

In Vivo Inhibition of Lipid Accumulation in *Caenorhabditis elegans*

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Abstract. This is a preliminary research report on the use of *Caenorhabditis elegans* as a model to establish anti-obesity screening assay of the natural plant resources. Nematode *C. elegans* has been used as experimental animal model for understanding lipid accumulation. The objective of this research was to investigate the effect of selected plant extracts on lipid accumulation in *C. elegans*. Currently no report could be found regarding lipid accumulation in *C. elegans* treated with ethanolic leaf extracts of jabon merah (*Anthocephalus macrophyllus*), jati belanda (*Guazuma ulmifolia*), and Mindi (*Melia Azedarach*) plants. Lipid accumulation was determined qualitatively using lipid staining method and quantitatively by colorimetry using sulpho-phospho-vanillin reagent. Data showed that lipid accumulation was inhibited up to 72% by extract of *M. azedarach*, about 35% by both of *A. macrophyllus* and *G. ulmifolia* extracts, and up to 25% by orlistat (a synthetic slimming drug). Ethanolic extract of *A. macrophyllus*, *G. ulmifolia*, and *M. azedarach* leaves were shown to inhibit lipid accumulation in *C. elegans* and *M. azedarach* leaves extracts was the most effective inhibitor. *C. elegans* were shown to be an effective model for in vivo lipid accumulation mechanism and potential to be used as a rapid screening assay for bioactive compounds with lipid accumulation inhibitory activity.

1. Introduction

C. elegans is a nematode that has been used as experimental animal for studying lipid metabolism [1]. The lipid metabolism in this nematode is similar to that of mammals, but it lacks leptin-like molecules and their receptor, which regulates lipid storage through appetite control. Therefore, *C. elegans* model could provide information regarding lipid storage and mobilization without the influence of hormonal inhibition of food intake [2]. Lipid storage in *C. elegans* is regulated by *tub-1* gene. Mutation on this gene will result in excessive lipid accumulation as shown by a mutant strain RB1600 of *C. elegans*. Kim et al. [2] used this mutant strain for studying anti-obesity activity of taurine.

Both *Anthocephalus spp.* and *G. ulmifolia* have been used as slimming ingredients in traditional medicine of Indonesia [3,4]. Administration of *Guazuma ulmifolia* leaves ethanolic extract has been reported to reduce abdominal fat of Sprague Dawley rats as much as 27.06% [5]. No reports regarding the effect of *Anthocephalus macrophyllus* on lipid metabolism, only recent report by Acharyya et al. [6] that methanolic extract of the root of jabon putih (*Anthocephalus cadamba*) reduced low density lipoprotein (LDL) concentration in Wistar rats and increased the high density lipoprotein (HDL) concentrations. Similarly, methanolic leaf extract of *Melia azedarach* has been shown to possess antioxidant activity [7]. Very few reports were found on the lipid metabolism activity of *M. azedarach*.



According to Indian traditional medicine (Ayurveda), *M. azedarach* can stimulate the excretion of urine [8]. The water- and methanolic- extracts of *M. azedarach* was reported to reduce the body lipid mass of hyperlipidemic albino Wistar rats significantly [9].

2. Materials and Methods

Plant materials of *A. macrophyllus*, *G. ulmifolia*, and *M. Azedarach* were obtained from Tropical Biopharmaca Research Center (Trop BRC) Conservation and Cultivation Station at Cikabayan, IPB Dramaga Campus, Bogor. *C. elegans* strains of N2 (wild type) and RB1600 along with *Eschericia coli* strain OP50 were provided by the Caenorhabditis Genetics Center (CGC) at University of Minnesota, Minneapolis (USA). Orlistat (Xenecal®), a commercially available slimming agent, was purchased from a general drugstore in Bogor. Culture media and chemicals used for nematode cultivation, maintenance, and experiments were Luria Bertani (LB) broth (Himedia), agar (Himedia), S Medium (NaCl, pepton, CaCl₂ 1M, MgSO₄, and buffer KPO₄ 1M pH 6), M9 medium (KH₂PO₄, Na₂HPO₄, NaCl, MgSO₄ 1 M), cholesterol (Sigma), NaCl, pepton, CaCl₂, MgSO₄, bufer KPO₄ 1M pH 6, antifungal nistatin (Kimia Farma), and streptomycin (Kimia Farma). Reagents and other chemicals for sample extraction, nematode staining, and quantitative lipid determination were all purchased from Merck, Darmstad, Germany.

2.1. Preparation of Ethanolic Extracts

Dried leaves was prepared as reported by Sulistiyani et al. [10]. Samples were dried at 50°C and then the dried leaves were mashed into 40 mesh. Ethanolic leaf extracts of these plants were prepared by maceration of each dried leaves with ethanol 96% (1:10) at room temperature for 2x24hours. The extracts were filtered and concentrated with rotary vacuum evaporator at 50°C to produce semi-dried crude ethanolic extracts.

2.2. Experimental Design

The wild type (N2) and the mutant (RB1600) strains of *C. elegans* in this research were used in 6 experimental condition groups: 1) normal condition (in standard media contained 5 ppm cholesterol), 2) high lipid control (in media contained 10ppm cholesterol), 3) high lipid with addition of orlistat; high lipid treatment with addition of : 4) *A. macrophyllus*; 5) *G. ulmifolia*; and 6) *M. azedarach* ethanolic extracts. Each treatment groups of *C. elegans* were incubated with 3 doses of extracts: 25, 50, 100 ppm (orlistat and *M. azedarach*) and 250, 500, 1000 ppm for *A. macrophyllus* and *G. ulmifolia*. All assays were performed in triplicates. Liquid cultures of *C. elegans* were incubated at 25°C for 40 hours in each experimental medium until it reached life phase early L4. Additional 9.5 hour incubation were done to reach a complete L4 life phase before measurement of total lipid.

2.3. Culture of *C. elegans* and Extract Treatments

C. elegans were grown on Nematode Growth Medium (NGM) agar plates and cultivated according to Stiernagle [11] and were kept at 25°C. Nematodes were maintained on living *E. coli* strain OP50 as a food source which was seeded to the surface of NGM plates. The stock of *E. coli* was prepared by culturing it overnight in an LB medium until concentrated particles were formed. During each experiment, the nematodes were grown in liquid culture medium (S Medium) prepared as described [12 Lewis & Flemming 1995] that contained concentrated *E. coli* and were maintained at room temperature (25°C). The orlistat- and extract-containing experimental media were prepared by adding indicated concentration of orlistat or ethanolic plant extract into high lipid (10 ppm cholesterol)-containing S Medium. Liquid cultures of *C. elegans* were treated with aliquots of experimental media in microplates kept in incubator for 12-40 hours at 37°C.

2.4 Qualitative Analysis of Lipid Accumulation

Qualitative analysis of lipid accumulation in the nematodes was determined using lipid staining method of Kim *et al.* [2]. For the staining, the nematodes were removed from the experimental cultures with ose to be placed on the preparative slides and starved for 6 hours. After they were washed in M9 media, the nematodes were fixed with 1% paraformamide 1% in M9. Then the preparative slides were rised with 95% ethanol. The staining were performed in 70% ethanol saturated with Sudan black B. The effect of treatment was studied via microscopy on stained nematodes.

2.5 Total Lipid Quantitative Analysis

For quantitative analysis, total lipid of the *C.elegans* was extracted using the method of Bligh and Dyer [13]. Nematodes were washed off the experimental media by gentle centrifugation to pellet worms, and washed twice in 0.9% NaCl, After incubation with in 0.9% NaCl for 20 minutes at room temperature, nematodes were pelleted by centrifugation and resuspended in 0.5 mL H₂O. The worm suspension were mixed with 3 mL of methanol:kloroform (2:1) solution and vortexed for 5 minutes and then 1 mL of chloroform and 1.8 mL of H₂O were added. The mixture was vortexed again for 5 minutes until separated into 2 phases, the upper phase was the polar part and the bottom phase was the organic part that contained the cellular lipid (triglycerides, membrane phospholipid, cholesterol etc.). After the upper phase was discarded, the remaining lipid-containing fraction was dried until all the solvent was evaporated. Then mixture solution of methanol:chloroform (1:1) 0.2 mL was added to the sample and ready for total lipid determination.

Total lipid concentrations were determined by colorimetry using sulpho-phospho-vanillin reagent as described by Knight *et al.* [14] based on the reaction between lipid carbonium ion and active carbonyl group of the phospho-vanilin forming cherry-red color product. Pure olive oil was used as standard and was dissolved in chloroform at concentrations of: 2.5, 5, 10, 25, 50, and 100 ppm. Samples and standards (0.2 mL) each was mixed gently with 1 ml concentrated sulfuric acid to hydrolize the lipid ester. These mixtures were heated in waterbath at 100°C for 20 minutes under the acid/fume hood for a highly reactive lipid carbonium ion was formed in this reaction. Subsequently, reaction tubes were cooled down under running tap water for 5 minutes , and 2 mL of vanilin-fosphate was added prior to incubation at room temperature for 30 minutes. This reaction yielded cherry-red colored product which was stable within 60 minutes. The absorbance of standards and sample mixtures were measured at 525 nm using using a spectrophotometer.

3 Results and Discussions

3.1 Effect of High Lipid-Diet on Cellular Lipid of *C. elegans*

Experiments were conducted to examine the effect of medium with high cholesterol content on lipid accumulation in both the wild type N2 and RB 1600 mutant strains. The Rb1600 *C. elegans* strain has mutation in the tub-1 gene [2]. Functional loss of tub-1 in *C.elegans* leads to accumulation of lipids [15, 16]. Figure 1 shows that the lipid content of the wild type N2 strains (36 ppm) was slightly higher than that of the RB1600 strain (25 ppm) when incubated with medium contained standard amount of 5 ppm cholesterol. Incubation of the nematodes with higher concentration of cholesterol (10 ppm) in the medium increased the total cellular lipid in the wild type N2 as well as the RB1600 strains by 77% compared to those incubated with standard cholestesterol concentration. These quantitative data were supported by Sudan black staining , which showed no substantial difference appeared in the fat stores of both strains of nematodes incubated with a normal medium (data not shown). When cultured on a high lipid medium, however, the nematodes was seen to accumulate excess lipid as shown by the appearance of higher number of dark stained lipid droplets (Figure 2). Visually, the lipid droplets in the wild type N2 were more noticeable than the mutants (data not shown). In this study, the RB1600 mutants showed less response to high cholesterol regarding its cellular lipid accumulation. It is not clear whether this was due to experimental condition or other characteristic of this mutant. In *C. elegans*, the gene products of *tub-1* are thought to manage lipid accumulation in synergistic manner

with those of *kat-1*, which govern fatty acid beta-oxidation [17]. *kat-1* encodes a beta-oxidation enzyme, 3-ketoacyl-CoA and found to be expressed in intestine, body wall muscles, and in pharynx. The *tub-1* mutants demonstrate an obese phenotype especially under an impaired *kat-1* gene [2].

Incubation of the *C.elegans* with high cholesterol-content medium resulted in cholesterol accumulation in lipid droplets in the form of cholesteryl ester and other lipids. In *C. elegans*, cholesterol is converted to dafakronic acid which in turn will trigger translation of *daf-12* gene. The gene product is fatty acyl reductase-1 (FARD-1) which will catalyze the fatty acid catabolism[18]. Thus, incubation with cholesterol will not increase the accumulation of triglyceride. Triglyceride could be accumulated by increasing the amount of bacteria (i.e. *E. coli* OP50) thus increasing the bacterial fat [19]. *C. elegans* does not have pathway for cholesterol de novo synthesis from acetate [19] thus increase in total cellular lipid following incubation with cholesterol represents the efficiency in cholesterol absorption by the nematodes. In other words, any reduction in the amount of cellular lipid in this study may be attributed particularly to inhibition of cholesterol absorption.

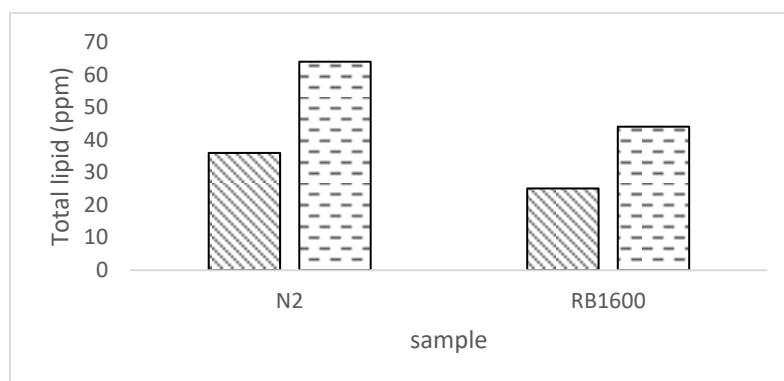


Figure 1. Lipid accumulation in wild-type N2 and mutant RB1600 *C.elegans* strains, each treated with medium contained normal cholesterol- (left bars) and high cholesterol- content (right bars) for 40 hours at 37°C.

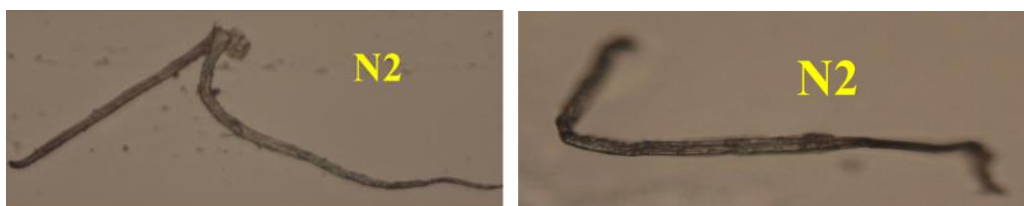


Figure 2. Sudan black staining of wild-type N2 *C.elegans* strains incubated in medium with normal cholesterol- (left) and high cholesterol-content (right) for 40 hours at 37°C.

3.2 Effect of Orlistat Treatment on Lipid Accumulation in Cholesterol-fed *C. elegans*.

Orlistat is a generic name for a commercial slimming agent [20]. Incubation of RB1600 mutant nematodes with orlistat at concentration of 25, 50, and 100 ppm cause reduction of total cellular lipid which shown as the appearance of dark spots of lipid droplets became less noticeable than the high lipid control (Figure 3 A). These observations are consistent with the quantitative data which show 33% to 70% reduction in total cellular lipid concentration compared to the cholesterol-fed control (Figure 3B). Similar effects of orlistat on the wild type N2 strains were observed, only in lesser degree. The data suggest that the mechanism of lipid stores in *C. elegans* of both strains was affected by orlistat. This is an important observation since orlistat often used as a positive control for body weight reducing (slimming) agent since its mechanism is to inhibit intestinal lipid absorption. Orlistat (tetrahydrolipstatin) is synthesized from lipase inhibitor derivative produced by *Streptomyces*

toxytricini [21]. Orlistat inhibits lipid absorption by inhibition of lipase, including carboxyl ester lipase (pancreatic cholesterol esterase) in mammals [22]. Whereas *C. elegans* also has cholesterol esterase [23]. Thus, it is thought that orlistat inhibit the lipid (cholesterol) accumulation by inhibition of lipase, particularly cholesterol esterase.

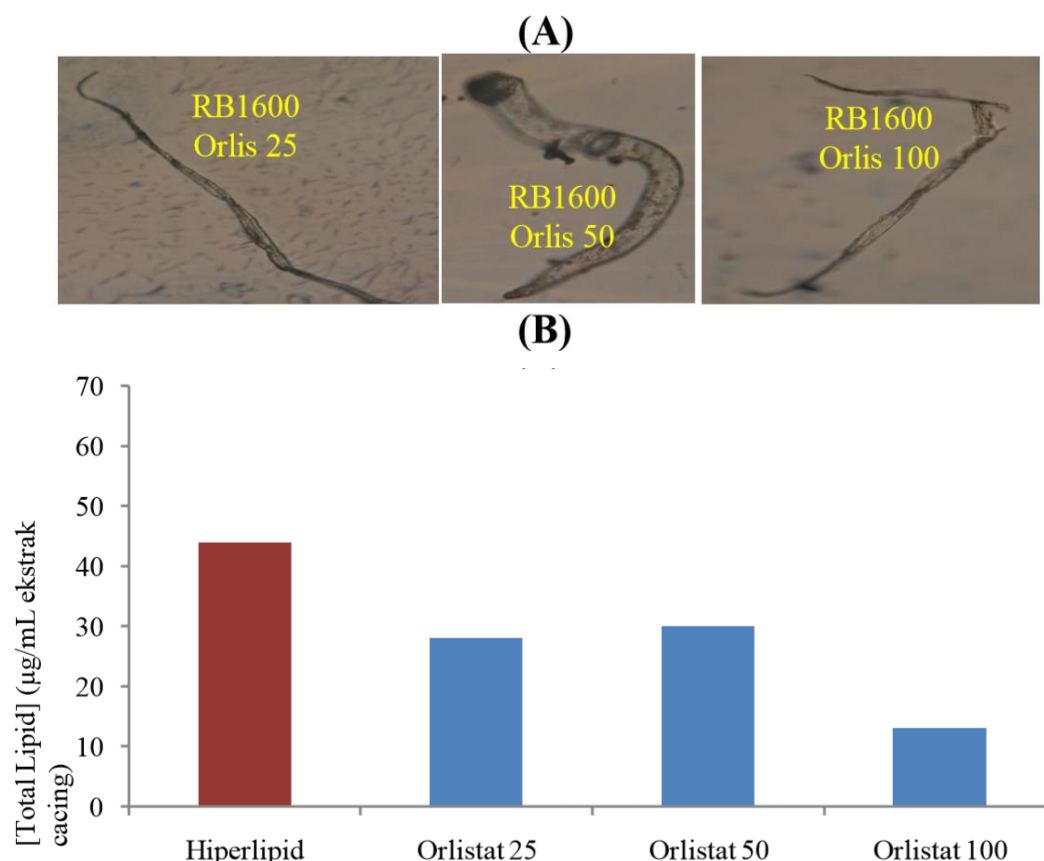


Figure 3. Lipid accumulation in *C.elegans* treated with orlistat: visually (A) and quantitatively (B)

3.3 Effect of Ethanolic Extracts of *A. macrophyllus* and *G. ulmifolia* on Lipid Accumulation in Cholesterol-fed *C. elegans*

Data showed that lipid accumulation was higher in cholesterol-fed (the high lipid) control N2 strain of *C.elegans* than those treated with *A.macrophyllus* or *G. ulmifolia* extracts at concentration of 250 ppm (Figure 4). Quantitative analysis showed that the lipid accumulation was inhibited about 35% by both *A. macrophyllus* and *G. ulmifolia* leaf extracts. This inhibition was higher than those of N2 treated by orlistat at concentration of 100 ppm which resulted only in 25% inhibition (data not shown). Sudan black staining indicated that the nematodes treated with *G.ulmifolia* showed no dark spots, while those treated with *A. macrophyllus* were seen to accumulate excess lipid as shown by the appearance of higher number of dark stained lipid droplets (Figure 4A). Visually, the lipid droplets in the high lipid control of wild type N2 were more noticeable than the others.

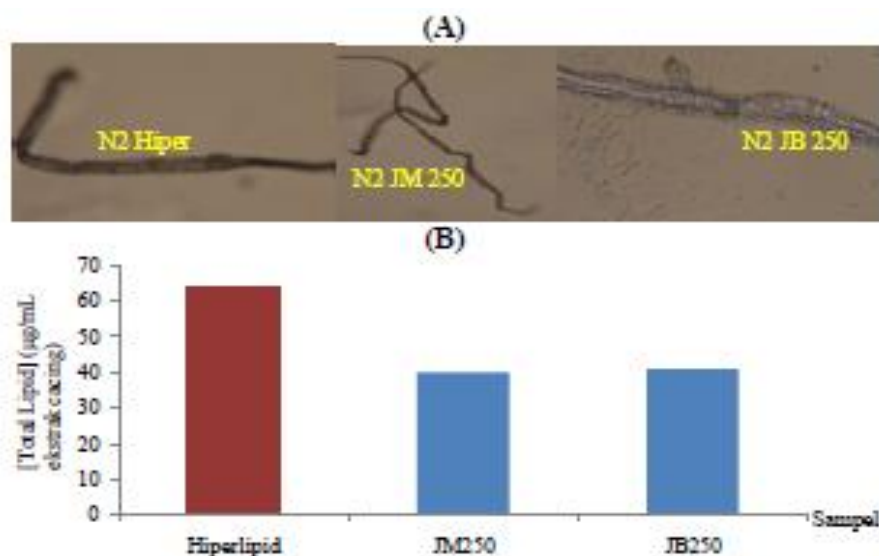


Figure 4. Lipid accumulation in wild type N2 of *C.elegans* treated with leaf extracts of *A.macrophyllus* (JM) and *G.ulmifolia* (JB) at concentration of 250 ppm each: visually (A) and quantitatively (B)

3.4 Effect of Ethanolic Extracts of *M. azedarah* on Lipid Accumulation in Cholesterol-fed *C. elegans*
Treatment with *M.azedarach* leaf extract resulted in much lower lipid accumulation, since this extracts were able to reduce lipid accumulation at concentrations of 25-50ppm as much as 72-78% (Figure 5). Sudan black staining also supported the reduction of lipid stores as showed by the lack of dark spots (data not shown).

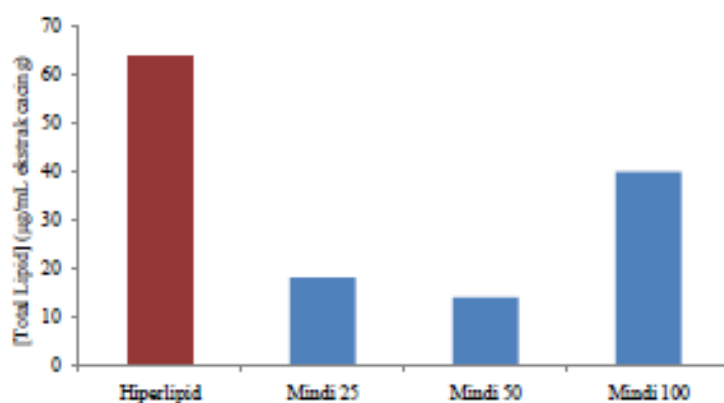


Figure 5. Lipid accumulation in *C.elegans* treated with *M.azedarach* leaf extract in various concentrations (ppm)

Taken together, these data showed that lipid (cholesterol) accumulation in *C. elegans* were inhibited by ethanolic extracts of *G. ulmifolia* and *A. macrophyllus* as much as 35% at concentration of 250 ppm, and by extracts of *M. azedarach* at much lower concentration (25 ppm) and stronger inhibition effect (72%). Recently, we reported the strong cytotoxicity effect of *M.azedarach* leaf extracts [24], which explained the small concentration it needed to exhibit its effect on the nematodes. The mechanisms by which these plant extracts exert their action may be mediated through their activity as lipase inhibitors. Our recent studies on ethanolic extracts of these plants indicated that all of them were potential lipase inhibitors as shown by their inhibitory activity against crude extract of fungal lipase [25]. Their inhibitory activity at concentration of 100

ppm each was 59.7%, 61.9%, and 33.8% for *A. macrophyllus*, *G. ulmifolia*, and *M. azedarach*, respectively. In conclusion, cultures of *C. elegans* can be used as potential rapid screening assay for natural compounds with lipid reduction activities.

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