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Original research article

Effects of Ethanol Crude Extract of *Helicteres Isora* Fruit on Adipogenesis and Fat Accumulation in 3T3-L1 Cells

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ABSTRACT

Helicteres isora Linn. (*H. isora*) has revealed great potential in antioxidant, anti-inflammatory, antidiabetic, and hypolipidemic activities in diabetic rats. Fruits and roots of this plant previously exhibited the antihyperglycemic and hypolipidemic activities. There has been no research study about the direct effects of *H. isora* extract on lipid metabolism in adipocytic cells. Thus, the objectives of this study were to determine if ethanol crude extract from *H. isora* fruit would affect 3T3-L1 adipogenesis and fat accumulation within mature adipocytes as well as hormone-sensitive lipase (HSL) and adiponectin gene transcription. Adipogenesis and fat accumulation were assessed by measuring the amount of triglyceride within fat droplets formed in the cytoplasm of 3T3-L1 mature adipocytes. It was found that ethanol crude extract of *H. isora* fruit (500 μg/mL) significantly induced adipocyte differentiation and slightly increased fat accumulation (no significance) within 3T3-L1 mature adipocytes presented as triglyceride/protein ratio (%fold change). In addition, adiponectin and HSL mRNA levels, determined by real-time RT-PCR, were significantly reduced in ethanol crude extract-treated cells. These results indicate that the ethanol crude extract of *H. isora* fruit probably induces obesity by increasing of both number (adipogenesis) and size (fat accumulation) of adipocytes. Ethanol fruit extract of *H. isora* might not be useful for diabetic prevention.

Keywords: Adipogenesis; Fat accumulation; Adiponectin; *H. isora* fruit; Hormone sensitive lipase

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1. Introduction

Helicteres isora Linn. (*H. isora*) is tropical trees and shrubs with axillary flowers and fruits consisting of five twisted carpels found in Asia, including the Indian Subcontinent, South China, the Malay Peninsula, Myanmar, Sri Lanka, and Saudi Arabia. Also, *H. isora* has been found in Thailand, named as Porkabid or Porbid. *H. isora* is a medicinal plant belonging to the family of Sterculiaceae which exhibits several pharmacological effects. This plant contains several compounds such as phenols, flavonoids, alkaloids, glycosides, phytosterols, carotenoids, and tannins [1]. Different parts of this plant exhibit different compounds, which have different properties [2]. Stems of this plant are used as anthelmintic, while fruits are used as colic, anticonvulsant, and abdominalgia [3]. In addition, the water extract of *H. isora* fruit inhibits reverse transcriptase from avian myeloblastosis virus [4] and has an anti-HIV-1 activity [5]. Acetone fruit extract of *H. isora* has a strong antioxidant activity (96.44%) compared to hexane, and isopropyl alcohol extracts [6]. This plant also has antidiabetic and hypolipidemic activities. Hot water extracts of *H. isora* fruit cause significant antioxidant activity and moderate antidiabetic activity by increasing the glucose uptake activity in the isolated rat hemidiaphragm model [7] and in the rodent skeletal muscle cells [8]. The hypolipidemic effect from an aqueous extract of *H. isora* bark is observed in streptozotocin (STZ)-induced diabetic rats. After 3 weeks of bark extract treatment, the rats exhibit a significant reduction in serum and tissue cholesterol, phospholipids, free fatty acids, and triglycerides [9]. The antihyperglycemic and hypolipidemic activities of *H. isora* root extracts are also found in alloxan-induced diabetic rats. Blood glucose, total cholesterol, triglycerides, and urea levels are reduced in alloxan-induced diabetic rats after 10 days of treatment with butanol and aqueous ethanol extracts of *H. isora* root. Both extracts are able to restore the pancreatic islets, kidney glomeruli, and liver to its normal size [10]. Ethanol crude extract from *H. isora* fruits is able to restore the lipid levels of diabetic rats to near normal levels after 45 days of treatment [11].

Obesity is an enlargement of adipose tissue to store excess energy intake. Hyperplasia (increasing cell numbers by adipocyte differentiation) and hypertrophy (increasing cell size by fat accumulation) are two possible growth mechanisms [12]. Obesity is a risk factor for type 2 diabetes caused by insulin resistance. Previous studies show that *H. isora* has great potential in terms of antidiabetic, anti-inflammatory, antioxidant and hypolipidemic activities. However, there has been no study about the direct effects of *H. isora* extract on fat accumulation and adipocyte differentiation (adipogenesis) in vitro. Thus, the effects of ethanol crude extract from *H. isora* fruit on 3T3-L1 adipogenesis and fat accumulation, including changing of adipocyte-specific genes expression such as hormone-sensitive lipase (HSL) and adiponectin genes, were investigated in this study. These results will provide some information to understand the effects of ethanol crude extracts from *H. isora* fruits on lipid metabolism, which may be useful for type 2 diabetes prevention and treatment in the future.

2. Materials and Methods

2.1 Materials

Mouse 3T3-L1 pre-adipocytes were obtained from American type culture collection (ATTC®CL173; Manessas, VA, USA). Dulbecco modified Eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco (Gibthai, Thailand). Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), and insulin were purchased from Sigma-Aldrich (St. Louis, Mo, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA).

DNAse and the ImProm-II™ Reverse Transcription System were purchased from Promega (Madison, WI, USA). The iTaq™ Universal SYBR® Green Supermix was from Bio-Rad (Hercules, CA). Stanbio LiquiColor® triglyceride reagent was obtained from Stanbio Laboratory (Boerne, Texas, USA). Pierce® BCA Protein Assay Kit was from Thermo Scientific (Rockford, IL, USA)

2.2 Ethanol crude extract from Helicteres isora fruits

Dried *Helicteres isora* Linn. fruits were purchased from Vejpong Pharmacy Company, Bangkok, Thailand. They were ground, passed through sieve no.60, and kept in a well-closed container before use. Dried and crushed fruits (500 g) were macerated in 95% ethanol (3.0 L) for 1 day at room temperature and filtered (1st filtrate). Sludge was macerated in 95% ethanol again for two days and filtered (2nd filtrate). The second filtrate was mixed with the first one before it was dried by rotary evaporation and kept in an amber well-closed container at 4°C. The amount of powder crude extract was calculated (1.85% yield) and kept at 0-4°C until used by dissolving with 95% ethanol or dimethyl sulfoxide (DMSO).

2.3 Effects of ethanol crude extract on 3T3-L1 adipogenesis

Pre-adipocyte 3T3-L1 cells were cultured at 37°C, 5% CO2 in adipocyte medium; 10% FBS in DMEM containing 25 mM glucose (DMEM/High glucose) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Ten thousand pre-adipocyte 3T3-L1 cells per well were seeded in 6-well plates and cultured in adipocyte medium until there were 80-100% confluent cells. Then, pre-adipocyte 3T3-L1 cells were started the adipocyte differentiation by adding the adipocyte medium supplemented with adipogenesis inducer (0.5 mM IBMX, 250 nM dexamethasone) for two days. Then, adipogenesis inducer was substituted with insulin (5 µg/mL) for two days. After that, cells were further incubated in adipocyte medium without insulin supplement for three days. During seven days of adipocyte differentiation, cells were treated with ethanol crude extract (25, 50, 100, 250 and 500 µg/mL) and DMSO as a control. After seven days of differentiation, cells were harvested and kept at -70°C for triglyceride and protein determination.

2.4 Effects of ethanol crude extract on fat accumulation in 3T3-L1 mature adipocytes

Pre-adipocyte 3T3-L1 cells were grown to confluence in 6-well plates and stimulated after two days of post-confluence with the adipogenesis induction medium (adipocyte medium supplemented with 0.5 mM IBMX, 250 nM dexamethasone, and 5 µg/mL insulin) for 2 days. Then, cells were cultured in 3T3-L1 adipocyte medium supplemented with 5 µg/mL insulin for 4-6 days. The 3T3-L1 adipocyte medium was changed every two days. After 3T3-L1 adipocytes attained maturity in at least 80 percent of the cells, mature adipocytes were treated with ethanol crude extract (25, 50, 100, 250 and 500 µg/mL) and DMSO as a control for 24 hours. After that, cells were harvested and kept at -70°C for triglyceride and protein determination.

2.5 The triglyceride and protein ratio

Cells were lysed with 5%Triton X-100 at 95-100ºC for 30 min. The cell lysate determined the triglyceride contents using Stanbio LiquiColor® triglyceride reagent (Quantitative enzymatic colorimetric method) according to the manufacturer's protocol. Pierce® BCA Protein Assay Kit was used to determine protein in the cell lysate based on the bicinchoninic acid (BCA) colorimetric method. Protein concentration in each lysate indirectly represented the numbers of cells in each well. The fold changes of lipids within the cells during adipogenesis or fat

accumulation were normalized with proteins and presented as the triglyceride/ protein ratio.

2.6 The effects of ethanol crude extract on the expression of adipogenic genes by Real-time PCR

3T3-L1 mature adipocytes, obtained from pre-adipocyte 3T3-L1 adipogenesis as mentioned above, were treated with ethanol crude extract (250 and 500 µg/mL) and DMSO as a control for 24 hours. After that, cells were harvested and kept at -70°C for RNA isolation and adipogenic gene expression by real-time RT-PCR.

Total RNA was isolated from 3T3-L1 mature adipocytes using TRIzol reagent according to the manufacturer's protocol, and residual DNA contamination was removed by DNAse. cDNA synthesis was performed by the ImProm-II™ Reverse Transcription System according to the manufacturer's protocol.

PCR amplifications were done by Real-time PCR using the iTaq™ Universal SYBR® Green Supermix. Specific primer sequences (synthesized by Integrated DNA technologies, Singapore) used in this experiment are shown in Table 1. Quantification of gene expression was enabled using CFX96 Touch-Real time PCR Systems (Bio-Rad, USA) and Bio-Rad CFX manager software. Briefly, the cDNA was denatured at 95°C for 3 min followed by 35 cycles of PCR (95°C, 5 sec for denaturation step, 60°C, 30 sec for annealing and extension steps). Melting curve analysis for PCR products was performed at the final step, beginning from 65°C to 95°C (0.5°C increment, 5 sec/step). The mRNA levels of all genes were normalized using actin as a reference gene. The differences in gene expression between ethanol crude extract-treated cells and control were presented as the relative expression ratios (R) or fold changes calculated using the delta-delta method ($R=2^{-\Delta\Delta Ct}$). All results were obtained from at least three independent experiments.

2.7 Statistical analyses

The results are expressed as the mean±SEM for triplicate of three independent experiments. Treatments were compared by one-way ANOVA using Tukey's posthoc test or Dunnett's Multiple Comparison Test to identify statistical differences at $p<0.05$.

Table 1. Primer sequences and PCR product sizes for Real time-PCR.

Name	primer sequences	PCR (bp)	Ref.
HSL	F: 5'-AGDCACCAGCCAACGGATAC-3'	239	Using Primer3 and BLAST
	R: 5'-ATCACCCTCGAAGAAGAGCA-3'		
Adiponectin	F: GAAGATGACGTTACTACAAC	704	[13]
	R: GGTAGTTGCAGTCAGTTGGT		
Actin	F: AAGAGAGGTATCCTGACCCT	218	
	R: TACATGGCTGGGGTGTTGAA		
	R: TGGCCCTAAGTATTCAAGTTCTG		

3. Results and Discussion

Since *H. isora* fruit exhibits the anti-diabetic and hypolipidemic activities [10, 14], the direct effects of ethanol crude extract from *H. isora* fruit on 3T3-L1 adipogenesis and fat accumulation in mature 3T3-L1 adipocytes, including the gene expression of adiponectin and hormone sensitive lipase, were studied.

3.1 The effects of ethanol crude extract on 3T3-L1 adipogenesis

3T3-L1 pre-adipocytes were treated with the adipogenesis induction medium (see Methods and Materials). During adipogenesis, fat droplets are accumulated, and pre-adipocytes become mature. Therefore, to detect adipogenesis, the triglyceride within the fat droplets were determined, and amounts of the cells per well were estimated by protein determination. The adipogenesis control, ethanol crude extract-untreated cells, showed the triglyceride/protein ratio as 100 percent. For ethanol crude extract-treated cells, the fold changes (percent) of the triglyceride/protein ratio compared to adipogenesis control were 150.54±16.73, 186.12±22.63, 193.88±22.63, 211.09±22.71 and 290.60±54.69 for 25, 50, 100, 250 and 500 μg/mL ethanol crude extract treatments, respectively. Increasing the concentration of ethanol crude extract to 500 μg/mL increased the triglyceride/protein ratio. Particularly, 500 μg/mL ethanol crude extract-treated cells were significantly increased in the triglyceride/protein ratio compared to adipogenesis control ($p>0.05$) (Fig. 1).

For the 3T3-L1 adipogenesis study, the higher the triglyceride/protein ratio, the higher the mature adipocyte production. The result showed ethanol crude extract of *H. isora* fruit (500 μg/mL) was able to induce the adipocyte differentiation. It is known from the previous study that ethanol (80%) crude extract from *H. isora* fruit can induce the cell differentiation of human colon cancer cells (RCM-1) by increasing the ducts resembling villiform structures [15]. They found that the ethanol extract of *H. isora* effectively induced RCM-1 cell differentiation, while other solvent extracts (hexane, dichloromethane, and water) showed no significant effect. This finding suggests that some specific compounds, for example, mainly phenolic compounds in ethanol crude extract of *H. isora* fruits [16], might induce the 3T3-L1 adipogenesis. However, there were no previous studies about the other major compounds in ethanol crude extract from *H. isora* fruits.

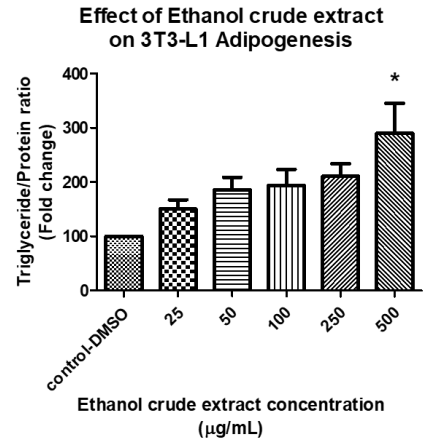

Fig.1. Effect of ethanol crude extract of *H. isora* fruit on adipocyte differentiation (Adipogenesis) presented as fold change (percent) of triglyceride/protein ratio. These results are shown as mean ±SEM from three experiments. * $p<0.05$ vs Control-DMSO.

3.2 The effects of ethanol crude extract on fat accumulation in 3T3-L1 mature adipocytes

The fold changes of fat accumulation within the 3T3-L1 mature adipocytes between ethanol crude extract treated- and untreated-cells (control) were determined by triglyceride/protein ratio. The ethanol crude extract-untreated cells were set the triglyceride/protein ratio as 100 percent.

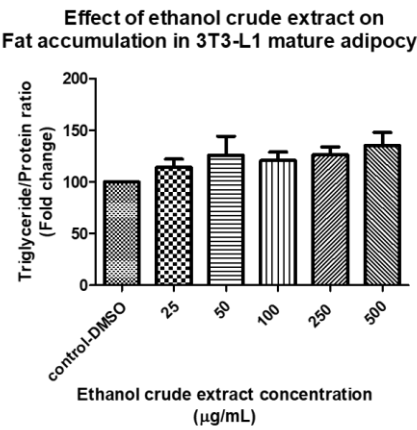

Fig. 2. Effect of ethanol crude extract of *H. isora* fruit on fat accumulation within 3T3-L1 mature adipocyte presented as fold change (percent) of triglyceride/protein ratio. These results are shown as mean ±SEM from three experiments. * $p<0.05$ vs Control-DMSO.

The percent of fold changes in ethanol crude extract treated-cells were 114.14±7.88 (25 μg/mL), 125.93±18.27 (50 μg/mL), 120.74±8.23 (100 μg/mL), 126.40±7.55 (250 μg/mL) and 135.49±12.51 (500 μg/mL) compared to control. However, there was no significant difference in fat accumulation between these ethanol crude extract-treated cells and control ($p>0.05$) (Fig. 2).

Although ethanol crude extract from the fruit of *H. isora* has revealed the hypolipidemic activity in streptozotocin-induced diabetic rats [11], our results showed that ethanol crude extract of *H. isora* fruit slightly increased fat accumulation within the mature 3T3-L1 adipocytes (no significance).

3.3 Real-time RT-PCR and primer specificity

RT-PCR experiments yielded a single band of expected PCR product size on 2% agarose electrophoreses gels (Table 1 and Fig. 3 D). Additionally, a melting curve analysis revealed single product-specific melting temperatures as follows: adiponectin (83.0°C), HSL (81.0°C), and actin (82.4°C) (Fig. 3 A-C). No primer-dimers or non-specific PCR products were observed during 35 cycles of the real-time PCR amplification protocol. Optimal and identical real-time amplification efficiencies (E) of target genes (adiponectin, HSL) and the reference gene (actin) are presumed as E=2.

3.4 The effects of ethanol crude extract on adipogenic genes in 3T3-L1 mature adipocytes

For the study of gene expression in mature adipocytes, two adipogenic genes were investigated; adiponectin and hormone sensitive lipase (HSL) in this study. The relative adiponectin mRNA levels (mean±S.E.M. values) in cells treated with 250 and 500 μg/mL were 0.43±0.06-fold, 0.30±0.06-fold, respectively (Fig.4A). The significant decrease was found only at 500 μg/mL of ethanol crude extract compared to controls ($p<0.05$). The relative expression ratios of HSL mRNA levels in cells treated with 250 and 500 μg/mL were 0.83±0.22-fold, 0.31± 0.06-fold, respectively. There was a significant decrease in ethanol crude extract-treated cells at a concentration of 500 μg/mL compared to untreated controls ($p<0.05$) (Fig.4B).

Our results showed that ethanol crude extract of *H. isora* fruit slightly increased fat accumulation within the mature 3T3-L1 adipocytes. Furthermore, the hormone-sensitive lipase mRNA transcription was decreased in mature adipocytes treated with ethanol crude extract compared to untreated control. HSL regulates adipocyte lipolysis by catalyzing the triglyceride, a storage lipid within the cell, into glycerol and free fatty acids [17]. Thus, a possible explanation for this finding of reduced HSL mRNA transcription might be the reason for a slight increase in fat accumulation within the mature adipocytes [18].

Adiponectin is a peptide hormone mainly synthesized by adipocytes in adipose tissues [19]. Decreased plasma adiponectin

is associated with insulin resistance, type-2 diabetes [20] and cardiovascular diseases [21]. In 500 μg/mL ethanol crude extract-treated mature adipocytes, adiponectin mRNA transcription significantly decreased, whereas fat accumulation slightly increased. It is possible that the more increased fat accumulation within the mature adipocyte, the more decreased adiponectin mRNA transcription [22-23]. Our results were consistent with decreased adiponectin level in obesity [24] and diabetics [25-26]. These results indicate that the ethanol crude extract of *H. isora* fruit probably causes obesity because of the increasing of both number (adipogenesis) and size (fat accumulation) of adipocytes. Ethanol fruit extract of *H. isora* might not be useful for obesity and diabetic prevention.

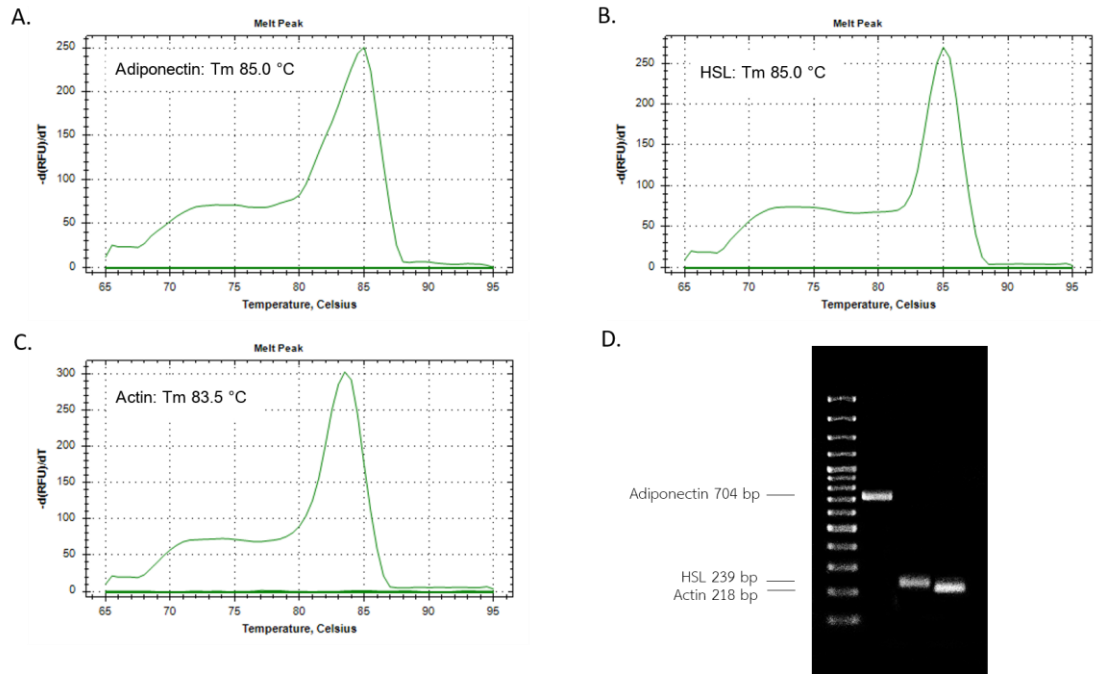

Fig. 3. Melting peak analysis of PCR products for adiponectin (A), HSL (B), and actin (C). Ethidium bromide-stained agarose gel separation of specific amplicons of adiponectin, HSL and actin (D).

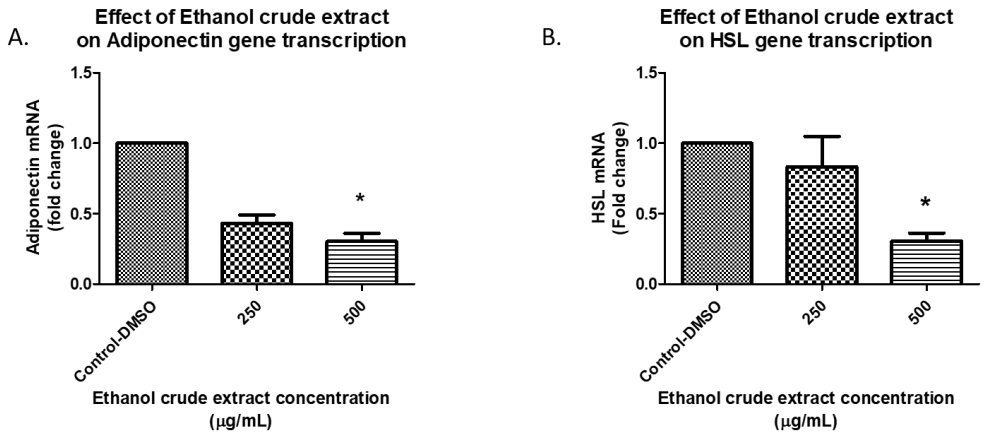

Fig. 4. Effect of ethanol crude extract of *H. isora* fruit on adiponectin (A) and hormone sensitive lipase (B) transcription within 3T3-L1 mature adipocyte reported relative to that of actin mRNA (reference gene) as means ±S.E.M. (n=3), **P*<0.05, relative to control.

4. Conclusion

Ethanol crude extract of *H. isora* fruit might not be suitable for diabetic prevention and treatment due to its effects on adipocyte differentiation, fat accumulation, and adiponectin transcription.

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