# Anti-Adipogenic Activity of Fractions of *Guazuma ulmifolia* Leaf

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Abstract : This study purposed to investigate the anti-adipogenic activity of the chloroform, ethyl acetate, and residual ethanol fractions of *Guazuma ulmifolia* leaf extract. The fractionation of ethanol extract was carried out by solvent-solvent partition using chloroform and ethyl acetate. Inhibition of fractions to the proliferation and differentiation of primary cultures of rat preadipocytes were tested to investigate the anti-adipogenic activity. Separation of ethanol extract yielded three fractions, i.e. fraction of chloroform, fraction of ethyl acetate, and fraction of residual ethanol. The results of anti-proliferation and anti-differentiation activity test showed that the highest activity was demonstrated by ethyl acetate fraction, followed by residual ethanol fraction and chloroform fraction. The highest total flavonoid content was also shown by the fraction of ethyl acetate. The fraction of ethyl acetate showed the highest anti-adipogenic activity and the highest total flavonoid content

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## 1 INTRODUCTION

Nowadays obesity is not only seen as a performance problem but has become a pandemic and a major health problem resulting in increased risk of comorbidity that contributes significantly to mortality (Castanon, Lasselin and Capuron, 2014). Obesity has become a major risk for diabetes, hypertension, coronary heart disease, dyslipidemia, and certain tumors (Pi-Sunyer, 2002). The World Health Organization (WHO) defines obesity as an abnormal or excessive accumulation of fat that harms the health of human (Word Health Organization, 2018). In fact, obesity can be controlled by reducing the fat content of food accompanied by an increase in physical exercise. However, it is estimated that over 90% of people who lose weight with diet and increase physical exercise, within 2-5 years will return to their original weight. Increased adipose tissue mass involves an increase in the number of adipocytes formed from precursor cells, which in turn occurs enlargement of adipocyte size. The formation of the adipocytes from precursor cells and enlargement of their size is a life cycle of adipocyte, and treatment that can regulate adipocyte counts and measures can be used as a therapeutic approach to treat obesity (Rayalam, Della-Fera, and Baile, 2008).

Flavonoids, polyphenol compounds, are widely present in plants and are known to inhibit proliferation in some cell cultures and have anti-adipogenic effects on 3T3-L1 line cells. The results of Park *et al* (2009) research show that luteolin inhibits the differentiation of preadipocyte and regulates the early stages of adipogenesis. Other flavonoids myricetin, a flavonoid found in various foods, can inhibit adipogenesis as indicated by decreased intracellular lipid droplet accumulation (Bin and Choi, 2012). Flavonoids present in *Citrus aurantium* L. suppress adipogenesis by decreasing PPARg and C/EBPa expression (Kim *et al.*, 2012). The anti-adipogenic effects of this 3T3-L1 cell, coupled with anti-proliferative activity, proposes the presumption that flavonoids may inhibit the increase of adipocytes or signals that promote adipogenesis.

*G. ulmifolia* is a plant originating from tropical America and is a plant belonging to the family Sterculiaceae. In Indonesia, this plant commonly called Jati belanda and traditionally has been used to lose weight and reduce excessive fat content (Mardisiswojo and Rajakmangunsudarso, 1985).



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The leaf of *G. ulmifolia* contain a number of phytochemical constituents i.e. colistin, colatannins, caffeine, tartaric acid, theobromine, xanthan gum, catechins, kaempferol, some of procyanidin (procyanidin B-2, B-5, and C-1), and tiliroside (Sharma and Prasad, 2014; Departement of Health Republic of Indonesia, 2008). Among these compounds, namely catechins, kaempferol, prosianidin and tiliroside including flavonoid derivatives compounds. The presence of this compounds gives the estimate of *G. ulmifolia* leaf as anti-obesity by inhibiting adipogenesis.

In this study, the ethanolic extract of *G. ulmifolia* leaf was fractionated by means of the liquid-liquid partition using chloroform, ethyl acetate. The obtained fractions (chloroform, ethyl acetate, and last remaining ethanol) were tested for inhibiting the proliferation and differentiation of rat preadipocytes.

# 2 MATERIALS AND METHODS

## 2.1 Materials

Organic solvents of n-hexane, chloroform, ethyl acetate and ethanol were in proanalytical grade (Merck), TLC plate (Merck), collagenase type I (Sigma), culture media DMEM, HEPES, $NaHCO_3$, biotin, D-pantothenate, FBS, Penicillin and Streptomycin (Sigma), differentiation induction materials insulin, dexamethasone, IBMX (Sigma)

## 2.2 Collection and Drying of G. ulmifolia Leaf

Leaf of *G. ulmifolia* was collected from Meru Betiri National Park with an altitude of 900 - 1,223 m asl and an average rainfall of 2,300 mm/year in October 2016. Prior to collection, the plants were determined in LIPI Botanical Gardens, Purwodadi, East Java. The leaf was sorted, i.e. removed the damaged leaf and other impurities then washed with running water. The clean leaf was dried and then pulverized (grounded) to powder.

## 2.3 Extraction and Fractionation

*G. ulmifolia* leaf powder weighing 800 g was defatted with n-hexane four times (each 1000 mL). The residue was collected, air dried, and macerated in 70% ethanol (2000 mL) for 24 hours. This procedure was repeated three times using the same powdered leaf. The filtrate then concentrated by using a rotary evaporator at 45°C under reduced pressure to obtain a less 70% ethanol extract. Successively, the extract was fractionated using chloroform and ethyl acetate (3 x 350 mL of each solvent) to obtain chloroform, ethyl acetate, and residual 70% ethanol fraction. The fractions solvent was completely removed under the vacuum to obtain dry fractions and preserved in vials and kept at 4 °C before use.

## 2.4 Determination of Total Flavonoid Content

Total flavonoid content was measured by the aluminum chloride colorimetric assay. An aliquot (150 µL) of fractions or standard solution of quercetin (5, 10, 20, 40, 60, 80 and 100 mg/L) was added to 1.5 ml cuvet containing 0.4 ml of aqua distilled water. To the cuvet was added 0.03 mL 5 % $NaNO_2$ and 0.03 mL 10 % $AlCl_3$. After 6 min, 0.2 mL 1 N NaOH and 0,24 of mL distilled water were added. The solution was stirred until homogeneous, then the absorbance was measured at 415 nm. Total flavonoid content of fraction was expressed as mg quercetin equivalents (QE)/g fraction. Samples were analyzed in triplicates (Ratnadewi *et al.*, 2018).

## 2.5 Preparation of Cell Culture

Preadipocytes were isolated from mice adipose tissue aged 4-8 weeks. The visceral fat tissue was sliced in a sterile condition and cleaned as much as possible from surrounding tissues. The tissue was washed with PBS and chopped into small pieces. Chopped tissue was digested by type I of collagenase at 37 °C for an hour. After that, the suspension was filtered through 250 µm nylon mesh. The suspension containing isolated cells were centrifuged at 1000 rpm for 7 minutes, and the two types of cells were separated. Mature adipocytes were found at the top layer of the suspension and the pellet at the bottom of a tube containing preadipocytes cells. Furthermore, the pellet was resuspended in culture medium containing FBS 10%, homogenized, and plated on plate culture, then incubated at 37˚C, 5% $CO_2$ (Duarte, *et al.*, 2012). After two days, differentiation was induced by the addition of induction medium ((DMEM/F12 added by 66 mM insulin, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine) and incubated at 37°C in a 5% $CO_2$ incubator for 24 hours.

The cell culture was incubated with chloroform, ethyl acetate, and ethanol fractions of *G. ulmifolia* leaf fraction for up to 24 hours (Lin, Della-Fera, and


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Baile, 2005). All experiments were performed at least in triplicate at concentrations of 500 ppm.

### 2.6 MTT Assay

Cell proliferation was examined by MTT assay. In brief, 20µl assay medium containing MTT was added to each well of 96 well plates. The incubation continued at 37 ℃ for 4 hours , adding 150 µl DMSO to dissolved the colored formazan. The absorbance of each sample was measured by a microplate reader at 490 nm.

### 2.7 Determination of The Cells Differentiation

After the treatment with fractions, the amount of differentiated and non-differentiated cells were calculated under the microscope at a 400 magnification (Hemmrich *et al.*, 2005). Cells were calculated at 25 fields of view. Cell differentiation was calculated based on the number of cells undergoing morphological changes in adult adipocytes.

### 2.8 Oil-Red-O Staining

Cells were fixed in 10% formalin and washed a moment with running tap water. After rinsed with propylene glycol, the freshly prepared Oil-Red-O working solution was added with agitation for 7 minutes. Then, the cells were rinsed with 85% propylene glycol and stained with hematoxylin. Finally, cells were washed with running tap water, dried, and observed under the microscope at 400 magnification (Lin, Della-Fera, and Baile, 2005).

## 3 RESULTS

### 3.1 Extraction and Fractionation

The yields of all the fractions corresponding to the initial dry leaf material are shown in Table 1. The extractive yield varied among the solvents used. Chloroform and ethyl acetate fractions showed less extractive yield as compared to residual 70% ethanol fraction.

Table 1: Fractions yield (%) of 800 g *G. ulmifolia* leaf powder in the different solvent

| Fractions | Extractive value (g) | % Yield |
|---|---|---|
| Chloroform Fraction | 3,61 | 0,45 |
| Ethyl acetate Fraction | 5,98 | 0,75 |
| Ethanol Fraction | 48,41 | 6,05 |

### 3.2 Total Flavonoid Content

The results obtained in the estimation of flavonoid content (Table 2) showed that all fractions had a certain amount of total flavonoid content. The fraction of ethyl acetate showed the highest total flavonoid content (314,50 ±4,50 mg QE/g fraction). The total flavonoid contents exhibited the descending order with fractions of ethyl acetate > chloroform > ethanol.

Table 2: Total flavonoid content of fractions

| Fractions | Total flavonoid content (mg QE/g Fraction) ± SD |
|---|---|
| Chloroform Fraction | 109,92 ± 2,15 |
| Ethyl acetate Fraction | 314,50 ± 4,50 |
| Ethanol Fraction | 10,22 ± 1,08 |

### 3.3 Anti-proliferation and Anti-differentiation Activities of Guazuma ulmifolia Leaf Fractions

Anti-proliferation activity indicates the ability of fractions to inhibit the proliferation of preadipocyte cells. Anti-proliferation activity of each fraction is shown in Figure 1. Cell differentiation is characterized by changes in adipocyte morphology. Inside the cell, there is a lipid drop as shown in Figure 2. The anti-differentiation activity showed the ability to inhibit preadipocyte cell changes in adipocyte mature cells. The anti-differentiation activity of the fractions is shown in Figure 3.

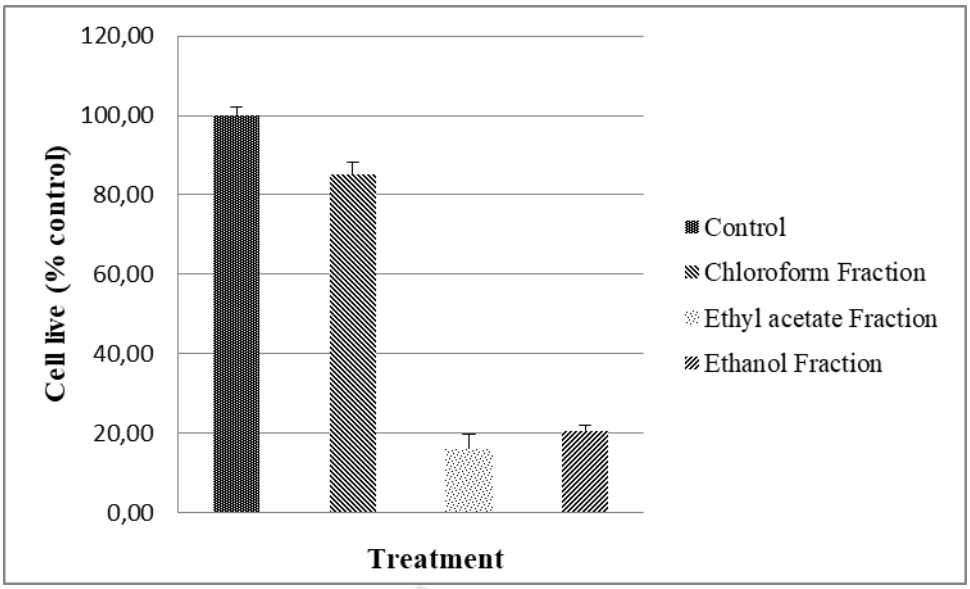


Figure 1: Anti-proliferation activity of *G. ulmifolia* leaf fractions

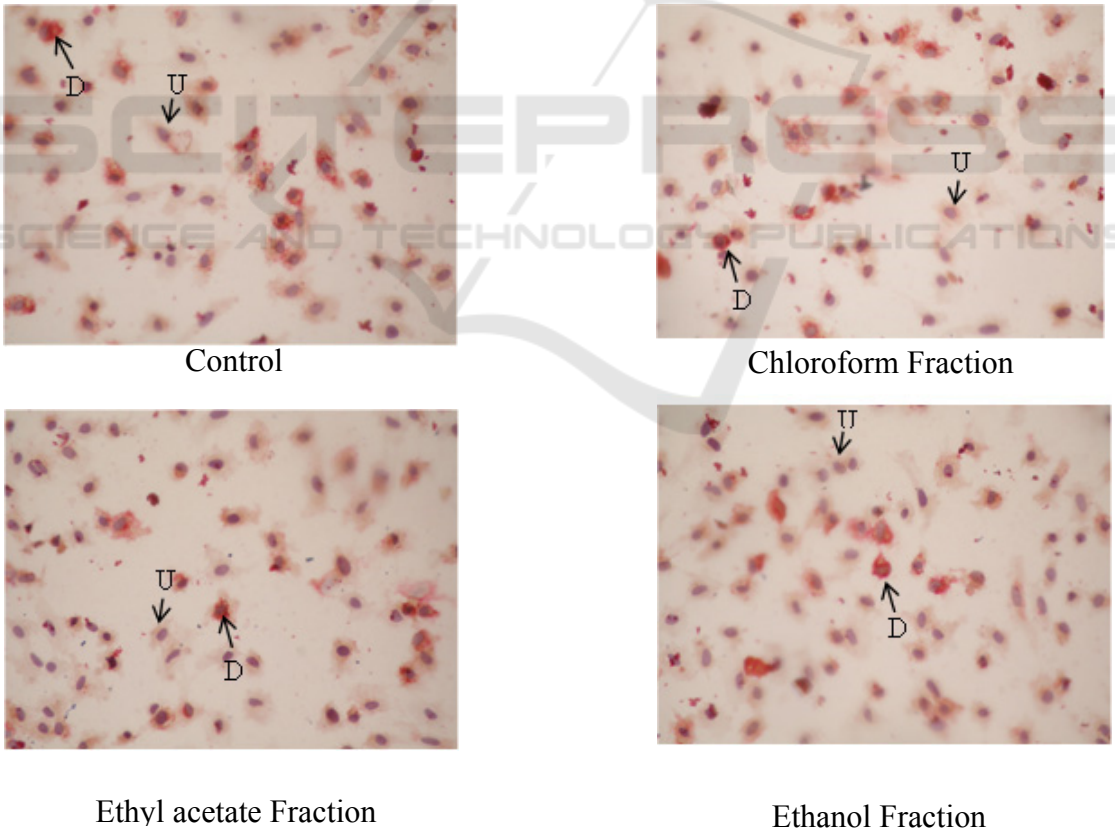


Figure 2: Preadipocyte morphology after incubation with *G. ulmifolia* leaf fractions for 24 hours. D = Differentiated cells, U = Undifferentiated cells



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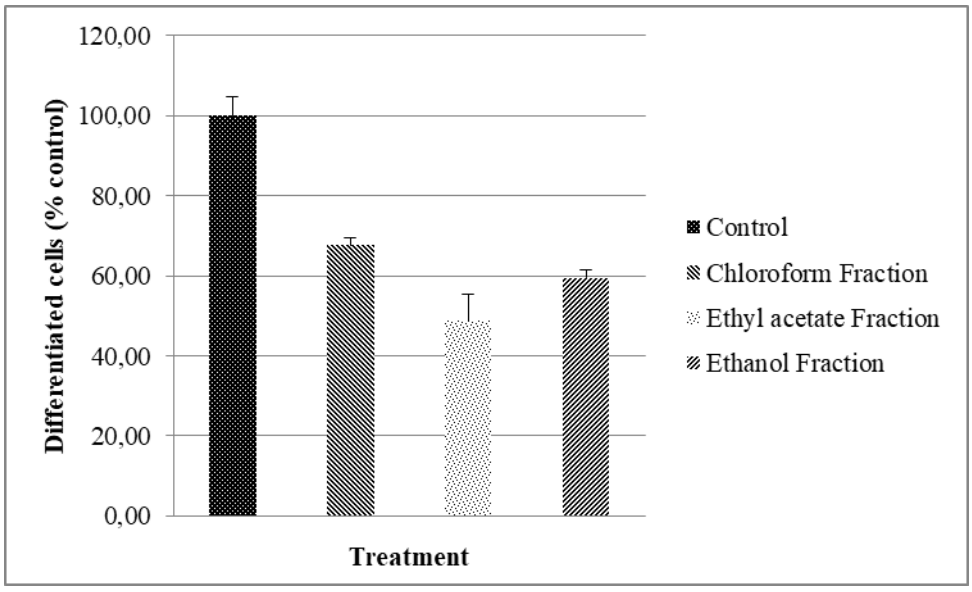


Figure 3: Anti-differentiation activity of *G. ulmifolia* leaf fractions

## 4 DISCUSSION

Fractionation of ethanol extract of *G. ulmifolia* leaf using chloroform and ethyl acetate showed the most yield was the residual 70% ethanol fractions (Table 1). These results indicated that most of the extracted content was polar compounds.

The leaf of *G. ulmifolia* contain flavonoids, i.e. catechins, kaempferol, procyanidin B-2, procyanidin B-5, procyanidin C-1 and tiliroside. Numerous studies reported that flavonoids inhibited adipogenesis (Park *et al.*, 2009; Bin and Choi, 2012; Hsu and Yen, 2007; Chien *et al.*, 2005)(5,6,15,16). The fractionation of ethanol crude extract resulted in fractions with different total flavonoid content. The sequence of the fractions based on total flavonoid content detected were ethyl acetate fraction > chloroform fraction > ethanol fraction (Table 2). This results indicated that flavonoids compound which was found in the leaf of *G. ulmifolia* prefer to be extracted with solvent possessing moderate polarity degrees (semi-polar) such as ethyl acetate and chloroform than solvents with strong polarity (ethanol) as seen in Table 2. Based on the principle of like dissolves like, it was thought that most flavonoids were in the form of aglycons (Sarker, Latif, and Gray, 2006).

Anti-proliferation activity of ethyl acetate fraction was higher than other fractions (Figure 1). This result was consistent with the total flavonoid content of the ethyl acetate fraction as mentioned above, that higher than other fractions. Other studies have shown that flavonoid of *G. ulmifolia* leaf (tiliroside) can inhibit the proliferation of T47D and MCF7 cancers cell lines (Da'i *et al.*, 2016).

Cells differentiation were indicated by preadipocytes morphological changes into mature adipocytes that were shown by the formation of fat droplets in the adipocytes. Fat droplets can be observed with Oil-Red-O staining. This staining has been widely used to exhibit the differentiation of preadipocytes to adipocytes because it is positively correlated with the amount of lipid stored in the cell. Hence it is used to indicate potential anti-obesity effects of natural products (Poudel *et al.*, 2015). Figure 2 showed the morphology of adipocytes after the treatment with chloroform, ethyl acetate, and residual ethanol fractions for 24 hours. The differentiated cells (D) were characterized by orange lipid droplets and otherwise, these droplets were not observed in undifferentiated cells (U). The ethyl acetate fraction also showed the highest anti-differentiation activity among the other fractions (Figure 3).

In the case of anti-proliferation activity of chloroform fraction and residual ethanol fraction, ethanol fraction showed higher activity than the chloroform fraction. Similarly, by anti-differentiation activity, ethanol fraction showed higher activity than chloroform fraction. This may be due to only certain flavonoids that can inhibit proliferation and differentiation of preadipocytes. For example, ganistein may inhibit the differentiation of preadipocytes of the 3T3-L1 cell

line, but naringenin had no effect on this process (Harmon and Harp, 2001).

## 5 CONCLUSION

Based on the result of above research can be concluded that the ethyl acetate fraction contains the highest total flavonoid contents among other fractions. The ethyl acetate fraction also showed the highest anti-proliferation and anti-differentiation of rat preadipocytes.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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