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Lipid ameliorating effect of Eugenol in *Caenorhabditis elegans*

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Abstract

Eugenol, is a major volatile constituent of clove essential oil and active component of many medicinal plants. It has been reported as a potential drug to encompass life threatening indispositions including hyperglycemia, neural disorders and cancer. Due to oil characteristic of EUG, we intend to evaluate its effect on the lipid metabolism using the *Caenorhabditis elegans* animal model.

Worms (N_2, wild type) were exposed to different concentrations of eugenol (1, 10 and 50µM). Lipid accumulation was determined quantitatively by colorimetry using sulpho-phospho-vanillin reagent and qualitative analysis was carried by staining the nematodes with Sudan black B and Oil Red O. The graph was statistically analyzed using one-way ANOVA and t-test using Graphpad Prism 8.2.0.

Eugenol dose dependently reduced overall lipid accumulation in *C. elegans* (N_2, wild type). Eugenol (50µM) significantly reduced lipid accumulation by 55.03%. We further confirmed that lipid reduction up on treatment in *tub-1* mutant (RB1600) and *daf-16* mutant was less than that in wild type (N_2) *C. elegans*. Overall results indicate that lipid ameliorating effect of eugenol supplementation was independent of *tub-1* and *daf-16* in *C. elegans*.

The present work demonstrated eugenol as a potential compound which ameliorates lipid accumulation and exerts its effect independent of *tub-1* and *daf-16* in *C. elegans*. So, it can be assumed that eugenol can be beneficial to humans as well, albeit further research is necessary before declaring it for human consumption.

Obesity is a complex disorder that increases risk of diseases and health problems such as heart disease, diabetes, high blood pressure, osteoarthritis and certain forms of cancer[16]. According to WHO, in 2016, more than 1.9 billion adults were overweight of these over 650 million adults were obese[12]. Obesity arises when there is an energy imbalance between calories consumed and calories expended. However, there are also genetic, behavioral and hormonal factor that influences body weight[17]. One of the desirable approaches in the treatment of obesity is the use of traditional medicinal plants or natural organic compound rather than using drugs due to its limited effects[11]. Phenolic phytochemical compounds play a preventive role in maintenance of good health; provide protection from many diseases and it delays absorption of fat, suppression of enzymatic activities and increase lipolytic effects[12].

So, our study focused on Eugenol (1-hydroxy-2-methoxy-4-allyl benzene) (EUG), which is naturally occurring phenolic compound. It holds the title of "King of Antioxidants" [US Dept. Agriculture] and is used for treatment of heart disease, cancer, Alzheimer's disease, diabetes, reduce risk of gingivitis and purify the blood[3,14,15]. Previous studies showed that EUG exhibits lipid lowering potential in various animal models. In our study, for the first time we investigated the effect of EUG on fat accumulation using *Caenorhabditic elegans* as a model organism. *C. elegans* is a good experimental model to study lipid metabolism as it has a short lifespan, its genome can be easily maneuvered under laboratory conditions and it portrays similarity to humans, which include conserved fat regulatory pathway.

In *C. elegans*, lipids are primarily stored in intestinal and hypodermal cells as triacylglycerols (TAG) in the form of droplets while, in mammals lipid droplets are stored in adipocytes. Fat regulatory pathways are conserved in both *C. elegans* and humans. About, 305 genes were identified in *C. elegans* by RNA_i Screening that reduced fat deposits. TUBBY acts as a regulator of fat storage, functional loss of *tubby* called *tub-1* in *C. elegans* leads to accumulation of lipids. *tub-1* gene is responsible for maintaining lipid homeostasis in *C. elegans*[4]. Lipid metabolism in *C. elegans* is regulated by insulin signaling. Down-regulation of insulin signaling confers fat accumulation instead of its degradation[7]. Therefore; we investigated the effect and mechanisms of EUG treatment on the lipid accumulation by using *tub-1* and *daf-16* mutants.

The wild type N_2 strain and mutant strains *tub-1* (ok1972) and *daf-16* were obtained from *Caenorhabditis* Genetics Center for Research Resources (NCRR) and these worms were maintained on nematode growth medium (NGM) agar plates seeded with *E. coli* strain OP-50 as food source at 20°C[20]. EUG and other reagents and chemicals for sample preparation, sample staining and quantitative lipid determination were all purchased from Sigma-Aldrich. EUG, concentrations of 1µM, 10µM and 50µM in methanol were made. The EUG of indicated concentrations was added on OP-50 seeded NGM plates. Eggs from gravid L_4 synchronized worms were then transferred in equal amount to each plate and incubated for 48 hrs at 20°C.

Experiment was conducted on three experimental conditions. Worms (N_2) were grown on normal cholesterol concentration (10ppm), higher cholesterol dose (20ppm) and without cholesterol (control). Different concentrations of EUG were added to cholesterol fed worms. All assays were performed in triplicates

Quantitative analysis of lipid accumulation in *C. elegans* was determined by using Bligh and Dyer method[6]. For, total lipid analysis the nematodes were washed off in PBS (Phosphate Buffer Saline) by centrifugation and collected. The worms suspension were mixed with solution of chloroform: methanol (2:1) and homogenized until the mixture is separated into 2 phases, the bottom part contained the cellular lipid while, upper part contained polar part. Supernatant was removed and the lower portion was incubated with 0.9% NaCl for 20mins at room temperature. After incubation mixture was vortexed, centrifuged and the whitish layer was siphon off. Methanol: chloroform (0.2ml) + H_2SO_4 (1ml) were added and heated in water bath at 100^{o}C for 20mins. Reaction tubes were cooled down and 2ml of phospho-vanillin was added and incubated for 30mins at room temperature. The absorbance of standard and test samples was measured at 525nm using spectrophotometer.

Qualitative lipid accumulation analysis was carried by staining the nematodes with Sudan black B and Oil Red O. Sudan black B staining was done by Kim *et al.*[9] method using freeze-thaw technique[9]. Nematodes to be stained were starved by removing from experimental cultures and placing them on the preparative slides. Worms were washed in 1X PBS (Phosphate buffered saline) 2-3 times by centrifugation. The pellet along with 1ml PBS was incubated on ice for about 10mins to stop pharyngeal pumping. 50µl of freshly prepared 10% PFA (Para formaldehyde) was added. Alternate freezing and thawing were done for three times (freezing in ethanol bath and simultaneously thawing in warm water). Worms were then washed in cold 1X PBS (3-4 times). It was then dehydrated in increasing concentration of ethanol 25%, 50% and then 70% for 2mins each. Ethanol was aspirated leaving 300µl in the tube to which 250µl of saturated Sudan black B (in 70% ethanol) was added and left overnight in shaker. Saturated solution of Sudan black B was prepared by adding 16mg/ml in 70% ethanol and then filtering with 0.2µm filter syringe to remove un dissolved dye. The worms were then centrifuged to remove the dye leaving 100µl pellet of worms. It was washed once in 70% ethanol to remove excess dye and then suspended in PBS. Finally, stained worms were mounted on smear made of 2% agarose and images were taken in bright field microscope (Nikon ECLIPSE, E100, VproImage software).

Oil Red O staining was done as previously described by Wang *et al.,* Soukas *et al.*, methods[22,19]. The treated worms to be stained were age synchronized and one day adult L_4 worms were taken. The worms were washed twice in chilled 1X PBS and then suspended in PBS. Equal volume of 20% PFA was added. The mixture was then passed through three series of freeze thaw in ethanol bath alternating with warm water. It was then centrifuged and washed in PBS to remove PFA followed by dehydration in 60%

isopropanol for 15mins. 60% Oil Red O in deionized water (prepared from stock of 0.5% Oil Red O in 100% isopropanol, equilibrated for one day) was used to stain the worms. The working solution of the dye was filtered through 0.2µm syringe filter. Isopropanol from the worms was replaced with equal volume of Oil Red O solution and allowed to stain overnight in shaker. The unbound dye was removed through centrifugation and washed with 0.01% Triton in 1X PBS. The stained worms were mounted onto a smear of 2% agarose and imaged in bright field microscopy (Nikon ECLIPSE, E100, VproImage software) as well as through 510nm fluorescence emission filter.

Statistical analysis:

The graph was statistically analyzed using one-way ANOVA and t-test using Graphpad Prism 8.2.0.

Effect of EUG on lipid accumulation in wild type (N_2) C. elegans fed with high cholesterol diet :

Effect of EUG on fat accumulation was determined both quantitatively and qualitatively under Sudan black and Oil red O dye. We also explored the possible pathways by which EUG might be exhibiting its effects on key genes in lipid metabolism by using mutants such as *tub-1* and *daf-16*. Quantitative analysis of lipid accumulation in *C. elegans* was determined by using Bligh and Dyer method[6]. Total lipid content when supplemented with high cholesterol diet (20ppm) was 58.6% (**$p<0.01$, ANOVA, Figure 1A). While, significant reduction in lipid content of 55.03% (**$p<0.01$, ANOVA, Figure 1B) was observed upon treatment (50µM) as compared to that of untreated control worms fed with high cholesterol diet (20ppm). The most effective dose that significantly reduced lipid content was 50µM. EUG significantly lowered total lipid content in dose dependant manner and result was statistically significant as compared to the control nematodes. Total lipid content was calculated from the equation obtained by calibrating olive oil as a standard. The straight line equation thus obtained was $y=0.363x–0.175$ ($R^2=0.999$).

Effect of EUG on key regulatory genes in lipid metabolism using tub-1 (RB 1600) and daf-16 (mu-86) mutant strains :

We also explored the possible pathways by which EUG might be exhibiting its effects on key genes in lipid metabolism using available mutants such as *tub-1* and *daf-16*. TUBBY protein encoded by *tub-1* gene acts as a regulator of fat storage, functional loss of *tubby* called *tub-1* in *C. elegans* leads to accumulation of lipids. *tub-1* gene is responsible for maintaining lipid homeostasis in *C. elegans*. EUG (50µM) significantly reduced lipid accumulation by 50.12% in *tub-1* mutant (RB1600) which demonstrates that reduction in fat content is *tub-1* independent (Figure 2).

Lipid metabolism in *C. elegans* is regulated by insulin/insulin like growth factor (IIS/IGF) signaling and DAF-16 is responsible for fat accumulation and mutation in it results in fat degradation. In addition, we further studied whether *daf-16* is involved in reduction of fat accumulation in *C. elegans* or not. Total

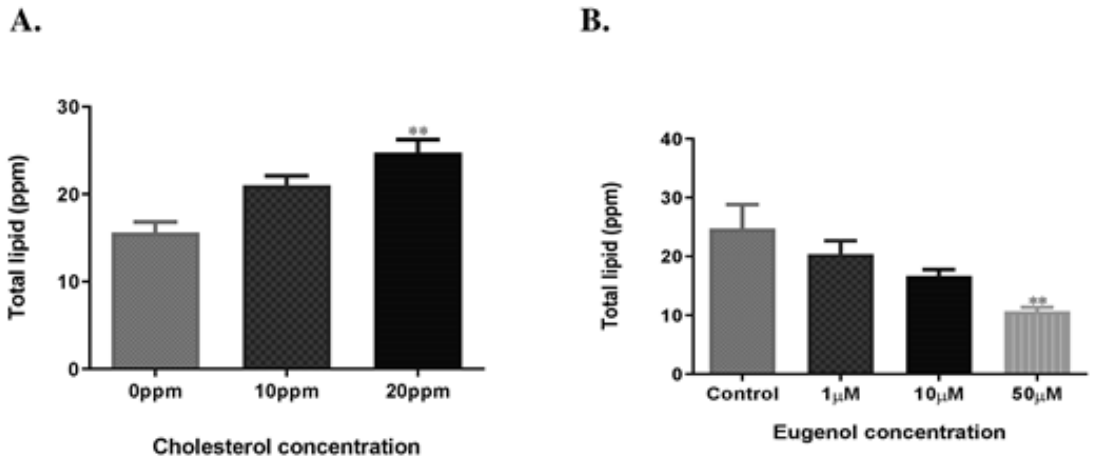


Figure 1: Effect of EUG on lipid accumulation in *C. elegans* (N_2)
(A) Effect of Cholesterol concentration on total lipid content in *C. elegans*
(B) Effect of EUG treatment on lipid accumulation when fed with high cholesterol diet.
The graph was statistically analyzed using one-way ANOVA in GraphPad Prism 8.2.0
(**signifies $p<0.01$ value). Error bar represents S.E.M (Standard error of mean).

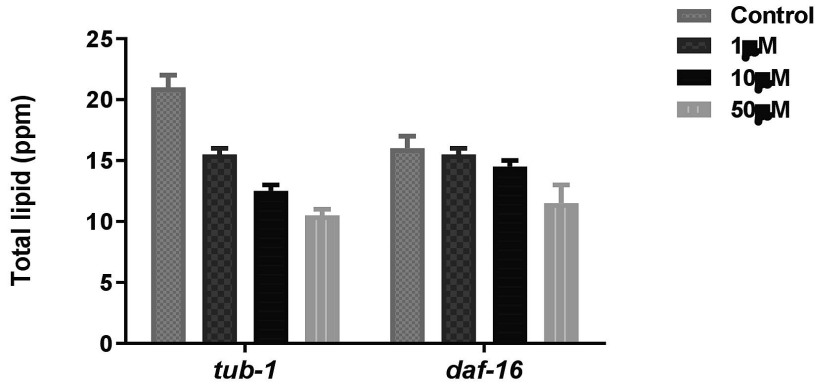


Figure 2: Effect of EUG on key regulatory genes in lipid metabolism using *tub-1* (RB 1600) and *daf-16 (mu-86)* mutant strains of *C. elegans.*

lipid content in *daf-16* mutant was almost equal to wild type. EUG decreased total lipid content in *daf-16* mutant by 28.12% only and the % of lipid reduction in *daf-16* mutant was less than that in wild type (N_2) *C. elegans*. Overall results indicate that lipid ameliorating effect of EUG supplementation was independent of *tub-1* and *daf-16* in *C. elegans*.

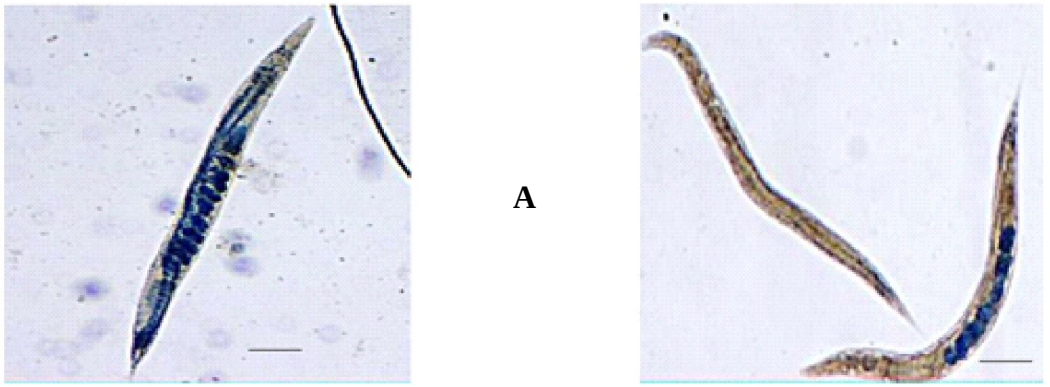
Effect of EUG treatment on Fat accumulation observed via Sudan black and Oil Red O staining in wild type worms:

Qualitative lipid accumulation analysis

was carried out by staining the nematodes with Sudan black B and Oil Red O stains. Results showed that worms (N_2) fed with high cholesterol diet accumulate excess triglycerides as lipid droplets in the hypodermal and intestinal tissue. Nematodes fed with high cholesterol appeared darkly stained as compared to nematodes fed with low cholesterol diet. EUG treated worms showed notable differences in lipid droplets reduction when stained with Sudan black B and Oil Red O staining among the worms (N_2) fed with high cholesterol diet (Figure 3A,3B,3C,3D). No difference was observed when fed with normal diet (data not shown). In addition, fluorescence images of Oil red O stained worms under fluorescence microscopy (Nikon, ECLIPSE T_i) also showed significant reduction in lipid droplets up on treatment (Figure 3E). In our study, we observed that higher concentration (50μM) reduced total lipid content in wild type *C. elegans* significantly but its effect was minimal for *tub-1* mutant strain. Quantification, staining and fluorescence imaging of total lipid content of wild and mutant strains showed that amelioration of lipid accumulation on EUG treatment is independent of *tub-1* and *daf-16* in *C. elegans*.

Phenolic phytochemical compounds play a preventive role in maintenance of good health; provide protection from many diseases and in lowering lipid content in various organisms. Lipids, is a major source of energy in an organism and co-ordination and regulation of lipid metabolism is a complex process and imbalance or abundance of nutrients leads to obesity. In *C. elegans,* lipids are primarily stored in intestinal and hypodermal cells as triacylglycerols (TAG) in the form of droplets while, in mammals lipid droplets are stored in adipocytes[19]. EUG is one such naturally occurring phenolic compound that has been reported to exhibit strong antioxidant potential and lowered total lipid content in various animal models. Due to oil characteristic of EUG, we intend to evaluate its effect on the lipid metabolism in *C. elegans*.

In our study, we investigated anti-obesity potential of EUG in *C. elegans* both quantitatively and qualitatively. EUG treatment significantly reduces fat accumulation in dose dependent manner and was statistically significant as compared to the control nematodes. Consistent with this result Triglycerides content also showed significant reduction in fat deposit in EUG treated worms as compared to untreated worms. Our results are consistent with previous findings that EUG treatment markedly reduced lipid contents in mice hepatocytes and EUG in combination with aspirin lowers lipids in rats[21]. In one of the other study, it has been reported that polyphenol like epigalocatechine-3-gallate and polyphenols present in grape, apple, olive oil and pomegranate juice have been reported to lowered lipid contents in various animal models[8,5]. Further, we evaluated EUG mediated lipid lowering effect in *C. elegans* by using Sudan black B and Oil red O staining techniques. Both are lipophillic dye that stains triglycerides of fixed worms[10]. Oil red O is fluorescent dye used for visualizing fat deposits. EUG treated worms showed notable differences in reduction in lipid droplets among the worms (N_2) fed with high cholesterol diet while, its effect was minimal for *tub-1* mutant strain. These consistent results strongly indicate that the EUG has more immediate effect on lipid accumulation rather than its synthesis.



staining in wild type worms at magnification 4X.

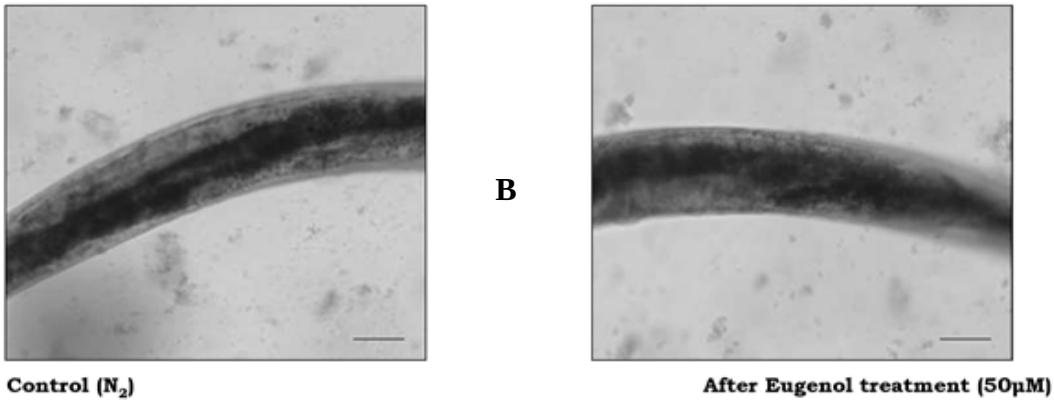


Figure 3: Effect of EUG treatment on Fat accumulation observed *via* (B) Sudan black staining in wild type worms at magnification 20X.

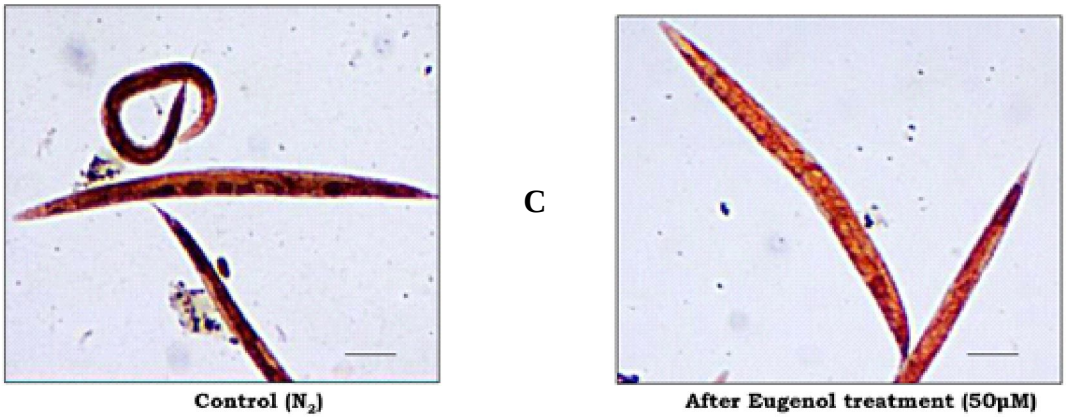


Figure 3: Effect of EUG treatment on Fat accumulation observed *via* (C) Oil Red O staining in wild type worms at magnification 4X.

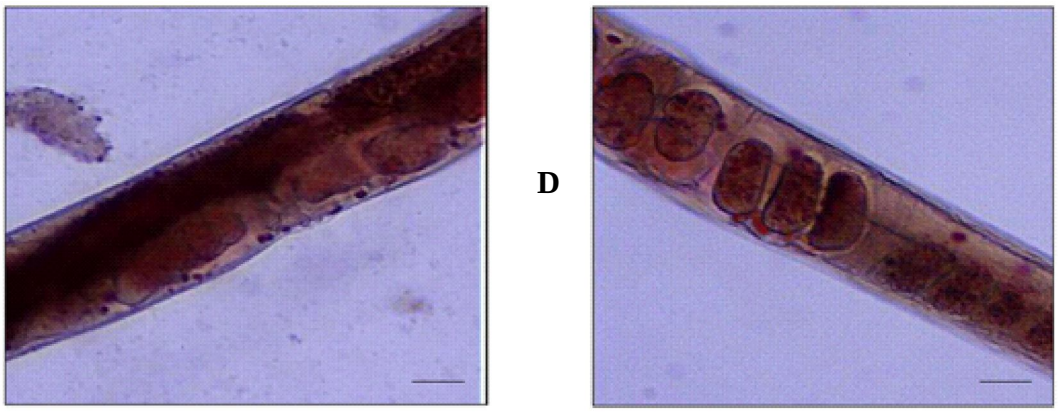


Figure 3: Effect of EUG treatment on Fat accumulation observed *via* (D) Oil Red O staining in wild type worms at magnification 20X.

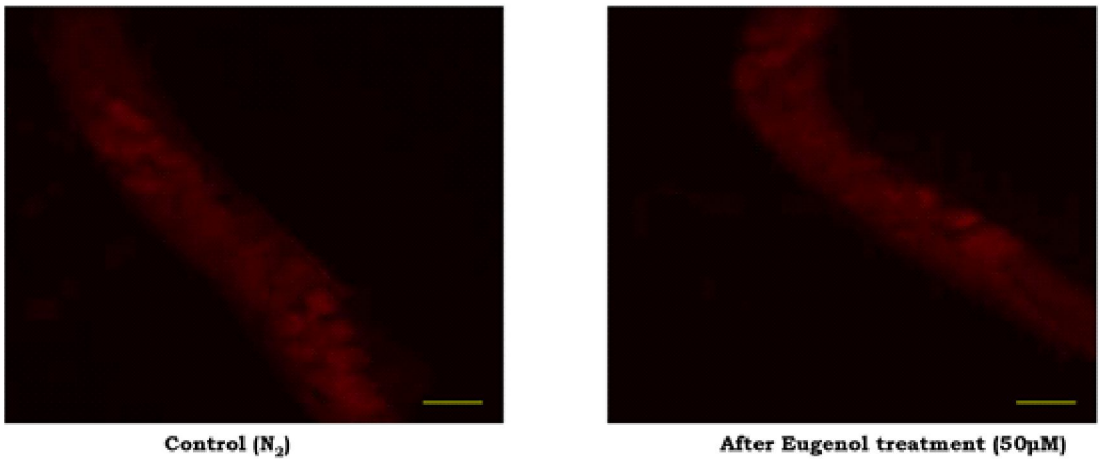


Figure 3: Effect of EUG treatment on Fat accumulation observed *via* (E) Florescence images of Oil Red O stained fixed worms. Scale bar represents 100µm.

To ascertain the pathway involved in lipid metabolism, we used mutant strains of *tub-1* and *daf-16* compared it with the results of wild strain N_2. The important genes involved in lipid metabolism are *tub-1*, *daf-16*, *aak-2*, *spb-1* or *nhr-49*[13]. Mutations in any of these genes lead to the accumulation of lipid. TUBBY acts as a regulator of fat storage, functional loss of *tubby* called *tub-1* in *C. elegans* leads to accumulation of lipids. *tub-1* gene is responsible for maintaining lipid homeostasis in *C. elegans*[18]. Treatment slightly reduced lipid accumulation in *tub-1* strains which indicate EUG anti-obesity potential is independent of pathway were *tub-1* is involved. Lipid metabolism in *C. elegans*

is regulated by insulin signaling and down regulation of it conferred an increased fat accumulation instead of its degradation[7]. Nutrient availability activates insulin signaling that allows inhibition of transcription factor DAF-16, this promotes growth and development, reproduction and fat metabolism. *tub-1* is independent of DAF-16 for maintaining lipid homeostasis in *C. elegans*[23].

We further testify the result by using *daf-16* mutant. Treatment slightly reduced lipid accumulation in *daf-16* mutant and the % of lipid reduction in *daf-16* mutant was less as compared to wild type (N_2) *C. elegans* which suggested that reduction in overall lipid content up on treatment is independent of *daf-16*. Overall results indicate lipid ameliorating effect of EUG treatment is independent of *tub-1* and *daf-16* in *C. elegans*. Our finding conferred that EUG mediated fat reduction is independent of insulin pathway, so instead it might modulate through other pathways.

To our knowledge, this is the first study reporting the lipid lowering effect of EUG in *C. elegans*. It exerts its effect independent of *tub-1* and *daf-16* which may curtail obesity. So, it can be assumed that EUG can be beneficial to humans as well, albeit further research is necessary before declaring it for human consumption.

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Ethical approval: Not required

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