ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Anti-Adipogenic compound from *Guazuma ulmifolia* Leaf

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ABSTRACT:
The purpose of this research was to test the anti-adipogenic activity and to identify the active compound of the leaf of *Guazuma ulmifolia*. The activity of anti-adipogenic of the compound of *G. ulmifolia* leaf was tested using primary cultures of Wistar strain white mouse preadipocytes. Isolation of the active compound was carried out in the following way. The powder of *G. ulmifolia* leaf was defated with *n*-hexane followed by extraction with 70% ethanol. The extract was concentrated and fractionated by liquid-liquid partition using chloroform and ethyl acetate solvents. The ethyl acetate fraction was loaded into a Vacuum Liquid Chromatography (VLC) followed by a reverse phase preparative layer chromatography to obtain a pure isolate. Isolated compound inhibited preadipocytes proliferation and differentiation. Intensive spectroscopic and spectrometric analysis suggested molecular structure of a tiliroside which agreed with previously reported data. The study successfully revealed the traditional claims of the anti-obesity agent of a leaf of *G. ulmifolia* through inhibition of preadipocytes proliferation and differentiation.

KEYWORDS: *Guazuma ulmifolia* Leaf, Anti-Adipogenic Compound, Tiliroside.

INTRODUCTION:
Locally named as Jati Belanda, *Guazuma ulmifolia* Lamk., was exist in Indonesian traditional medicinal formulation as weight loss agent in which previously, the old manuscript was exclusively accessed only by the Javanese royal family[1]. Previous studies reported that crude extract of leaf of *G. ulmifolia* possessed various pharmacological activities including antidiabetic, antihypertensive, antimicrobial, antioxidant, antiulcer, antiobesity agent and indicated as hair growth promoter[2]. The leaf of *G. ulmifolia* was reported to contain catechins, kaempferol, procyanidin, and tiliroside[3,4]. These compounds have received great attention because of their importance to human health[5,6]. Obesity has become a serious problem of health in both developing and developed countries which increases the risk of morbidity and mortality[7,8].

Received on 27.06.2019 Modified on 28.07.2019
Accepted on 29.08.2019
Research J. Pharm. and Tech. 2020; 13(1):411-415.
DOI: 10.5958/0974-360X.2020.00080.3

Obesity is characterized by an increase in the adipocytes size and number, derived from preadipocytes that undergo proliferation and differentiation. Natural products has been a good source for inhibitor of adipocytes proliferation and differentiation[9]. Previous studies showed polyphenols, including flavonoids, to possess a strong anti-obesity activities by inhibiting the proliferation and differentiation of preadipocytes[10]. In this research, we aimed to isolate the flavonoid constituents of leaf of *G. ulmifolia* leaf, and to conduct test against preadipocyte proliferation and differentiation for the first time.

MATERIAL AND METHODS:
Medicinal plant sample:
G. ulmifolia leaf was obtained from National Park of Meru Betiri Jember Indonesia at 900-1,223 m.a.s.l in average rainfall of 2,300 mm/year in October 2016. The sample was identified at Botanical Gardens of Indonesian Institute of Sciences, Purwodadi, East Java. The leaves are sorted, washed, and dried prior pulverization (blended) into powder.

Extraction and Isolation:
The powder of *G. ulmifolia* leaf (700 g) was macerated with *n*-hexane (1000 mL) for 24 hours at room temperature three times to remove the fat. The remaining part was dried then macerated with 70% ethanol (3x2000 mL) for 24 hours. Obtained filtrate (5000 mL) was volume reduced through rotary evaporation (1000 mL). The filtrate is sequentiallya partitioned with chloroform (3x350 mL each) followed by ethyl acetate (3x350 mL). The ethyl acetate fraction was rotary evaporated to produce crude ethyl acetate fraction (3465 mg). Ethyl acetate fraction (1250 mg) was loaded into a Vacuum Liquid Chromatography (silica gel 60, 40 g) and developed with chloroform: methanol: water (40: 10: 1) to produce 7 fractions. The fraction 2 was purified using preparative layer chromatography (PLC silica gel 60 RP-18 F_{254}, 1 mm) with methanol : water (1:1, 100 mL) to produce compound **1** (5.1 mg).

Cell Culture Preparation:
Preadipocyte was isolated from visceral adipose tissue of mice 4-8 weeks aged. The tissue of visceral fat was cut into small pieces in sterile conditions and was cleaned from the surrounding tissue. The tissue was then washed with PBS (Phosphate Buffer Saline) and was cut into smaller pieces. The chopped tissue was hydrolyzed with collagenase type I (0.2% w/v) at 37 °C for 1 hour. After hydrolysis, the suspension was screened through nylon mesh 250 µm. The suspensions contained isolated cells, then were centrifuged for 7 minutes at 1000 rpm. These produced two cell layers consisting of mature adipocytes in the upper layer and preadipocyte pellets in the lower layer. The pellets were resuspended in a medium of culture containing 10% FBS (Fetal Bovine Serum) were homogenized and were then planted on a microwell plate followed by incubation at 37 ˚C under 5% CO2 [11]. After 48 hours, the suspension was added a differentiation-inducing medium (DMEM/F12 (Dulbecco′s Modified Eagle′s Medium/Nutrient Mixture F-12 Ham) supplemented with insulin, 3-isobutyl-1-methylxanthine and dexamethasone with a final concentration of 66, 500 and 100 nM, respectively). The mixture was then incubated at 37 °C for 24 hours in a 5% CO2 incubator. Then, cells culture was incubated with compound 1 for 24 hours[12]. All experiments were carried out in triplicate at a concentration of 62.5 ppm.

MTT assay:
The proliferation of cell was evaluated by MTT test. Into each well of 96 well plates, a medium containing MTT assay (20µl) was added and incubated at 37℃ for 4 hours and followed by addition of DMSO (150 µL). Each sample measured for its absorbance at λ 490 nm.

Cells Differentiation Determination:
The cells were fixed with 10% formalin after the incubation with compound 1 and washed with running water from the faucet water briefly. Propylene glycol was used to rinse cells. The newly working solution of Oil-Red-O was added with 7 minutes agitation. Propylene glycol was used again to rinse cells followed by hematoxylin staining. The cells were washed with running water from the faucet and dried. Lastly, it was observed under the microscope at 400 times magnification[12,13]. The 25 areas of view used to count the cells. The differentiation of cell was calculated based on the number of mature adipocytes wichs were undergoing morphological changes to total cells.

RESULTS AND DISCUSSION:

Isolation of compound 1:
The compound was assigned as kaempferol 3-O-(4",6"-O-di-*p*-coumaroyl)-glucoside (Figure 1). It was isolated as an amorphous yellow powder. TLC based purity test showed single spot in both UV and citrate-borate staining visualisation (Figure 2).

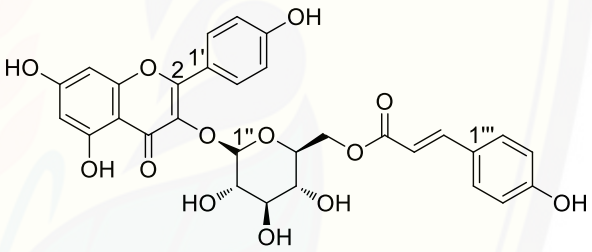

Fig 1: The structure of compound 1

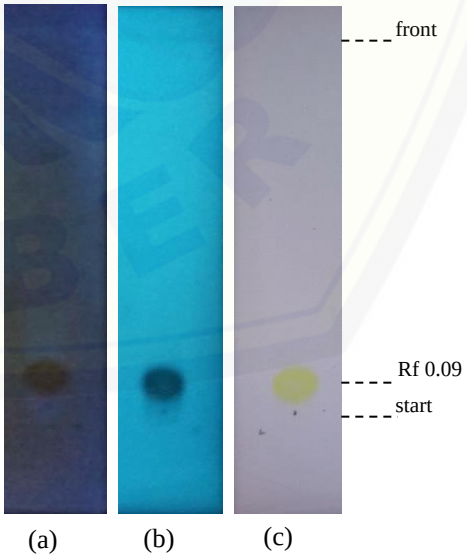

Fig 2: Chromatograms of compound 1 (RP18-TLC, MeOH/H_2O (1:1)) at a λ 254 nm (a), at a λ 365 nm (b), at a visible light with citrate-borate staining (c).

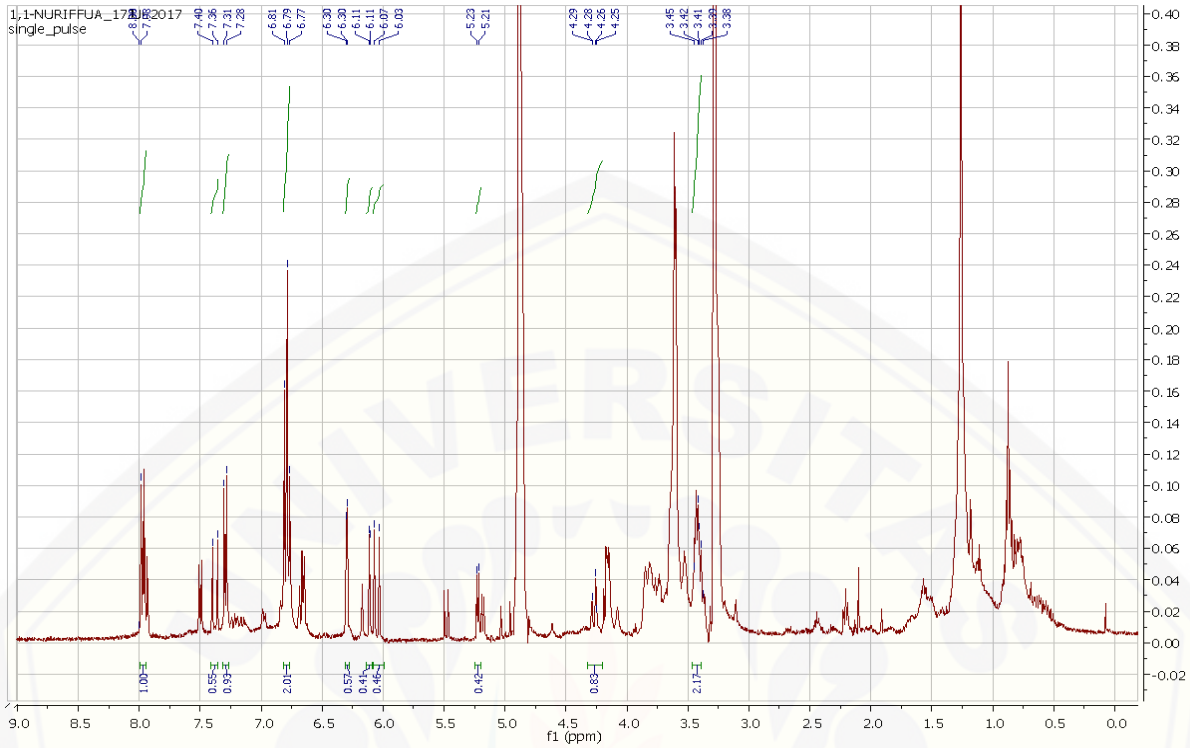

Fig 3: ^{1}H NMR spectrum of compound 1

Table 1: ^{1}H NMR chemical shift values for the isolated compound recorded in CD_3OD (400 MHz)

Carbon No.	Carbon Type	δH,	
		J Exp.	*J* Lit.[11]
Flavonoid Moiety			
2	C		
3	C		
4	C		
5	C		
6	CH	6.11 (d, ^{2}J = 2.0 Hz)	6.14 (d, *J* = 2.1 Hz)
7	C		
8	CH	6.30 (d, *J* = 1.6 Hz)	6.37 (d, *J* = 2.1 Hz)
9	C		
10	C		
1′	C		
2′	CH	7.97 (d, *J* = 8.8 Hz)	7.98 (d, *J* = 8.8 Hz)
3′	CH	6.80 (d, *J* = 8.8 Hz)	6.77 (d, *J* = 8.8 Hz))
4′	C		
5′	CH	6.80 (d, *J* = 8.8 Hz)	6.77 (d, *J* = 8.8 Hz)
6′	CH	7.97 (d, *J* = 8.8 Hz)	7.98 (d, *J* = 8.8 Hz)
Sugar Moiety			
1″	CH	5.22 (d, *J* = 6.8 Hz)	5.20 (d, *J* = 7.6 Hz)
2″ - 5″	CH	3.38–3.45 (m)	3.35–3.51 (m)
6″a	HCH	4.29 (dd, *J* = 12.0, 1.6 Hz)	4.35 (dd, *J* = 12.6, 2.0 Hz)
6″b	HCH	4.26 (dd, *J* = 12.0, 1.6 Hz)	4.21 (dd, *J* = 12.8, 6.8 Hz)
Phenylpropanoid Moiety			
1 ‴	C		
2 ‴	CH	7.30 (d , *J* = 8.8 Hz)	7.36 (d, *J* = 9.2 Hz)
3 ‴	CH	6.78 (d, *J* = 8.8 Hz)	6.77 (d, *J* = 9.2 Hz)
4 ‴	C		
5 ‴	CH	6.78 (d , *J* = 8.8 Hz)	6.77 (d, *J* = 9.2 Hz)
6 ‴	CH	7.30 (d, *J* = 8.8 Hz)	7.36 (d, *J* = 9.2 Hz)
7 ‴	C = C	7.38 (d, *J* = 16.0 Hz)	7.44 (d, *J* = 15.6 Hz)
8 ‴	C = C	6.05 (d, *J* = 16.0 Hz)	6.07 (d, *J* = 15.6 Hz)
9 ‴	C		

The UV spectral analysis of compound **1** showed a shoulder at λ_{max} 268 nm and a broadband at λ_{max} 315 nm which is typical for acylated glycoside flavonol[14,15]. The ^{1}H-NMR spectrum (400 MHz, CD_3OD) of compound **1** (Figure 3) showed a typical signals of kaempferol aglycon[16,17,18,19]. A set of meta coupled aromatic methines were present at 6.11 and 6.30 ppm assigned respectively to the H-6 and H-8 protons. Two pairs of A2B2 aromatic systems, each integrating for two protons, were found respectively at 7.97 ppm and 6.80 ppm assigned to the H-2′, H6′ and H3′, H5′ protons, and at 7.30 ppm and 6.78 ppm assigned to the H-2‴, H-6‴ and H-3‴, H-5‴ protons. Two olefinic methines with trans coupling were present at 7.38 ppm and 6,05 ppm assigned to the H-7‴ and H-8‴ protons. Finally, the presence of a sugar moiety was clearly the peak at 5,22 ppm assigned to anomeric proton H-1″. The remaining peaks assigned to the sugar appear in the region 3.38-4.29 ppm (Table 1).

Figure 4 showed an $[M-H]^-$ ion at m/z 593. The loss of a coumaroyl-glucoside moiety (-308 amu) was shown a fragment ion at m/z 285 $[M\text{-kaempferol}]^-$. Finally, fragmentation at m/z 447 $[M\text{-p-coumaroyl}]^-$ proposing the compound was kaempferol-3-O-(6-p-coumaroyl)-glucoside (tiliroside)[20].

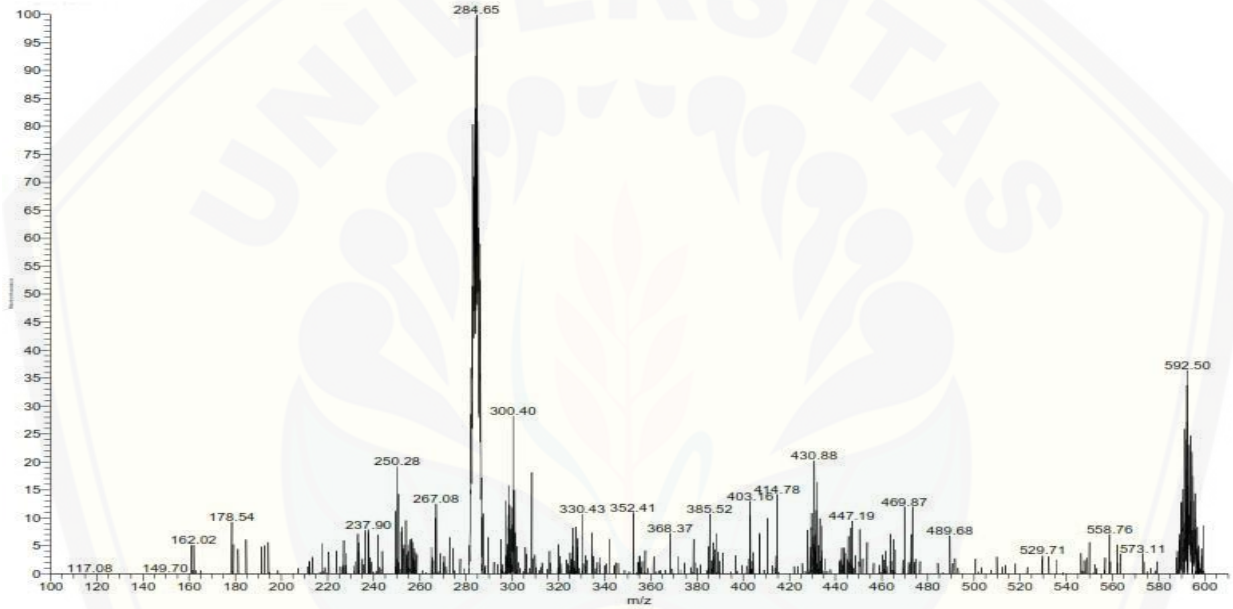

Fig 4: MS/MS spectrum of compound 1

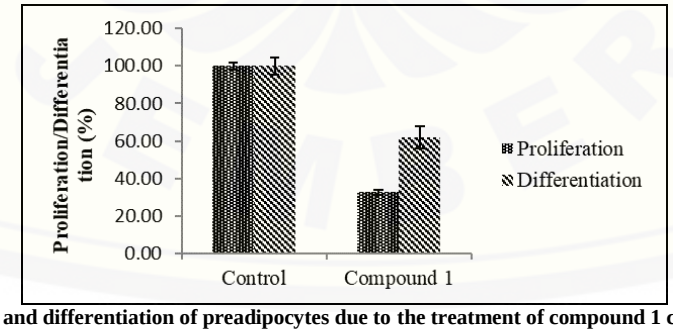

Fig. 5: Percent of proliferation and differentiation of preadipocytes due to the treatment of compound 1 compared with control

Based on the UV spectrum, a ^{1}H NMR spectrum, and the MS/MS spectrum comparison with the reported compounds in the literature, the structure of the isolated compound **1** was tilirisode.

Anti-proliferation and Anti-differentiation Activities of Compound 1:

Cell proliferation was calculated based on the result of MTT assay. While the differentiation of cells was calculated based on the cells number that grow into mature adipocytes (Figure 5).

The treatment with compound **1** caused the preadipocytes proliferation and differentiation to decrease by 32.6% and 61.9% respectively compared to controls. The anti-proliferation activity demonstrates the capability compound **1** to inhibit the proliferation in which further experiment test resulted the compound **1** to

effect on preadipocytes proliferation. These results were consistent with previous studies, that flavonoids can inhibited cell proliferation[21,22]. Furthermore, kaempferol and catechins also suppressed cell growth[5]. While anti-differentiation activity demonstrates the capability compound **1** to inhibit cell morphology changes into mature adipocytes. These inhibitions mechanism confirmed compound **1** to possess significant activity on preventing fat accumulation.

Previous research on closed related to compound **1**, for example 3″-(*E*)-feruloylquercitrin also indicated an anti-adipogenic activity in 3T3-L1 cell culture, kaempferol (the core stucture of compound **1**) also exhibited anti-adipogenic activity as well as *p*-coumaric acid[23,24,25,26]. Thus, the anti-adipogenic activity of compound **1** possibility was contributed by the presence of molecular fragaments of compound **1,** agliconic kaempferol and the *p*-coumaroyl moiety.

CONCLUSION:

According to this research, it can be concluded that compound **1** was kaempferol-3-O-*β*-D-(6-*p*-coumaroyl)-glucopyranoside (tiliroside), which may inhibit preadipocyte proliferation and differentiation.

ACKNOWLEDGEMENT:

This work was supported by Ministry of Research, Technology and Higher Education of the Republic of Indonesia under contract number 0513/UN25.3.1/LT/2017.

CONFLICT OF INTEREST:

The authors hereby declare that regarding the publication of this paper there is no conflict of interests.

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