver, reduces body weight gain, adiposity, and microvessel density in adipose tissue, and reduces leptin resistance (González-Castejón and Rodriguez-Casado, 2011; Gupta *et al.*, 2012; Tabatabaei-Malazy *et al.*, 2015). *In silico* docking analysis demonstrated that curcumin suppresses the action of obesity proteins comparable to standard drugs in the treatment of obesity (Archana *et al.*, 2010).

Unfortunately however, the health benefits of *C. xanthorrhiza* are not supported by its strong aroma and taste that may affect consumers' acceptability thereby limiting its applications in the food industry. The formulation of curcuminoid fraction from *C. xanthorrhiza* in cider functional beverage through fermentation is an alternative method to improve its taste and acceptance. The present work demonstrated that oral administration of curcuminoid fraction and its cider product exerted significant inhibitory effects on the expression of oxidative stress related-genes, including cluster of differentiation 44 (CD44), intercellular adhesion molecule 1 (ICAM-1), and inducible nitric oxide synthase (iNOS) and lipoxygenase-1 (LOX-1), for preventing hypercholesterolemia-induced atherosclerosis *in vivo* (Mauren *et al.*, 2016).

Obesity is known as one of among risk factors that promote atherosclerosis. It is noted that interactions between the innate immune system with lipid-derived products seem to play a major role in the pathophysiology of atherosclerosis in relation with obesity. The role of pro-inflammatory cytokines and adipokines in triggering immune response may lead to the development of plaque activation in atherosclerosis. Therefore, therapeutic candidates that specifically target obesity prevention should be the focus of deeper investigation. In the present work, it was further tested whether curcuminoid cider functional beverage could affect the expression of various genes involved in obesity-induced inflammation, including PPARγ, CCAT/enhancer binding protein-alpha (C/EBPα), fatty acid-binding protein 4 (FABP4), IL1β, TNFα and chemokine in hypercholesterolaemic rats *in vivo*.

Materials and methods

Preparation of curcuminoid fraction from Curcuma xanthorrhiza and curcuminoid cider

Curcuminoid fraction extracted from *C. xanthorrhiza* and curcuminoid cider was prepared following the method described by Mauren *et al.* (2016). Briefly, 1 kg fresh *C. xanthorrhiza* was purchased from a local market in Bogor, Indonesia. The fresh rhizomes were cleaned, sliced and freeze-dried for 3 d. Afterward, the dried rhizomes were blended and filtered to obtain the same size particle powder. The powder of *C. xanthorrhiza* was macerated with 80% ethanol (1:4, w/v) at room temperature for 2 d. The supernatant was pooled and concentrated under reduced pressure at 45°C using a rotary vacuum evaporator. The crude extract was then freeze-dried to obtain curcuminoid fraction.

Curcuminoid fraction from *C. xanthorrhiza* was dissolved in hot water with final concentration of 1% and 2% (w/v). Acetobacter xylinum culture was added to curcuminoid solution with ratios of 2:1 and 4:1, and 2.5% sugar (w/v) was added to provide nutrients for bacterial growth as well as providing taste and aroma to the cider. Next, the solution was filtered and incubated for 14 d at 30°C. The cider was further pasteurised at 50–60°C for 20 min, and then filtered to obtain curcuminoid cider functional beverage. The cider product was then tested for its antiobesity efficacy using *in vivo* rat model.

Animal treatment and sample preparation

The antiobesity effect of curcuminoid cider was tested by using hypercholesterolaemic rat model following the method described by Mauren *et al.* (2016). Six groups of male Sprague-Dawley rats (±140-150 g; four rats per group) consisted of negative control (C-) were fed with a

standard diet; the remaining five groups received hypercholesterolaemic diets for four weeks (Table 1). Standard diet for negative control group consisted of 20% protein, 67% carbohydrate, 10% fat and 3% crude fibre. High cholesterol diet (HCD) for modelling obese groups contained 20% protein, 48% carbohydrate, 27.5% fat, 1% cholesterol, 0.5% cholic acid and 3% crude fibre. Curcuminoid fraction as the main material for curcuminoid cider production was also included in the experiment. The curcuminoid cider was administrated by oral gavage into the rats during the treatment. Meanwhile, the standard diet and water were always given to the rats ad libitum.

Table 1. Treatment of rat diets.

Group	Treatment
C-	Normal diet
C+	High cholesterol diet (HCD)
CID1	HCD plus administration of 1% v/v curcuminoid cider
CID2	HCD plus administration of 2% v/v curcuminoid cider
CUR1	HCD plus administration of 100 mg/kg bw curcuminoid fraction
CUR2	HCD plus administration of 300 mg/kg bw curcuminoid fraction

CID, curcuminoid cider; CUR, curcuminoid fraction

The rats were maintained in individual cages, under controlled temperature, humidity and illumination conditions, with water and diet ad libitum during treatment. Their body weight and lipid profiles (LDL, HDL, triglyceride and total blood cholesterol levels) were recorded weekly. At the end of four weeks, the animals were sacrificed. Blood was collected through the retro-orbital puncture and organs (lung and liver) were collected for further assay. This experiment was conducted at animal facility, Faculty of Veterinary, Bogor Agriculture University, Bogor, Indonesia, and approved by Animal Care and Use Committee, Atma Jaya Catholic University, Jakarta (Indonesia).

Isolation of mRNA and quantitative real-time polymerase chain reaction (qRT-PCR)

The gene expression related to obesity (PPARγ, C/EBPα, and FABP4) and pro-inflammatory cytokines (IL1β, TNFα, chemokine) from organs after treatment with curcuminoid cider and curcuminoids was analysed by using qRT-PCR. The total RNA was isolated using TRIzol® reagent (Life Technologies, California, US) according to the manufacturer's

protocol. The cDNA was synthesised using iScript One-Step RT-PCR Kit with SYBR® Green and KAPA SYBR® FAST qPCR Kit for analysis by RT-PCR. The primer sequences for qRT-PCR were designed according to a PCR primer selection program at the website of the Virtual Genomic Centre from the GenBank Database (Table 2). Beta-actin was used as an internal control. All gene expression values were normalised to β-actin housekeeping gene. The 2-ΔΔCt method of relative quantification was referred to estimate the copy numbers in genes (Livak and Schmittgen, 2001).

Table 2. Oligonucleotide primers for qRT-PCR

Target gene	Forward primer	Reverse primer
PPARγ	TCTGGGAGATCCT CCTGTT	CAATCGGATGGTT CTTCGGA
C/EBPα	CGACTTCTACGAG GCGGAG	TGGCTTTATCTCGG CTCTTG
FABP4	GGACCTGGAAAC TCGTCTCC	GGACCTGGAAACT CGTCTCC
IL1β	GGAACCCGTGTCT TCCTAAAG	CATCCCATACACA CGGACAA
TNFα	ATGTACCTGGGAG GAGTCTT	GGCTCTGAGGAGT AGACGATAA
Chemokine	CCAAGGCACATTC CACTACA	GTCTTCTTTGACT CCGGATGG
β-actin	GTGCTATGTTGCC CTAGACTTC	TAGGAGCCAGGGC AGTAAT

Statistical analysis

Triplicate experiments from different organs (lung and liver) were performed. Data were presented as mean ± standard deviation. The significant difference between untreated and treated groups was statistically analysed by the paired Student's *t*-test ($p \leq 0.05$, $p \leq 0.01$).

Results

Effect of curcuminoid cider on the gene expression of obesity in hypercholesterolaemic rats

The antiobesity effect of curcuminoid cider was analysed from rat organs (lung and liver) against the gene expression of PPARγ, C/EBPα and FABP4 by qRT-PCR (Figures 1a-1c). Curcuminoid cider at 1 and 2% dose-dependently reduced >80% of PPARγ, C/EBPα and FABP4 gene expression in hypercholesterolaemic rats. Curcuminoid fraction at 100 and 300 mg/kg only inhibited up to 80% of PPARγ gene expression.
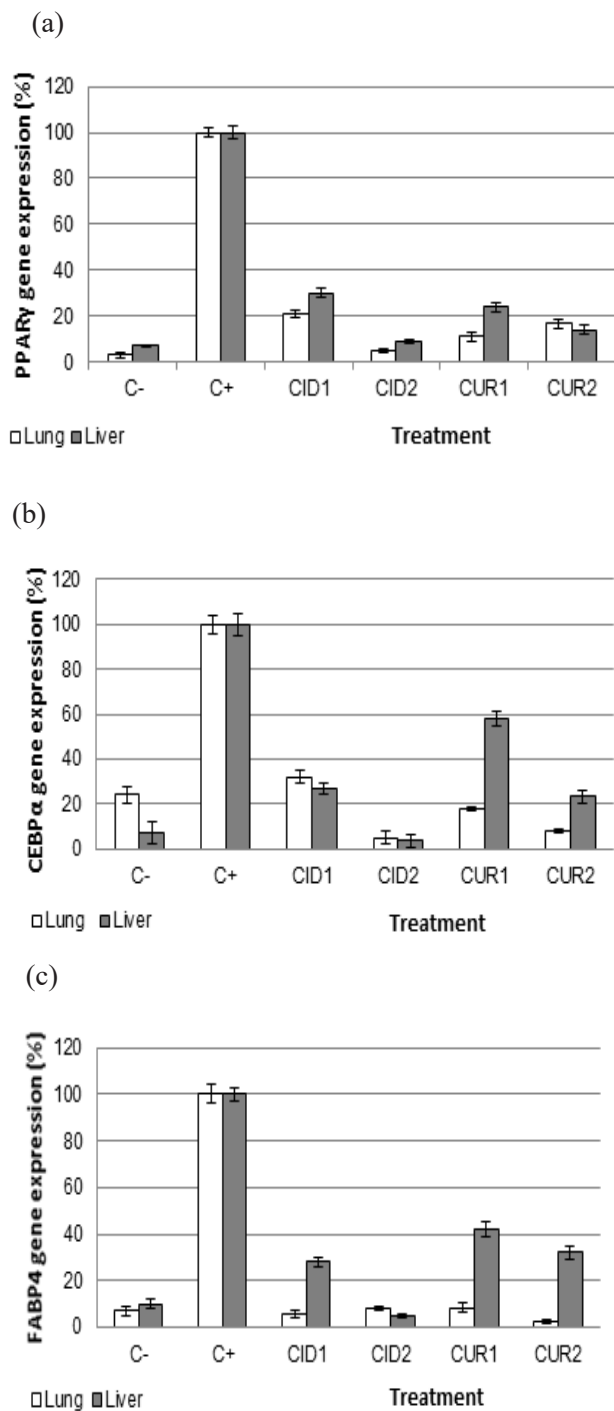
(a)

(a)

(b)

(b)

(c)

(c)

Figure 1. Effect of curcuminoid cider on the gene expression of obesity, including PPARγ (a), C/EBPα (b), and FABP4 (c) in hypercholesterolaemic rats in vivo by qRT-PCR. Six groups of male Sprague-Dawley rats were divided into: normal group diet (negative control; C-), high cholesterol diet 2% (positive control; C+), HCD+ 1% v/v cider (CID1), HCD + 2% v/v cider (CID2), HCD + 100 mg/kg bw curcuminoid fraction (CUR1), and HCD + 300 mg/kg curcuminoid fraction (CUR2). Data are mean ± SD from triplicate experiments (*n* = 3) and different organs (lung and liver). *$p < 0.05$ and **$p < 0.01$ were significantly different as compared to positive control (C+).
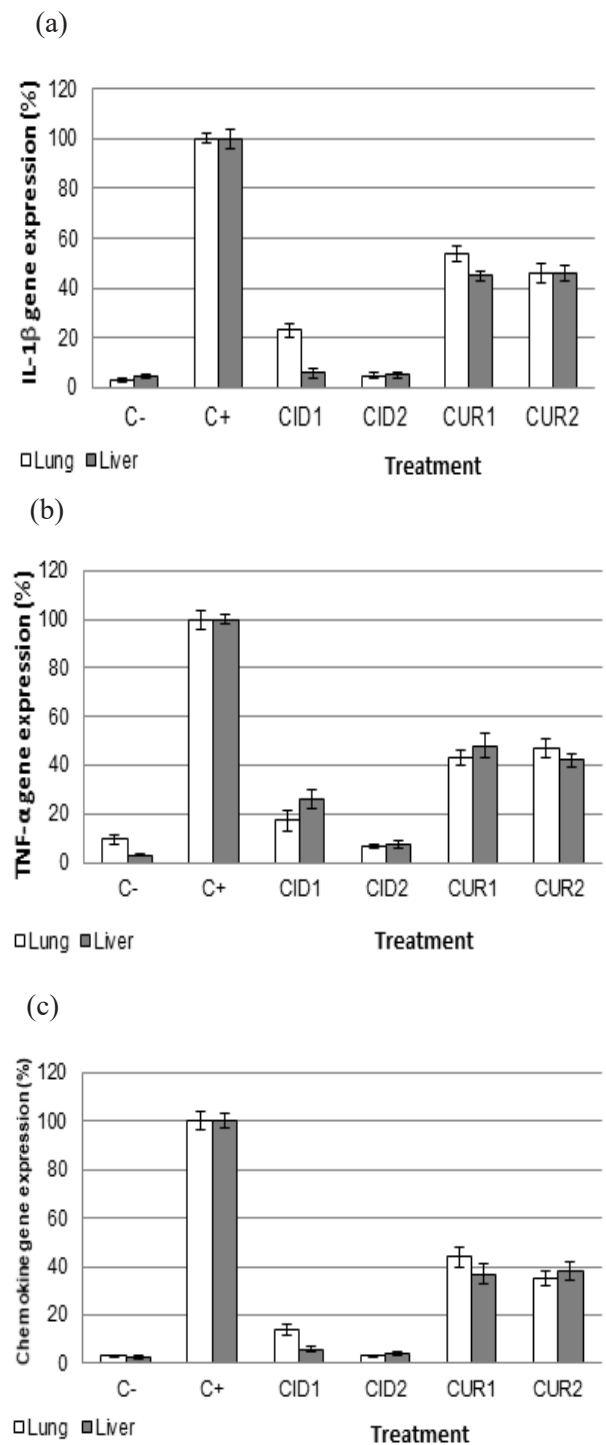
Figure 2. Effect of curcuminoid cider on the gene expression of pro-inflammatory cytokines, including IL-1β(a), TNF-α (b), and chemokine (c) in hypercholesterolaemic rats in vivo by qRT-PCR. Six groups of male Sprague-Dawley rats were divided into: normal group diet (negative control; C-), high cholesterol diet 2% (positive control; C+), HCD+ 1% v/v cider (CID1), HCD + 2% v/v cider (CID2), HCD + 100 mg/kg bw curcuminoid fraction (CUR1), and HCD + 300 mg/kg curcuminoid fraction (CUR2). Data are mean ± SD from triplicate experiments (*n* = 3) and different organs (lung and liver). *$p < 0.05$ and **$p < 0.01$ were significantly different as compared to positive control (C+).

Effect of curcuminoid cider on the gene expression of pro-inflammatory cytokines in hypercholesterolaemic rats

The curcuminoid cider was further tested for its efficacy on modulating gene expression of pro-inflammatory cytokines (IL1β, TNFα, chemokine) in hypercholesterolaemic rats by qRT-PCR (Figures 2a-2c). The results showed that curcuminoid cider at 1 and 2% strongly suppressed >80% of IL1β, TNFα and chemokine gene expression in hypercholesterolaemic rats. Compared to cider, curcuminoid fraction (100 and 300 mg/kg) only blocked 50-60% of these pro-inflammatory genes.

Discussion

Cider is known as one of the traditional fermented beverages with aroma-forming volatile compounds. The volatile compounds and phenols influence the taste and aroma that are linearly contributed to the quality of cider (Riekstina-Dolge *et al.*, 2012). The identification of chemical compounds in curcuminoid cider by pyrolysis GC/MS revealed that cider consisted of major organic acids, phenols and aldehydes (Mauren *et al.*, 2016). *A. xylinum* used in curcuminoid cider production is a group of acetic acid bacteria that has the ability to partially oxidise sugar and then release aldehydes and organic acids into the media (Mamlouk and Gullo, 2013). A fermented beverage made from *C. aromatica* extract and *Aspergillus oryzae* exerts higher organic acids that is responsible for antioxidant activity as compared to that of the unfermented one (Ra and Kim, 2016). As fermented beverage, cider has been reported for its several pharmacological effects, such as cardioprotective, antimicrobial, antitumor, anti-inflammatory, antidiabetic and antiobesity activities (Budak *et al.*, 2014). In terms of cider production from curcuminoid fractions isolated from *C. xanthorrhiza*, we explored whether the cider exerted antiobesity effect by modulating various gene expression related to obesity-induced inflammation in hypercholesterolaemic rats.

For obtaining the dried curcuminoid fraction, the methanol residue in curcuminoid extract was evaporated, thus, only the dried fraction was used in *in vivo* treatment. The odour of curcuminoid fraction was reduced for *in vivo* oral administration by diluting the curcuminoid fraction in 0.5% w/v carboxymethyl cellulose (CMC) to obtain the soluble liquid sample. In addition, liquid sample was directly administered into the stomach of rats by oral gavage. Therefore, we assume that the odour effect of curcuminoid fraction was less reduced than the dried one. Recent studies

have been reported about the safety use of curcumin and curcuminoid fraction for oral treatment in animal models (Hsieh *et al.*, 2014; Mauren *et al.*, 2016).

The *in vivo* data of lipid profile and body weight after treatment with curcuminoid cider and curcuminoid fraction has been previously reported by Mauren *et al.* (2016). High cholesterol diet induced the increase of body weight and serum cholesterol level in rats. Among the lipid profile data, results demonstrated that serum level of cholesterol in rats was significantly reduced after treatment with curcuminoid cider and curcuminoid fraction. However, there was no significant effect of both on modulating other parameters, including LDL, HDL and triglyceride levels.

A high-cholesterol diet or high-fat diet is known as a major cause of metabolic diseases, including obesity and diabetes; vascular diseases, including hypertension, stroke and arteriosclerosis; and liver diseases, including hepatic steatosis and cirrhosis. Alternative strategic therapeutics using antiobesity agents targeting proteins and genes related to obesity and obesity-related inflammation have been the focus of investigation. The present work revealed that curcuminoid cider significantly inhibited obesity gene expression, including PPARγ, C/EBPα and FABP4 in hypercholesterolaemic rats (Figures 1a-1c). Both cider and curcuminoid fraction had similar action on suppressing PPARγ gene expression (Figure 1a). Meanwhile, for C/EBPα and FABP4 genes, cider was found to have higher efficacy on inhibition of these genes as compared to that of curcuminoid fraction (Figures 1b-1c).

PPARγ and C/EBPα are the major transcription factors in adipogenesis and lipogenesis. Using high fat-fed mice model, dietary curcumin supplementation was proven to suppress the expression of PPAR and C/EBPα genes in subcutaneous adipose tissue (Ejaz *et al.*, 2009). In line with the results obtained in the present work (Figure 1a), the PPARγ gene expression was significantly reduced as compared to those of other genes by curcuminoid fraction. Among key adipogenic transcription factors, PPARγ is known to be highly expressed in adipocytes during adipogenesis (Bhandari *et al.*, 2013). As adipogenesis causes hypertrophy and hyperplasia of adipocytes, the blocking of PPARγ gene expression by curcuminoid fraction may indicate its potential for prevention of obesity. Other studies also showed that fermented C. longa and rambutan peel extracts attenuated gene expression of PPARγ and FABP4 in rats (Lestari *et al.*, 2015; Kim *et al.*, 2016). Decaffeinated green coffee bean extract has also been reported to reduce genes regulating adipogenesis, including PPARγ and

C/EBPα in obese mice (Song *et al*., 2014). Therefore, the results obtained in the present work indicate that both curcuminoid cider and curcuminoid fraction may act in obesity genes regulating adipogenesis, in particular PPARγ gene.

FABP4 has been recognised as a specific protein in adipocytes, macrophages and endothelial cells (ECs) that is expressed in various tissues and organs, including heart, lung, liver and kidney. The knockdown of FABP4 expression in ECs reduced proliferation both under baseline conditions and in response to vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Therefore, FABP4 emerged as a novel target of the VEGF/VEGFR2 pathway and a positive regulator of cell proliferation in ECs (Elmasri *et al*., 2009). VEGF and VEGF-R2 are also associated with angiogenesis progression. Adipose tissues require the formation of new blood vessels (angiogenesis) for expanding their size and numbers. The inhibition of angiogenesis is an alternative strategy to reduce adiposity (Ejaz *et al*., 2009). Recent studies reported that lipid chaperone is secreted from adipocytes and its level is associated with obesity, insulin resistance and atherosclerosis. In addition, FABP4 level is not only a marker of metabolic syndrome, but also a novel predictor of cardiovascular mortality in patients at high risk of atherosclerotic cardiovascular events (Furuhashi *et al*., 2011). In line with the results obtained in the present work, the blocking of FABP4 gene expression by curcuminoid cider may indicate its potency as an angiogenesis inhibitor that leads to the decrease in adiposity.

Curcuminoid cider also showed potential inhibitory effects on several genes related to obesity-induced inflammation in hypercholesterolaemic rats. Cider had higher efficacy on suppressing gene expression of IL1β, TNFα and chemokine as compared to that of curcuminoid fraction (Figure 2a-2c). These data are in line with another study from Aggarwal (2010) who investigated the efficacy of curcuminoid extract from C. longa as anti-inflammatory candidate on modulating pro-inflammatory cytokines of TNFα and IL6 at protein and gene levels in adipocytes *in vitro*. The inhibition of TNFα and IL6 by curcumin is mediated by Kβ protein that regulates these pro-inflammatory cytokines, suggesting that curcumin acts as an inhibitor of IKβ kinase that causes the inactivation of Kβ protein. In terms of obesity-induced inflammation, results obtained in the present work indicate that regular diet of curcuminoid cider strongly blocked the gene expression of pro-inflammatory cytokines including TNFα that are correlated to the decrease of PPARγ (adipogenic gene)

and indirectly contributed to the insulin sensitivity. In addition, Budak *et al*. (2011) showed that apple cider had antiobesity effect *in vivo* by decreasing a significant steatosis in rats fed with high-cholesterol diet when compared to the control group.

According to Feve and Bastard (2009), insulin resistance is linked to obesity, in particular lipid accumulation. Obesity is also associated with inflammation response that is characterised by altered cytokine production and the activation of inflammation signalling. The development of insulin resistance involves the overproduction of pro-inflammatory cytokines, including TNFa that affects the cell differentiation into hypertrophy and hyperplasia conditions, and finally causes lipid accumulation. Blocking the pro-inflammatory cytokine gene expression contributes indirectly to the insulin sensitivity. In AMPK signalling, the decrease in pro-inflammatory cytokine gene expression, the inactivation of lipogenic enzymes and the up-regulation of lipolytic proteins, could stimulate lipolysis through promoting fatty acid oxidation and reducing fatty acid and cholesterol synthesis that lead to the indirect effect on the increase in insulin sensitivity (He *et al*., 2012; Zhang *et al*., 2013). Therefore, it is proposed that curcuminoid cider may offer as one of molecular mechanism targets for the prevention and modulation of obesity directly and diabetes indirectly.

The consumption of Japanese vinegars such as Kurosu and Kibize with high phenolics, acetic acids and alcohol fermentations has also been reported to reduce cancer risk due to their ability to inhibit the proliferation of various cancer cells and their potent radical scavenging activity (Mimura *et al*., 2004; Nanda *et al*., 2004). Pre- and clinical studies on curcumin from C. longa revealed that its anticancer ability were proven by various mechanisms, such as suppressing the proliferation of tumour cells, down-regulating transcription factors NFκB, AP-1 and EGR-1, down-regulating the expression of pro-inflammatory enzymes and cytokines (COX-2, LOX, NOS, MMP-9, uPA, TNFα, chemokines, cell surface adhesion molecules, cyclin D1), down-regulating growth factor receptors (EGFR and HER2), and inhibiting the activity of signalling proteins (c-Jun N-terminal kinase, protein tyrosine kinases and protein serine/threonine kinases) (Aggarwal *et al*. 2003).

In vivo atherosclerotic study showed that dietary acetic acid in vinegars enhanced lipid homeostasis and reduced serum cholesterol and triglycerides in rats fed with a cholesterol-rich diet (Fushimi *et al*., 2006; Yamashita *et al*., 2007). Curcumin

was reported to reduce ox-LDL-induced cytokine production, such as IL1β, IL6, TNFα and M1 cell apoptosis in macrophages. However, it also increased PPARγ expression that led to the promotion of CD36 and ABCA1 expression, indicating its potential as anti-atherosclerotic agent (Chen *et al*., 2015). Interestingly, Van der Vorst *et al*. (2015) also stated that chemokines were involved in all phases of atherosclerotic lesion development, and inhibition of these chemokines could occur directly by curcuminoid or indirectly by other cytokine, such as IL1β. This result is in line with that obtained in the present work in which curcuminoid cider attenuated the chemokine gene expression

Curcuminoid from C. comosa also exerted anti-atherosclerotic effect via inhibiting pro-inflammatory cytokine genes in rabbits fed with a high-cholesterol diet (Charoenwanthanang *et al*., 2011). Recent study from Singh *et al*. (2015) demonstrated that curcuma oil from C. longa rhizome possessed significant antiatherosclerotic effect by suppressing the gene expression of pro-inflammatory cytokines (TNFα, IL1β, IL6, IFNγ) in THP-1 macrophages *in vitro*, and atherosclerotic hamster *in vivo*.

Conclusion

Curcuminoid cider fermented from curcuminoid fraction derived from *C. xanthorrhiza* has potential antiobesity effect through inhibiting several genes related to obesity and pro-inflammatory cytokines in hypercholesterolaemic rats *in vivo*. Curcuminoid cider demonstrated similar efficacy with curcuminoid fraction on interfering obesity-induced inflammation at genetic level, indicating its potential application as a functional fermented beverage for daily consumption in the management of obesity.

Acknowledgement

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