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CYP Gene Expression and *In Vivo* Biological Effects of Khat Ethanol Extract, Cathinone and Cathine in *Caenorhabditis elegans*

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Abstract: The significant influence of khat, cathinone, and cathine on appetite suppression, reproduction impairment, and inhibitory effects on CYPs enzyme activity is widely recognised, but much remains unknown about its mode of action. This study demonstrated that exposure to high doses of KEE, cathinone, cathine and sibutramine at 12.5, 1.8, 1.8 and 1.8 mg/ml showed the most visible reduction in pharyngeal pumping rate, respectively. *C. elegans* showed an anomalous dynamic of reproduction rate upon exposure to these compounds but overall, showed a reduced amount of progeny. KEE, cathinone, and cathine extended the lifespan of *C. elegans* as compared to sibutramine and control with survival rate increments of 59.5%, 43.3%, and 15%, respectively. Quantitative RT-PCR results were insignificant, but *cyp-14A3*, *cyp-34A9*, and *cyp-35A2* across all treatments were upregulated, ranging from >1 to 3-fold as compared to control. *cyp* genes expression could be upregulated to enhance the metabolism of khat extract, cathinone, cathine, and sibutramine in *C. elegans*.

Keywords: *Caenorhabditis elegans*, Cytochrome P450, Khat, *cyps* gene, *cyp-35A2*

1. Introduction

Khat (*Catha edulis* Forsk), a natural amphetamine plant natively found in the Arabian Peninsula and the Horn of Africa, is well-known for its psychoactive stimulants cathinone and cathine [1]. Human and animal studies indicate that khat chewing brought about appetite

suppression [2] and anorexigenic effects, although the mechanism of this effect remains unmapped. Both cathinone and cathine share high similarity in terms of chemical structures to amphetamines [3]. Cathinone and cathine were claimed to exert anorexigenic effects by adopting amphetamine-like mechanisms leading to enhanced production of norepinephrinergic (NE) and dopaminergic (DA) in the human brain [4–6]. High levels of DA and NE stimulate the dopamine receptors, resulting in decreased food intake and induced weight loss [5]. Effects of khat on reproduction and fertility showed controversial outcomes as studies demonstrated that khat caused a reduction in libido [7] and causes sexual impotence following long-term use and impairs foetal growth [8], while others proved otherwise, where khat intake showed a significant increase in sperm count and motility in mice as well as their adult offspring [9]. Khat's effect on reproduction may depend on its concentration, as a high concentration of khat extract impairs reproductive function but a low concentration of khat enhances testosterone production in male mice [10]. Studies on the effects of khat, cathinone, and cathine on lifespan are limited. However, plant extracts were found to extend lifespan, improve healthspan, and enhance resistance to stress in nematodes [11]. Substances such as terpenoids [12] and polyphenols (flavonoids), which were also found in khat, contain anti-aging and lifespan-extending properties [13].

Caenorhabditis elegans (*C. elegans*), a small, transparent, and simple multicellular organism, has gained attention as a powerful *in vivo* model in bioactivity and toxicological research. It is highly evident that human disease genes and signalling pathways are conserved in *C. elegans* [14]. The first discovery of miRNA was in a simple organism nematode *C. elegans* [15]. Approximately 40 – 80% of human genes have orthologous genes in *C. elegans* genome and 40 – 50% of human disease genes have orthologs in the worm genome [16]. Availability of complete genome sequences, molecular biology tools including gene knockout and RNAi knockdowns, and zero ethical concerns have catapulted many breakthrough discoveries using *C. elegans* [17]. The *C. elegans* has 83 *cyp* genes, of which 77 are protein coding, and 5 are pseudogenes [18–20]. In contrast, an earlier study reported that the nematode has 86 cyp genes with 84 documented mRNAs and 9 documented phenotypes [21]. CYPs in *C. elegans* held crucial responsibilities in processes including xenobiotic metabolism, fatty acid regulation, dauer formation, and stress responses [22]. The *C. elegans cyp35* family is most homologous to the human CYP2 family [23] and *cyp-35A2* is an ortholog to human CYP2C9 [24,25]. The *C. elegans cyp-14A3* is an ortholog to human CYP1A2 [24,26] while *cyp-34A9* is orthologous to human CYP2C8/9/19 [27,28]. The WormBase, on the other hand, stated that *cyp-14A3* is an ortholog to human CYP2D6, *cyp-34A9,* and *cyp-35A2* are orthologues to human CYP2A6. The human CYP2 family was not only involved in drug metabolism but also in the development of several diseases [29]. However, *C. elegans* showed

functional deficiency in CYP1A-like metabolism, which is consistent with a lack of CYP1-like enzymes encoded in the genome [28].

Our previous *in vitro* studies demonstrated that khat extract, and its active compounds, cathinone and cathine, inhibited a series of major human liver CYPs enzymes, including CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4 and CYP3A5 [3,30–32]. Our *in silico* studies also warranted our *in vitro* findings, predicted and visualised how cathinone interacted in the active sites and inhibited CYP1A2, CYP2A6, and CYP3A5 [3], as well as cathine with CYP2A6 and CYP3A4 [32]. *In vivo* studies using mice showed that cathinone treatment blocked weight gain, while high-dose khat extract significantly reduced weight gain of mice on an obesogenic diet [33]. Studies on the effects of khat, cathinone, and cathine using *C. elegans* on appetite, reproduction, and lifespan are lacking. This opens new avenues to answer whether khat, cathinone, and/or cathine stirred an upregulation or downregulation of *C. elegans cyp* genes to enhance drug metabolism or elimination of these compounds out of their body, which can apply to how these mechanisms function in humans. Findings on CYP-herb interactions would be valuable to avoid concurrent use of herbs and drugs, thus minimizing the occurrence of herb-drug and/or drug-drug interactions [34].

Our objectives were to evaluate khat and its active compounds, cathinone, and cathine, on *C. elegans* biological activities, including pharyngeal pumping (feeding rate), reproduction (egg-laying) rate and lifespan, and their effects on *C. elegans cyp* gene expression: *cyp-14A3*, *cyp-34A9* and *cyp-35A2*. These effects could be comparable, conserved in humans and *C. elegans*, and potentially extend to uncover pathways to *cyp*-related diseases. In the sight of the pharmacological profile of khat, cathinone, and cathine, alongside our interest in the discovery of their effects on *C. elegans cyp* genes and biological process, we demonstrated the appetite-suppressing properties, reproduction reduction and lifespan extension of khat and its active compounds using *C. elegans* feeding rate, reproduction, lifespan assays, and RT-PCR work.

2. Materials and Methods

2.1. C. elegans culturing

All the experiments were performed using N2 wild-type *C. elegans* strain obtained from *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN, USA, https://cgc.umn.edu/) and maintained under standard conditions [35]. Worms or nematodes were grown at 16°C on Nematode Growth Medium (NGM) agar plates seeded with *E. coli*

OP50 as the source of food in an incubator (BINDER GmbH, Tuttlingen, Germany). Video of *C. elegans* feeding on *E. coli* OP50 lawn treated with khat ethanol extract (KEE), cathinone, cathine, sibutramine (positive control), and water (negative control) under Leica DM750 microscope with a built-in camera connected to the computer using the program Leica LAS EZ 3.4, are as shown in Supplementary material (A).

2.2. Turning off pos-1 by RNAi

Feeding rate and lifespan assays were carried out using N2 *wild-type* worms. To prevent them from laying eggs which could interfere with worms scoring, the worms were made sterile by knocking down the *pos-1* gene. *Pos-1* stands for "posterior segregation" which encodes a protein that regulates embryonic development [36]. *Pos-1* RNAi treated worms produced unhatched eggs. Mutant worms produce eggs that are arrested in embryonic development [37]. 1M IPTG (Invitrogen, MA, USA) and 100 mg/ml carbenicillin disodium (TargetMol, MA, USA) were added to the NGM preparation after autoclaving step [38]. The eggs of wild-type N2 worms were plated onto *pos-1* RNAi plates and grown for approximately 45 hours at 25°C until they reached the young adult stage.

2.3. Egg Prep

Age-synchronised worms were prepared by egg prep method. 2 ml of Clorox® Bleach (CA, USA), 0.5 ml 10N NaOH (Nacalai Tesque, Japan), and 2.5 ml distilled water were prepared to make 5 ml of lysis buffer. The 5 ml of distilled water was poured and resuspended using a pipette to wash all the worms on a single 6 cm plate of worms and subsequently, pipetted into 5 ml of lysis buffer and vortexed for 15 mins to break the worms. The washed worms were centrifuged (Eppendorf, USA) at 1,800 rpm, for 1 min at 25 °C. The supernatant was discarded, followed by the addition of 5 ml of distilled water before sending the washed worms' mixture to another round of centrifuge. The wash step is repeated once more. *C. elegans* eggs were sedimented and appeared slightly translucent to the naked eye. The eggs were plated onto *pos-1* RNAi plates, grown for ~45 hours in an incubator (Binder, Tuttlingen, Germany) at 16°C until they reached the young adult stage (as described above) and were ready to be used for the experiment.

2.4. Feeding rate assay

During optimisation, *C. elegans* were treated with 25, 12.5, and 5 mg/mL of KEE, and 5, 0.5, and 0.05 mg/ml of cathinone, cathine, and sibutramine. *C. elegans* treated with the highest concentration of 25 mg/ml KEE showed quiescence after a few minutes of

feeding, as observed under the microscope. Some of the worms curled up and remained in satiety quiescence until the end of the feeding rate assay and there was no visible pharyngeal pumping observed. The rationale behind the selected treatment concentration in this study largely depends on the aforementioned optimisation outcome. *C. elegans* treated with the 2nd highest KEE concentration at 12.5 mg/ml showed pharyngeal pumping. Hence, we selected three KEE dosages at 12.5, 6.25, 3.125 mg/mL, in 2-times dilution. The concentrations selected for cathinone, cathine, and sibutramine were 1.8, 0.9, and 0.18 mg/ml as the concentrations tested during optimisation at 5, 0.5, and 0.05 mg/mL, were too low and the pharyngeal pumping rate does not seem to be reduced as hypothesised that these compounds should exert appetite suppressing effects.

The heat-killed *E. coli* (80°C for 30 mins) [39] were added with treatment at different concentrations as follows: a) KEE (12.5, 6.25, 3.125 mg/ml), b) cathinone (1.8, 0.9, 0.18 mg/ml), c) cathine (1.8, 0.9, 0.18 mg/ml), d) sibutramine (1.8, 0.9, 0.18 mg/ml) and e) control – autoclaved ultrapure water. 5 age-synchronised N2 adult worms were individually picked for each treated assay plate. 3 plates (3 technical replicates) each for KEE, cathinone, cathine, sibutramine, and control were prepared. Pharyngeal pumping assay in the presence of KEE, cathinone, cathine, and sibutramine, and control plates was scored at indicated times after transferring worms to these treated plates at 4, 24, 48, and 72 hours (Figure 1). All assay plates with worms were kept in the incubator (Binder, Tuttlingen, Germany) at 25°C. Pharyngeal pumping was quantified visually by counting the number of grinder movements in every 5 seconds observed under a Leica DM750 microscope that comes with a built-in camera connected to the computer using the program Leica LAS EZ 3.4 to view and capture the pharyngeal pumping. This assay was repeated three times. The feeding or pharyngeal pumping rate was represented by the mean of three independent experiments and compared by 2-way ANOVA followed by Tukey's multiple comparisons method, using GraphPad Prism Version 9 (GraphPad Software, La Jolla, California, USA).

2.5. Reproduction rate assay

5 adult N2 worms were individually picked from the egg prep plates transferred and placed onto the NGM plate treated [40] with KEE (12.5, 6.25, 3.125 mg/ml), cathinone (1.8, 0.9, 0.18 mg/ml), cathine (1.8, 0.9, 0.18 mg/ml), sibutramine (1.8, 0.9, 0.18 mg/ml) and control each. The adult worms were allowed to lay eggs for a day before transferring them into similar treated NGM plates. The previously treated NGM plates with eggs will be kept at 25°C in the incubator, allowed to hatch, grown into L3 worms, and counted as the number of progenies (Figure 2). The reproduction assay was carried out in three biological replicates using three different batches of N2 worms. The egg laying or reproduction rate was

represented by means of three independent experiments and compared by 2-way ANOVA followed by Holm-Sidak's multiple comparisons method, using GraphPad Prism Version 9 (GraphPad Software, La Jolla, California, USA).

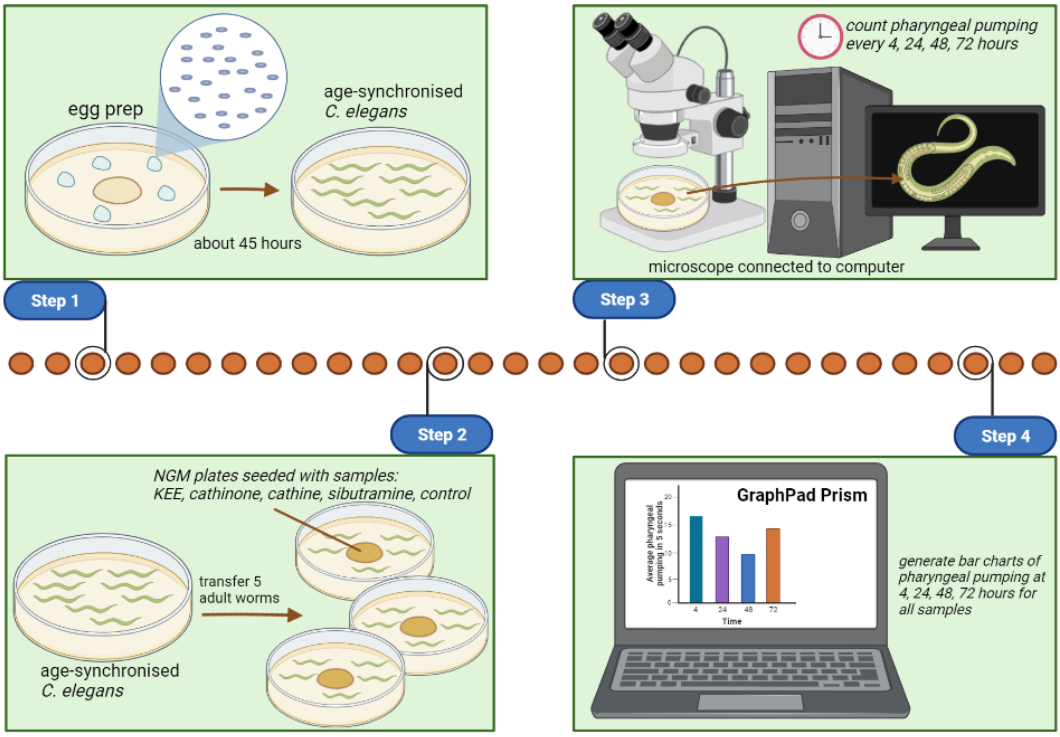

Figure 1. Overview of pharyngeal pumping assay.

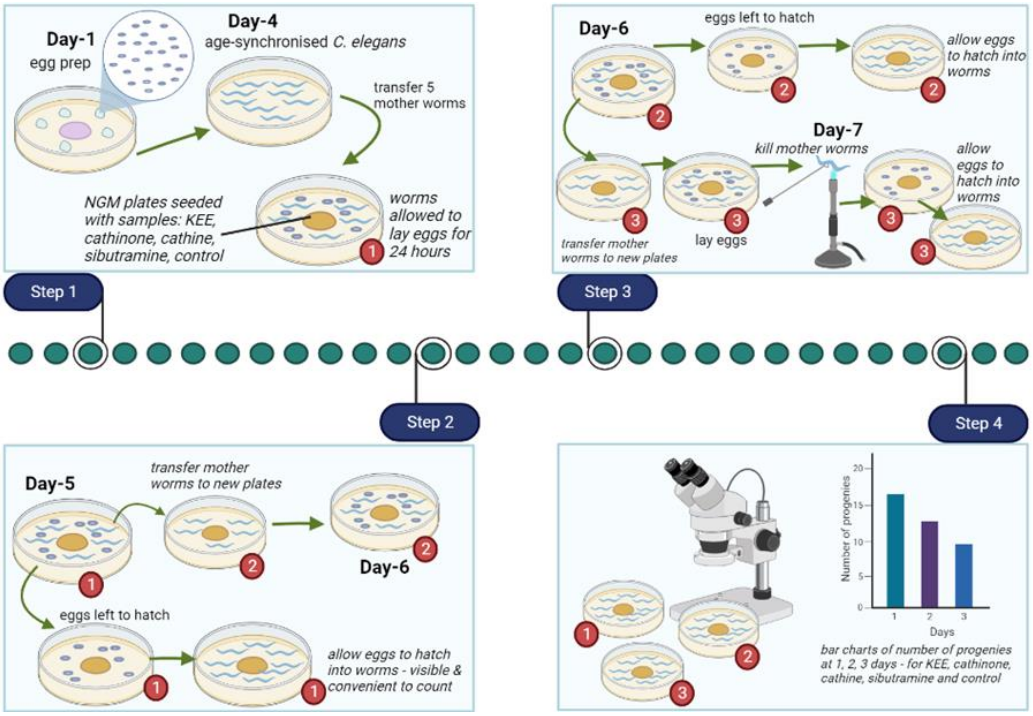

Figure 2. Overview of reproduction assay.

2.6. Lifespan assay

On Day-1, 30 adult N2 worms were individually picked from the egg prep plates and placed on the NGM plate treated with KEE (12.5, 6.25, 3.125 mg/ml), cathinone (1.8, 0.9, 0.18 mg/ml), cathine (1.8, 0.9, 0.18 mg/ml), sibutramine (1.8, 0.9, 0.18 mg/ml) and control each. 3 plates (3 technical replicates) each for KEE, cathinone, cathine, sibutramine, and control were prepared. The number of dead worms is scored every day for each treated plate until all worms die. This assay was repeated three times and maintained with the same assay conditions (e.g. incubation time and temperature) as described above. Animals that died from internal hatching or crawled off the plate were censored. Worms that did not respond to a tap using the picker were counted as dead [41]. The survival rate is presented as the mean of three independent experiments and compared by survival curves followed by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon curve comparisons method, using GraphPad Prism Version 9 (GraphPad Software, La Jolla, California, USA).

2.7. RNA extraction and RNA integrity

Based on the feeding, reproduction, and lifespan assays, the highest concentration for all treatments showed the highest impact on *C. elegans*, and therefore, worms are treated for 24 hours with the highest concentration of treatment as follows: 12.5 mg/ml KEE, 1.8 mg/ml cathinone, 1.8 mg/ml cathine, 1.8 mg/ml sibutramine and control, to capture the possible changes in its *cyps* gene. RNA extraction was carried out as previously described with minor modifications [40]. Initially, five 9 cm plates of age-synchronised treated *C. elegans* were washed using M9 solution into RNase-free Eppendorf tubes at least three times to remove bacteria, centrifuged briefly for 1 min and packed worms were transferred into a new Eppendorf tube. RNA was extracted with the addition of 1 ml TRIzol® Reagent (Invitrogen), vortexed for 30 seconds to break the outer cuticle of the worms, placed in liquid nitrogen, and thawed at 37 °C repeatedly for 3-6 times. The frozen worms in Trizol were thawed to permit dissociation of the nucleoprotein complex, 20 µl of chloroform (EMSURE® ACS, ISO, Reag. Ph Eur, Merck, Darmstadt, Germany) was added for lysis, incubated for 3 min and centrifuged at 12,000 rpm, 4°C for 15 min. The clear colourless upper aqueous phase layer of RNA was pipetted into the RNase-free Eppendorf tube, followed by the addition of 500 µl of isopropanol (ACS Grade, Merck, Germany). The tube was inverted several times, kept at room temperature for 10 mins followed by centrifuge at 12,000 rpm, 4°C for 10 min. The supernatant was discarded, 500 µL of ice-cold ethanol (75%) was added and centrifuged at 7,500 rpm, 4°C for 5 min. The supernatant was discarded, and the tube was left on ice for 15 min to let the ethanol evaporate and finally resuspended with 100 µl of RNase-free water

(Qiagen, Hilden, Germany). The tube can be stored at – 80 °C until used for the next RNA purification step. RNA purification step was carried out afterward using the RNeasy Mini Kit (Catalog No.NC9677589, Qiagen) following the manufacturer's instructions (https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en) and RNase-Free DNase Set (Cat. No. / ID: 79254, Qiagen). The RNA purity at A260/280 and A260/230 is quantified by NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, USA) with only RNA at ≥50 ng/μL are accepted to proceed with the RT-PCR step. Three experimental replicates were performed, with one biological replicate for each condition.

To assess the integrity of the total RNA, a run of aliquots containing 1 μl of the RNA samples for each treatment was carried out on a denaturing 2% agarose gel stained with SYBR Safe DNA Gel Stain (Catalog number: S33102, Invitrogen™, Carlsbad CA, USA). The intact total RNA run on a denaturing gel will show sharp, clear 28S and 18S rRNA bands in which the 28S rRNA band would be approximately twice as intense as the 18S rRNA band (Supplementary material B). The 2:1 ratio is a good indication that the RNA is completely intact [42].

2.8. Quantitative RT-PCR (qRT-PCR) & Pathway Analysis

Three technical replicate RT-PCR reactions were performed from each of the three biological replicates using one-step QuantiNova SYBR® Green RT-PCR Kit (Catalog No. 208152, Qiagen), 200 ng of total RNA, primers, StepOne Software v2.3 (Applied Biosystems, Waltham, Massachusetts, USA) and StepOnePlus™ Real-Time PCR System machine (Applied Biosystems, Waltham, Massachusetts, USA, Catalog No. 4376600). All reactions were performed with the standard two-step cycling according to the manufacturer's instructions (https://www.qiagen.com/us/resources/resourcedetail?id=c6452ea2-0ad1-4e7f-9d01-52799639f1a7&lang=en) or (www.qiagen.com/handbooks). Relative quantification was performed using the ΔCT method, with *pan-actin* (forward: 5′-TCGGTATGGGACAGAAGGAC-3′, reverse: 5′-CATCCCAGTTGGTGACGATA-3′) [43]. Primers for qRT-PCR were based on those previously published [24–28].

Results of the real-time PCR data were represented as C_T values, where C_T was defined as the threshold cycle number of PCRs at which the amplified product was first detected. There is an inverse correlation between C_T and the amount of target: lower amounts of target correspond to a higher C_T value, and higher amounts of target have lower C_T values. The average C_T was calculated for both the target genes and *pan actin* and the ΔC_T was determined as (the mean of the triplicate C_T values for the target gene) minus (the mean of

the triplicate C_T values for *pan actin*). Biological functions, upstream regulatory molecules/networks, network interactions, and pathways analysis of CYP genes were performed using QIAGEN Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, https://digitalinsights.qiagen.com/) and KEGG pathway (http://www.genome.jp/kegg/).

3. Results

3.1. Khat, Cathinone, and Cathine reduce the feeding rate of C. elegans

Feeding rate assay was carried out by manual scoring over short intervals of 5 seconds per worm, to estimate the average number of pharyngeal pumping rates. To maximise the accuracy of the pharyngeal pumping measurement, i) direct observation under the microscope and ii) videos of each worm in each treatment at every time point were captured, and the pharyngeal pumps were calculated later based on the video. The worms showed consistently diminished pharyngeal pumping in response to a high dosage of sibutramine (positive control) at 1.8 and 0.9 mg/ml from 4 to 24 hours (Figure 3). The average pharyngeal pumps of the worms at 1.8, 0.9 and 0.18 mg/ml of sibutramine were (mean ± SD) 10.04 ± 3.27, 14.27 ± 3.64 and 24.38 ± 1.57 pumps, respectively. Exposures to the highest dose of KEE at 12.5 mg/ml caused a reduction in the pharyngeal pumping rate of *C. elegans* at 4 hours with mean ± SD of 16.97 ± 1.85 pumps, slowly increasing from 24 hours onwards and plateaus suggesting possible tolerance. Exposures to 6.25 mg/ml of KEE showed irregular and slight reduction of pharyngeal pumping rate with mean ± SD of 20.10 ± 1.09 pumps whereas exposures to 3.125 mg/ml of KEE showed a drop from 4 to 24 hours followed by an increase from 24 hours onwards to 72 hours, with mean ± SD of 21.77 ± 1.55 pumps. In response to cathinone across all concentrations (1.8, 0.9, 0.18 mg/ml), pharyngeal pumping of *C. elegans* was reduced at 4 hours and similarly, the inhibitory effects of cathinone on pharyngeal pumping started to diminish from 24 to 72 hours, with mean ± SD of 24.20 ± 1.08, 26.17 ± 0.56 and 27.25 ± 0.46 pumps, respectively. Exposure to cathine on the other hand showed a visible reduction in pharyngeal pumping to 1.8 and 0.9 mg/ml at 4 hours, followed by an increase from 24 to 72 hours, with mean ± SD of 24.51 ± 1.44, and 24.88 ± 1.50 pumps, respectively. However, the lowest concentration of cathine at 0.18 mg/ml showed zero inhibiting effect on *C. elegans* pharyngeal pumping with mean ± SD of 27.25 ± 0.06 pumps. Overall, KEE, cathinone, cathine, and sibutramine across all concentrations caused a reduction in pharyngeal pumping of *C. elegans* especially at 4 hours after treatment, and later showed tolerance of the treatment. The inhibitory effects of KEE, cathinone, cathine, and sibutramine on *C. elegans* pharyngeal pumping rate are both dose-dependent and time-dependent.

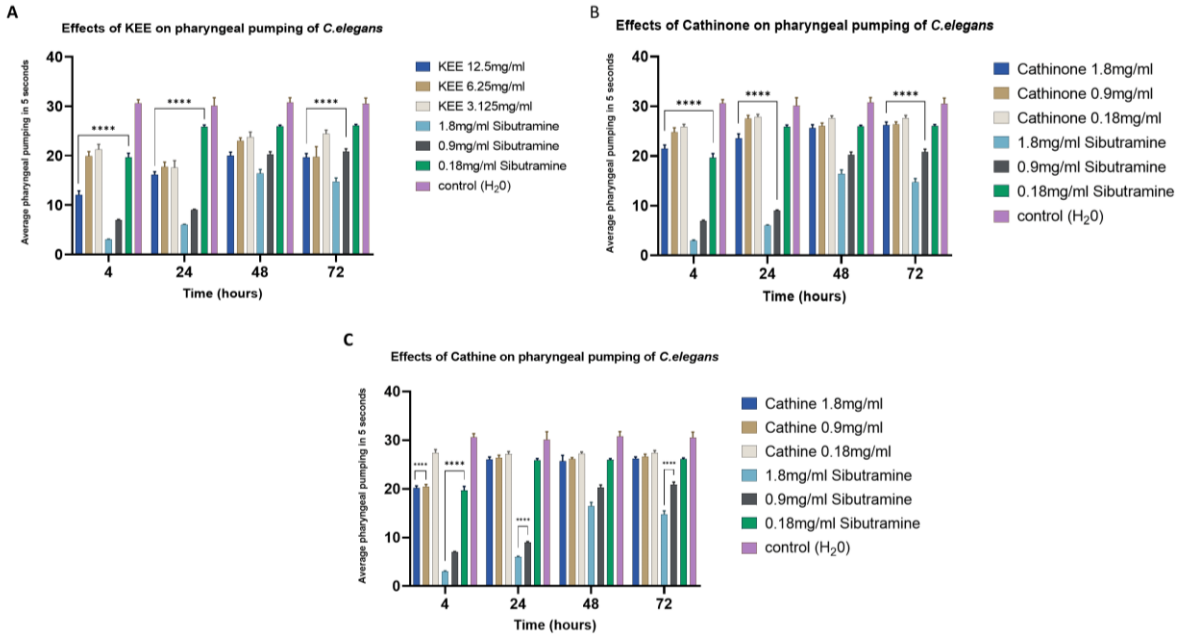

Figure 3. KEE, cathinone, cathine, and sibutramine induce a reversible inhibition of the pharyngeal pumping rate in *C. elegans*. The pharyngeal pumping rate of *C. elegans* exposed to different concentrations of (A) khat ethanol extract (KEE), (B) cathinone, and (C) cathine as compared to different concentrations of positive control, sibutramine, at different time-points; 4, 24, 48 and 72 hours. The pharyngeal pumping assay was carried out in three biological replicates for each concentration of KEE, cathinone, cathine, and sibutramine (*n = 15*). Exposures of KEE, cathinone, and cathine at 24 hour induces the strongest pumping inhibition in N2 (*wild-type*) worms as compared to other concentrations and time-point. Data represent the mean ± SD of pharyngeal pumping rate. Statistical analysis was performed using two-way ANOVA (Tukey's multiple comparisons test). ****A significant difference between 4 vs. 24, 24 vs. 48, and 24 vs. 72 hours for each treated sample across all concentrations ($p<0.0001$).

3.2. Khat, Cathinone, and Cathine reduce the reproduction rate of C. elegans

C. elegans showed an anomalous dynamic of reproduction rate on different treatments of KEE, cathinone, cathine, and sibutramine, with irregular rhythm of egg laying rate. Despite the irregular egg laying rate across all concentrations of all treatment, the inhibitory effects of KEE, cathinone, cathine, and sibutramine across all concentrations was most significant (****$p<0.0001$) at Day-1 as compared to negative control (Figure 4). Overall, KEE, cathinone, cathine, and sibutramine showed inhibitory effects on the egg-laying rate of *C. elegans* but their effects started to disappear on Day-2 as the number of eggs laid increased. However, on Day-3, the number of eggs laid under KEE, cathinone, cathine, and sibutramine treatment reduced. The number of eggs laid for control treatment was consistent and gradually increased from Day 1 to 3. At Day 1 the onset of egg-laying, the egg-laying rate of: i) 12.5 mg/ml KEE worms was (mean ± SD) 77.33 ± 29.60, ii) 1.8 mg/ml cathinone was

78.67 ± 40.70, iii) 1.8 mg/ml of cathine was 100.0 ± 44.26, and iv) 1.8 mg/ml of sibutramine was 20.22 ± 7.58, lower than that of the controls at 155.1 ± 24.25.

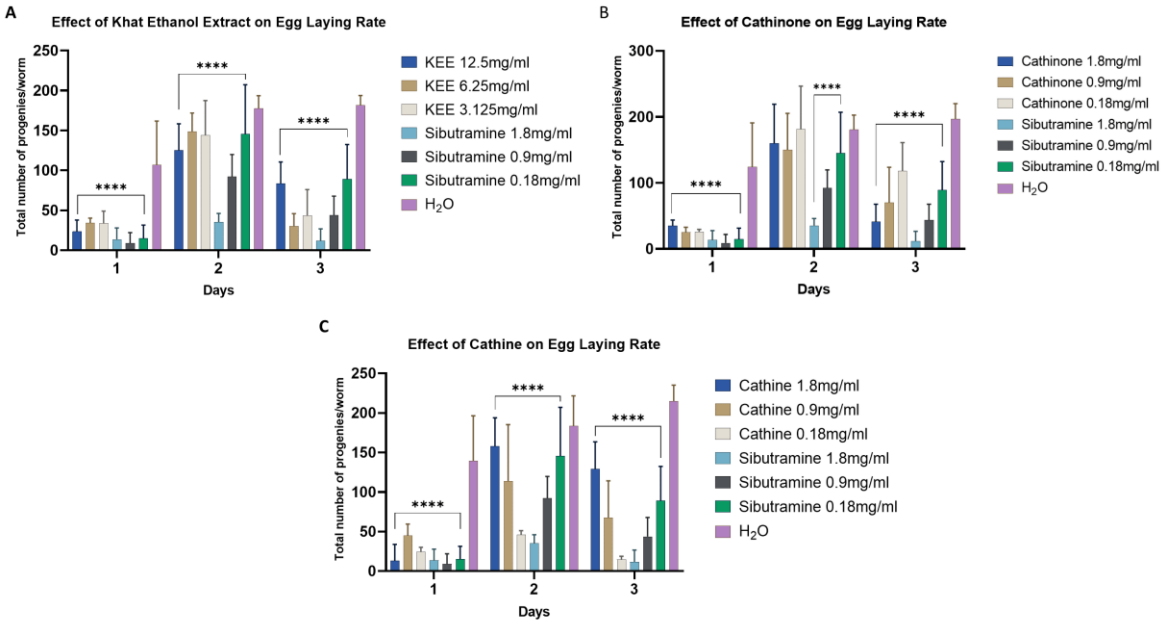

Figure 4. KEE, cathinone, cathine, and sibutramine reduce egg laying rate in *C. elegans*. Number of eggs laid per N2 worm each day on each plate were counted. The number of progenies was plotted as mean ± SD, **** $p<0.0001$; 2way ANOVA with Holm-Sidak's multiple comparisons test. Each egg-laying rate experiment was repeated under similar treatment using 5 worms each for at least three independent times with similar results. ****A significant difference between untreated (water) and treated worms ($p<0.0001$).

3.3. Khat, Cathinone, and Cathine extend the lifespan of C. elegans

The results illustrated that *C. elegans* treated with different concentrations of KEE, cathinone, and cathine showed right-shifted survival curves, especially the highest concentration of KEE, cathinone, and cathine at 12.5 mg/ml, and 1.8 mg/ml, respectively (Figure 5). The survival curves of all KEE, cathinone, cathine, and sibutramine are significantly different with ****$p<0.0001$. KEE at 12.5 mg/ml was observed to extend the worms' lifespan and/or survival rate most significantly with a median survival of 21 ± 2.31 days, as compared to cathinone, cathine, and sibutramine with a median survival of 15 ± 2.77, 10 ± 2.51 and 11 ± 3.17 days respectively. Compared to the control with a median survival of 8.5 ± 3.01 days, the highest concentration of KEE, cathinone, and cathine significantly extended the median lifespan to 12.5, 6.5, and 1.5 days and their survival rate increased by 59.5%, 43.3%, and 15%, respectively. Therefore, khat ethanol extracts could markedly and to a lower extent, cathinone and cathine, prolong the lifespan of *C. elegans* N2 in a dose-dependent manner.

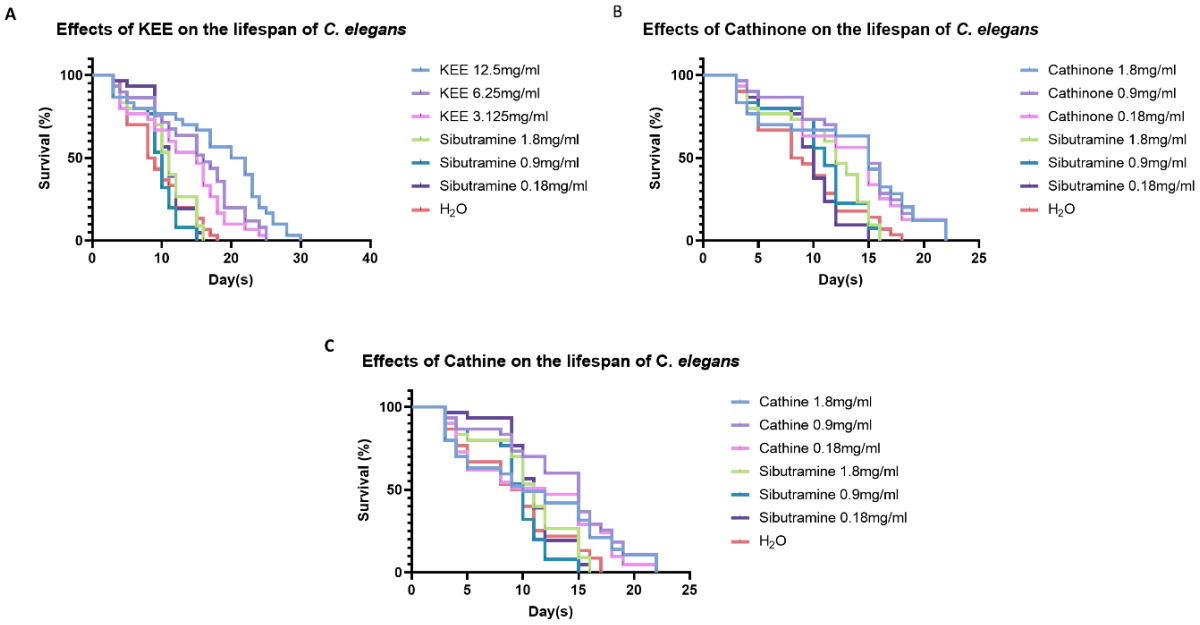

Figure 5. KEE, cathinone, and cathine extend lifespan in *C. elegans*. Kaplan-Meier plot for survival of *C. elegans* on different concentrations of (A) KEE, (B) cathinone, (C) cathine as compared to positive control, sibutramine. Survival curves are significantly different, determined by a log-rank test and Gehan-Breslow-Wilcoxon test, with ****p<0.0001. Each lifespan experiment was repeated under similar treatment using 90 worms each for at least three independent times with similar results.

3.4. Effects of khat, cathinone, and cathine on gene expression of selected C. elegans cyp genes

Based on the most significant effect of 12.5 mg/ml KEE, and 1.8 mg/ml of cathinone, cathine, and sibutramine on *C. elegans* pharyngeal pumping, reproduction, and lifespan, these concentrations were chosen to treat worm samples for RNA extraction and subsequently capture their effects on *C. elegans* CYP genes via RT-PCR. Although the fold change of *cyp-14A3*, *cyp-34A9*, and *cyp-35A2* across all treatments are not significant as compared to control, there is an increase in fold change as compared to the treatments (e.g. KEE, cathinone, cathine, sibutramine) (Figure 6). For *cyp-14A3*, KEE was upregulated about 2-fold, cathinone was upregulated about 3-fold, cathine was upregulated, approximately ≥ 3-fold, and sibutramine was upregulated > 3-fold. For *cyp-34A9*, KEE was upregulated > 2-fold, cathinone was upregulated about 2-fold, cathine was upregulated, approximately 1.5-fold, and sibutramine was upregulated > 1.5-fold. For *cyp-35A2*, KEE was upregulated about 1.5-fold, cathinone was upregulated > 1-fold, cathine was not upregulated, and sibutramine was upregulated > 1-fold.

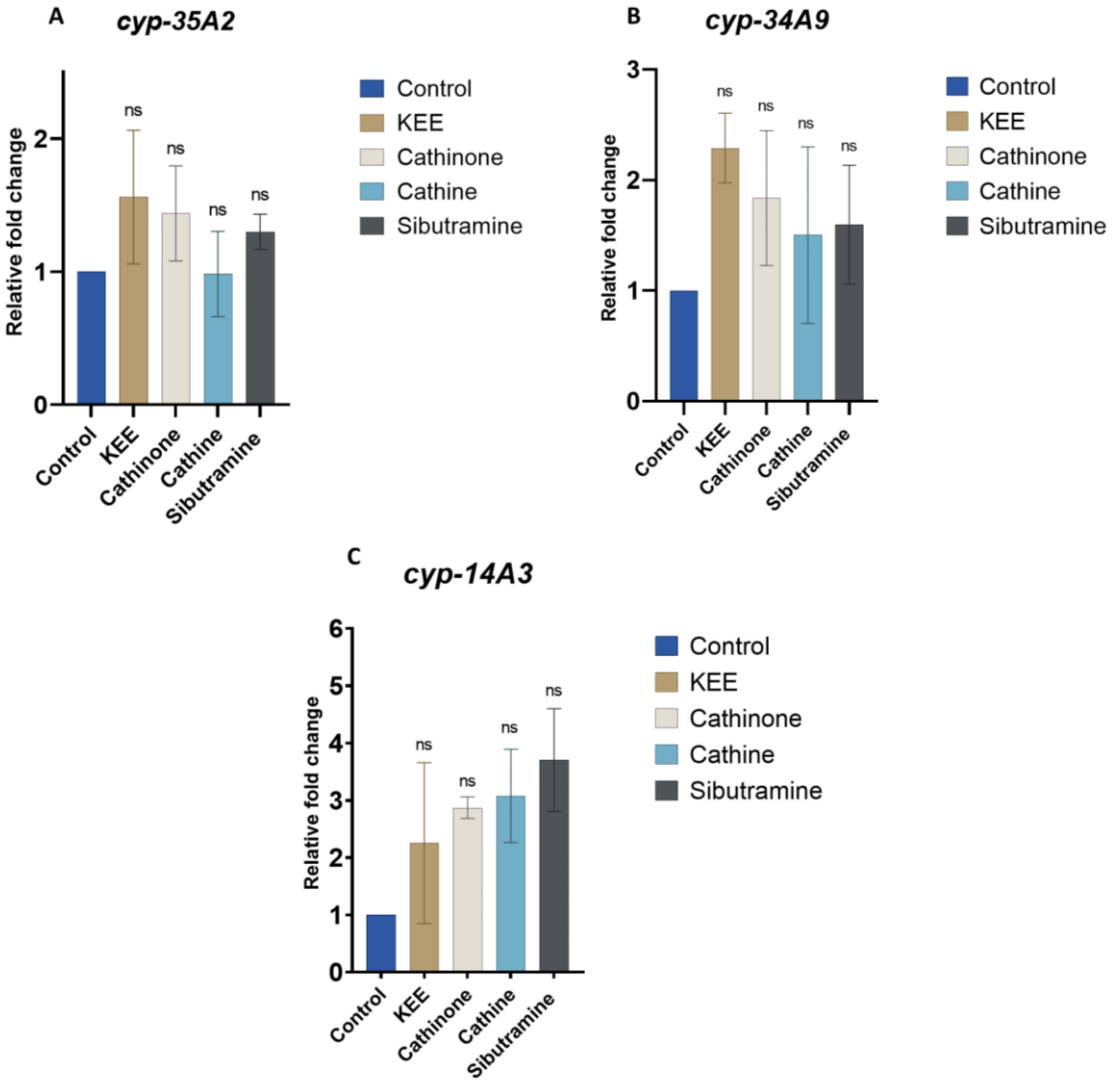

Figure 6. Real-time RT-PCR analysis of gene expression was performed for three CYP genes that encode for human CYP homologues CYP1A2, CYP2C8, and CYP2C19. The relative expression pattern of *C. elegans* (A) *cyp-14A3*, (B) *cyp-34A9*, and (C) *cyp-35A2* were captured after 24 hours of treating *C. elegans* with the highest concentrations of KEE, cathinone, cathine, sibutramine, and control. The expression level of target genes was normalised by that of *pan actin*. Values shown are the mean ± SD of $n = 3$. *ns refers to not significant* between untreated (control) and treated worms.

3.5. IPA Analysis

The IPA disease and biological functions tool identified several networks that these CYPs are linked to cellular and molecular functions, including drug metabolism, small molecule biochemistry, endocrine system development, and function, energy production, lipid metabolism, vitamin and mineral metabolism, nucleic acid metabolism, cell-to-cell signalling and interaction, cardiovascular system development and function, tissue morphology, cardiovascular disease, organismal injury and abnormalities, haematological

disease, immunological disease, cell morphology, cellular movement and hair and skin development and Function (Figure 7). Bar graphs in Figure 7 revealed the likelihood [-log(p-value)] that the specific molecular and cellular functions obtained from IPA disease and biological functional categories analysis to be affected by khat, cathinone, cathine, and sibutramine treatment. Table 1 displays the most significant pathways in our input data to be drug metabolism, small molecule biochemistry, energy production, lipid, vitamin, and mineral metabolism. Expression analysis under Drug Metabolism category for i) khat showed that CYP1A2, CYP2C9, and CYP2C19 were upregulated with a p-value of 1.84E-12, ii) cathinone showed that CYP1A2, CYP2C9, and CYP2C19 were upregulated with p-value 1.84E-12, iii) cathine showed that CYP1A2 and CYP2C19 were upregulated with p-value 1.84E-12 but CYP2C9 was downregulated and iv) sibutramine showed that CYP1A2, CYP2C9, and CYP2C19 were upregulated with p-value 3.87E-11. IPA network analysis also revealed that PXR (pregnane X receptor) acts as an upstream regulator of these CYPs expression (Table 1).

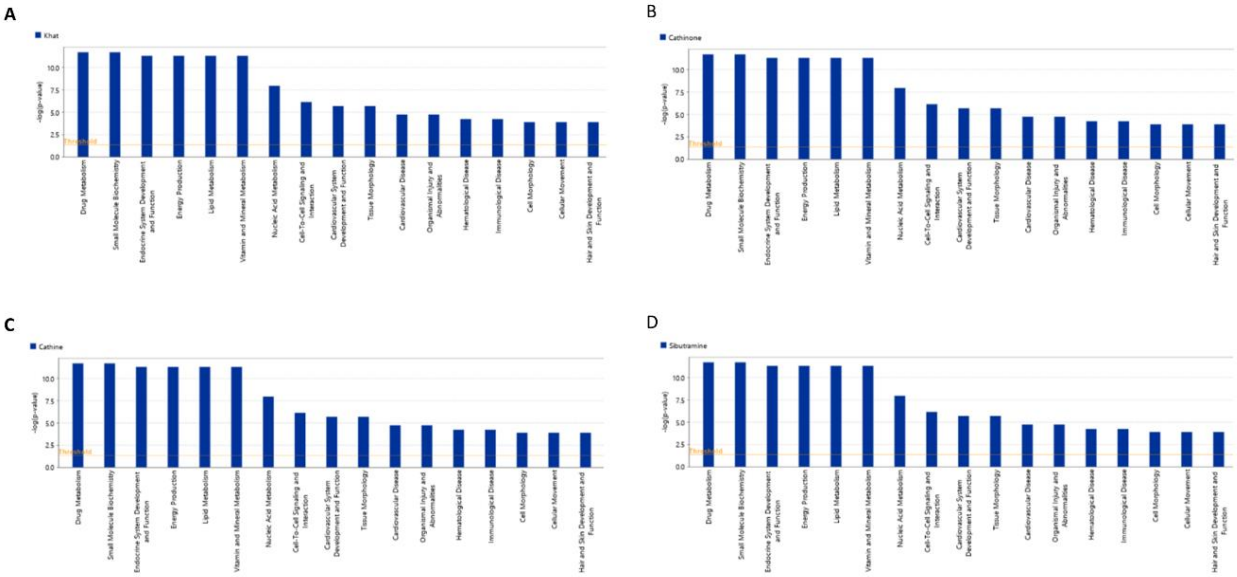

Figure 7. IPA analysis of upregulated human CYP1A2, CYP2C9, and CYP2C19 homologs to *cyp-14A3, cyp-34A9,* and *cyp-35A2* for (A) khat, (B) cathinone, (C) cathine, and (D) sibutramine treatment.

3.6. KEGG Analysis

The KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis revealed one or several CYPs could be involved in the metabolism of a single drug in *C. elegans* (Figure 8). CYP1A2, CYP2A6, CYP2C8, CYP2C9 and CYP2C19 are human orthologs to *C. elegans cyp-14A3*, *cyp-34A9* and *cyp-35A2*. Referring to the KEGG analysis in Figure 8 (https://www.kegg.jp/kegg-bin/show_pathway?cel00982), i) tamoxifen is metabolised by CYP2C9 (*C. elegans cyp-35A2*) and CYP2D6, ii) cyclophosphamide and ifosfamide are metabolised by CYP2C, CYP2B6, and CYP3A4, iii) citalopram is metabolised by CYP2C19, CYP2D6 and CYP3A4, iv) lidocaine is metabolised by CYP1A2 and CYP3A4, v) carbamazepine is metabolised by CYP2C8 and CYP3A4, and vi) valproic acid is metabolised by CYP2C9, CYP2B6, and CYP2A6 respectively.

Table 1. Top tox pathways and upstream regulators of CYP1A2, CYP2C9, and CYP2C19 human homologs of *C. elegans cyp-14A3*, *cyp-34A9*, and *cyp-35A2* predicted by IPA.

Pathway	***p-value***
Cytochrome P450 Panel – Substrate is a Xenobiotic (Human)	3.76E-10
PXR/RXR Activation	2.11E-08
Fatty Acid Metabolism	1.02E-07
Aryl Hydrocarbon Receptor Signalling	3.15E-07
NRF2-mediated Oxidative Stress Response	1.04E-06
Upstream Regulators	***p-value***
PXR ligand-PXR-Retinoic acid-RXRα	4.91E-09
phenobarbital	4.22E-08
NR1I3	8.35E-08
1,4-bis[2-(3,5-dichloropyridyloxy)]benzene	1.12E-07
rifampin	2.37E-07

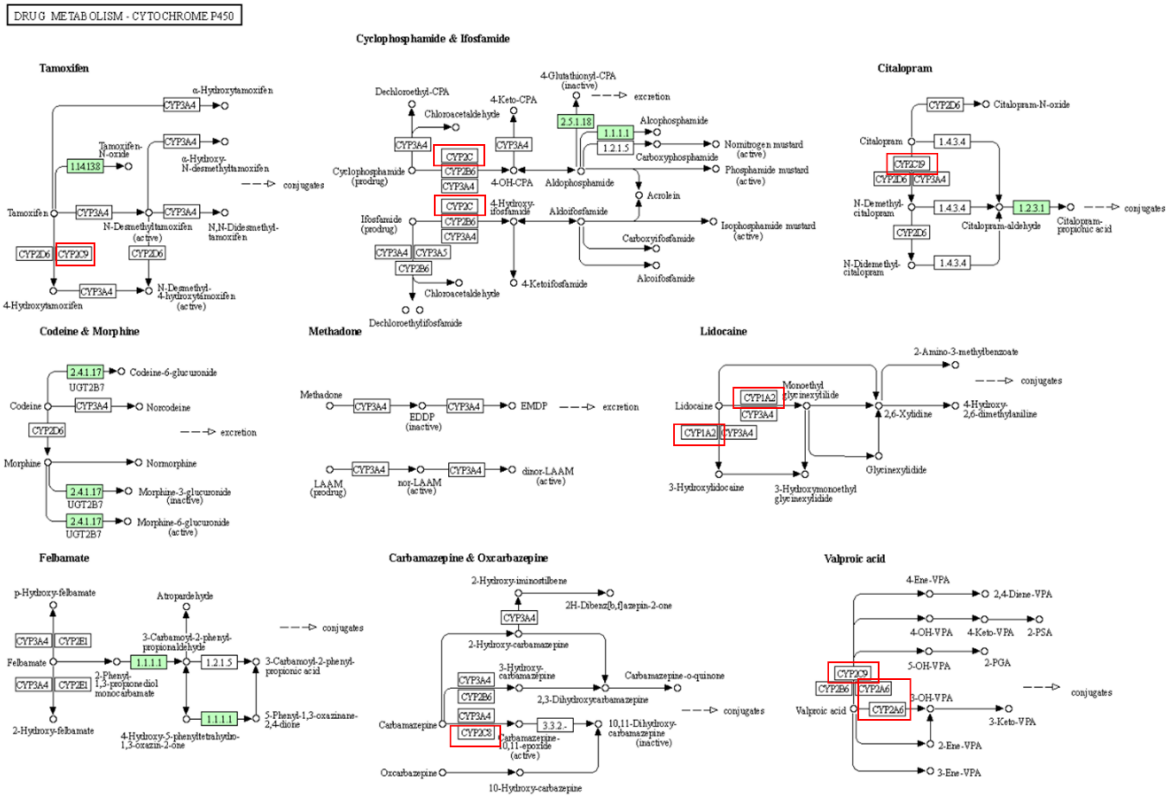

Figure 8. The KEGG analysis of drug metabolism in *C. elegans* cytochrome P450. The red rectangle highlighted the human orthologs of *C. elegans cyp-14A3*, *cyp-34A9*, and *cyp-35A2*.

4. Discussion

The present study framed three different assays to determine the biological effects of KEE and its active compounds, namely cathinone and cathine on *C. elegans* feeding, reproduction (egg-laying) rate, and lifespan, followed by gene expression study to explore the effects of KEE, cathinone, cathine, sibutramine on *C. elegans* CYP genes namely *cyp-14a3*, *cyp-34a9* and *cyp-35a2* in which their human homologues are CYP1A2, CYP2C8/9/19, and CYP2C9, respectively. CYP1A2, CYP2C8, CYP2C9, and CYP2C19 are major human liver drug metabolising CYPs responsible especially in drug or foreign substances metabolism and elimination. *C. elegans* feeding is regulated by a cycle of contraction and relaxation of the pharyngeal muscles that could be envisaged by the up and down movement of the grinder [44]. Under the microscope, pharyngeal pumping is a visually scoreable behaviour that is regulated by environmental signals that trigger sensory and integrative neural signalling to regulate pharyngeal movements [45]. The pharyngeal pumping rate of *C. elegans* mirrors the food intake and general health of the nematode [44]. The natural

amphetamine khat plant showed appetite-suppressing effects on *in vivo* animal models and human subjects despite little being known about its mechanism of action [5]. It was believed that the direct and indirect effects of khat may be branched from various factors, including behavioural, chemical, and neurophysiological effects on appetite and metabolism [5]. The consumption of khat was associated with delayed gastric emptying following ingestion of semi-solid meal in healthy human subjects [46]. The second major active compound of the khat plant, cathine (d-norpseudoephedrine, NPE), was extensively used as an anti-obesity medication due to its appetite-suppressing properties [4]. Cathine intake in rats demonstrated a reduction in 24-hour food intake, induced weight loss along with side effects including increased locomotor activity and wakefulness [4].

Equally untouched was the mechanism of cathinone or cathine in appetite-suppression pathways. Cathinone has been shown to possess similar effects on cardiovascular and behavioural parameters as amphetamine and therefore, cathinone may exert similar appetite-suppressing effects as amphetamine [47]. One hypothesis is that cathinone's anorectic effect is secondary to norepinephrinergic (NE) and dopaminergic (DA)-dependent mechanisms within the hypothalamus [5]. Possibly, similar to amphetamine, the anorectic effect of cathinone is mediated by neuropeptide Y (NPY) and requires co-stimulation of both dopamine receptors, D1 and D2 [5]. Modulation of the nucleus accumbens shell (NAcSh) spiking activity and both dopamine receptors, D1 and D2, by cathine, was found to contribute to cathine-induced food intake suppression and weight loss [4]. Another hypothesis would be that cathine and d-norpseudoephedrine, independently or in combination, facilitate the appetite suppressant effect of khat, therefore, illuminating their pharmacological effect on all direct and indirect feeding behaviours could find the solution to this puzzle [5]. The intake of khat, cathinone, cathine, and/or amphetamine-like substances may lead to the enhanced production of norepinephrinergic (NE) and dopaminergic (DA) in the human brain [5]. The high levels of DA and NE stimulate the D1 and D2 receptors together with the α and β receptors respectively, resulting in decreased food intake and weight loss.

Sibutramine, a serotonin and noradrenaline reuptake inhibitor, is an appetite-suppressing anti-obesity agent [48,49]. Sibutramine treated worms showed significantly reduced pharyngeal pumps of mean ± SD (67.3 ± 0.3) compared to control (70.0 ± 0.2)[50]. Malnutrition has been a persistent problem found among khat-chewer women in Sub-Saharan Africa due to loss of appetite, constipation, and increased gastric fullness [51]. In humans, sibutramine produces its therapeutic effects by inhibition of norepinephrine (NE) by 73%, serotonin (5-hydroxytryptamine, 5-HT) by 54%, and to a lesser extent, dopamine reuptake by 16%, at the neuronal synapse [52]. By inhibiting the reuptake of these neurotransmitters,

sibutramine promotes a sense of satiety and a decrease in appetite, thereby reducing food intake [53]. In *C. elegans*, serotonin is produced in nonneuronal tissues via phenylalanine hydroxylase and canonical biosynthesis via tryptophan hydroxylase in neurons [54]. Serotonin is released from neurosecretory-motor neurons (NSM) inside the pharynx or from extrapharyngeal neurons (e.g., ADF) [55] that stimulates pharyngeal pumping by affecting MC neurons to increase the frequency of pharyngeal pumping [56].

As studies suggest that amphetamine-like compounds including khat, cathinone, cathine, and sibutramine have appetite-suppressing properties and stimulate the release of serotonin and dopamine that reduce food intake in humans, we asked whether these compounds could exert similar appetite suppressing effects in *C. elegans*, knowing the fact that as opposed to humans, an increase in serotonin levels increases appetite in *C. elegans*. Interestingly, our findings showed that the highest concentrations of khat extract ethanol, cathinone, cathine, and sibutramine exposures reduced the average pharyngeal pumping rate by 5 seconds. It was observed that the average pharyngeal pumping rate in 5 seconds was 12, 21, 20, and 0 pumps at 4 hours, followed by a similar gradual increase from 24 – 72 hours across all concentrations (Figure 3). Adult worms pump 200–300 times per minute on average in the presence of food [57,58]. Hence, by estimation *C. elegans* would pump 3.33 – 5 pumps per second which is in agreement with the pharyngeal pumps shown in our control worm sample, which recorded an average of 30 pumps in 5 seconds (Figure 3). Serotonin treatment was found to increase the feeding rate when *C. elegans* was already well-fed [59]. Khat, cathinone, cathine, and sibutramine act as appetite suppressants in humans and stimulate serotonin and dopamine release, reducing food intake. Following this, khat, cathinone, cathine, and sibutramine treatment may increase intrinsic serotonin in *C. elegans* that increases pharyngeal pumping, equivalent to an increased feeding rate. However, this is not seen in our feeding rate outcome. We found that similar to humans, khat and its active compounds causes a reduction in pharyngeal pumping, reduced feeding and/or appetite loss in the worms. Therefore, we hypothesised that the appetite loss could be triggered by a different mechanism caused by khat, cathinone, cathine and sibutramine. However, this hypothesis is subject to the caveat that our study could not capture whether the intake of khat, cathinone, cathine and sibutramine led to the release of serotonin and dopamine that regulate the pharyngeal pumping or otherwise. Moreover, the treatment may release serotonin and dopamine that may upregulate or downregulate certain genes that controls pharyngeal pumping in the *C. elegans*.

Based on the WormAtlas, adult *C. elegans* lay 4 – 10 eggs per hour and one adult worm can produce 300 offspring over 3 days on average [60]. Ensuring the estimation of 4 –

10 eggs laid per hour by *C. elegans* under normal conditions, there would be 96 – 240 eggs produced in 24 hours/day. *C. elegans* egg-laying rate is very sensitive and is subjected to environmental cues alteration. For instance, more eggs will be produced in the presence of an abundant food supply or vice versa, while the sensation of touch receptors, high glucose, and carbon dioxide (CO_2) may inhibit egg-laying [61]. Remarkably, *C. elegans* responds to environmental stresses to augment its egg laying similar to other animals (e.g. *Acheta domesticus* crickets, *Pomacea canaliculata* snails) [61]. Exposures to low doses of teratogens such as nicotine, cadmium, and tributylin, in contrast, increase the *C. elegans* reproduction rate [62]. However, long term exposure to high doses of copper ($CuSO_4$) decreases worm brood size [63]. *C. elegans* generally have a high reproductive capacity in the first 4 days of the L4 phase and the reproductive capacity gradually decreases until the 5^{th} day [64]. The effects of khat on reproduction and fertility remain as ongoing research and the findings were controversial. Some studies revealed that khat negatively affected reproduction while others proved otherwise [65]. In humans, khat chewing is associated with lowered libido, and sexual impotence following long-term use in men. Meanwhile, in pregnant women, khat use is linked to inhibited utero-placental blood flow, foetal growth impairment [8] and is also associated with prelabour rupture of membranes [66]. The effects of khat may mainly be caused by its major constituent, cathinone, which was found to cause a significant decrease in sperm count, and motility, and an increase of abnormal sperm in cathinone-treated rodents [67]. Parallel to these findings, a recent study also found impairment of spermiogenesis (the final stage of sperm maturation) after administration of a high dosage (300 mg/kg body weight) of khat extract in rodents [68]. However, a study proved otherwise, as there was a significant increase in sperm count and motility after 4 and 8 weeks of khat treatment in rodents and the effects continued in their adult offspring as well [9]. Another rodent study showed that mild dose of khat may improve sexual motivation but high dose of khat reduced sexual motivation as testosterone levels reduces [69].

In line with that, we evaluated the effects of KEE, cathinone, cathine, and sibutramine on *C. elegans* reproduction rate for 3 days. Our findings exhibited that the egg-laying rate of KEE, cathinone, cathine, and sibutramine treated worms at the highest concentration on Day-1 was lower than the normal average number of eggs laid by *C. elegans* under normal conditions (e.g. 4 – 10 eggs/hour, 96 – 240 eggs/day), with an average of 23.3, 35.0, 13.0, and 13.7 eggs laid (Figure 4). All concentrations of KEE, cathinone, cathine, and sibutramine failed to impair *C. elegans* egg laying rate on Day 2 as the number of eggs laid increased to near normal, ranging from 125 – 148, 150 – 181, 46 – 157, and 35 – 145 eggs, respectively. On Day 3, the number of eggs-laid across all concentrations of samples treated was slightly lessened as compared to Day 2 (Figure 4) but remained within the normal range of the number

of eggs laid per day under normal conditions. It can be concluded that high concentrations of KEE, cathinone, cathine, and sibutramine impaired *C. elegans* egg laying rate but the inhibitory effects are not lasting, and the worms develop tolerance after 24 hours onwards. During assay optimisation (unpublished data), a high concentration of KEE at 25 mg/ml was found to cause satiety quiescence in the *C. elegans*. The worms fed actively right after being transferred into the khat-*E. coli* lawn for a few minutes and completely stop feeding afterward. This may indicate that our treatment may reduce worm movement and feeding which may also reduce its egg-laying rate. The regulation of *C. elegans* egg-laying was claimed to rely on cholinergic, serotonergic, GABAergic, and peptidergic circuits which our data could not capture [70]. Cathinone was found to induce serotonin release and serotonin reuptake inhibition [71] which is a paradigm that khat and its active compounds may exert their effects via serotonergic pathways to alter egg-laying and feeding rates. Similarly, assuming khat, cathinone, cathine, and sibutramine treatment causes an increase of serotonin release that increases the rate of egg-laying [72], this notion is proved otherwise in our reproduction rate outcome. Another explanation could be that the worms reduced their pharyngeal pumping upon exposure to new chemicals (e.g. khat, cathinone, cathine, sibutramine) and therefore, this is captured as a reduction in feeding rate. Worms could sense the taste or smell of novel food which negatively regulates the feeding response [73]. The reduction of pharyngeal pumping means the worms consumed less of these compounds, which reduces the release of serotonin and dopamine that stimulates feeding or reproduction rate, and thus, captured as a reduction of feeding and reproduction rate.

Phytochemicals in plants have antioxidant properties and could curb oxidative stress, which helps in aging and age-related disease prevention [11,74–79]. Other phytochemicals in khat including saponins [80,81], flavonoids, and tannins [82], also possessed antioxidant properties. Plant extracts were found to extend lifespan, improve health span, and enhance resistance to stress in *C. elegans* [11]. These findings support the notion that plant extracts promote anti-aging effects by extending lifespan and health span, besides enhancing stress resistance [64]. Data on the effects of khat and derivatives on lifespan are lacking in the literature. We postulated that the highest concentrations of KEE, cathinone, cathine, and sibutramine significantly extended the median lifespan of *C. elegans* to 12.5, 6.5, 1.5, and 2.5 days as compared to negative control. 12.5 mg/ml of KEE, 1.8 mg/ml of cathinone, cathine, and sibutramine induced a median lifespan increase of the worms' survival rate by 59.5%, 43.3%, 15%, and 22.7%, respectively (Figure 5). The extended lifespan of worms treated with KEE, cathinone, and cathine is an indication that khat has potent phytochemicals with anti-aging properties which are also observed by other studies such as: i) terpenoids, termed as geroprotectors (pharmacological agents that decrease the rate of aging and extend

lifespan) [12], and ii) polyphenols (flavonoids, tannic acid) [13]. The lifespan of *C. elegans* under normal condition was approximately 18 – 20 days [17] and thus it is clear that KEE, cathinone, cathine and sibutramine extended the *C. elegans* lifespan (Figure 5), but the exact mechanism requires further investigation.

Following the most significant impact of KEE, cathinone, cathine, and sibutramine at their highest concentrations on *C. elegans* feeding, reproduction, and lifespan, we proceed to determine the effects of these compounds on *C. elegans cyp* genes expression. In humans, hepatic CYP genes expression is upregulated to amplify liver drug metabolism capacity [83]. Our RT-PCR study, despite showing non-significant results, demonstrated that *cyp-14A3*, *cyp-34A9,* and *cyp-35A2* for all samples including khat, cathinone, cathine, and sibutramine are upregulated as compared to the control (Figure 6). As mentioned earlier, CYP1A2, CYP2C8, CYP2C9, and/or CYP2C19 are major human liver drugs metabolising CYPs. In *Caco-2* (cell line derived from colon carcinoma) cells treated with Aroclor 1254 (a polychlorinated biphenyl mixture) exhibited upregulated levels of CYP1A2 and CYP2C8-19 of 80-fold and 10-fold respectively [84]. Referring to WormBase, *cyp-14A3* is an ortholog to human CYP2D6, *cyp-34A9* whereas *cyp-35A2* are orthologues to human CYP2A6, all involved in catalysing oxidative metabolism of a variety of exogenous compounds and endogenous substrates. Additionally, literature stated that *cyp-14A3* is an ortholog to human CYP1A2 [24,26], *cyp-34A9* is an ortholog to human CYP2C8/9/19 [27,28] and *cyp-35A2* is an ortholog to human CYP2C9 [24,25]. Our *in vitro* findings and *in silico* prediction of khat, cathinone, and cathine showed inhibition on CYP1A2, CYP2C8, CYP2C9, and CYP2C19 which are human homologs of *C. elegans cyp-14A3, cyp-34A9* and *cyp-35A2*. Our *in vivo* findings may suggest that *cyps* were upregulated as compared to control samples despite being insignificant. The *cyps* upregulation could be to enhance metabolism upon exposure to novel chemicals (e.g. khat, cathinone, cathine, and sibutramine). However, these findings could not prove that the *cyps* upregulation was linked to *cyps* inhibition exhibited in our *in vitro* studies [3,30–32]. CYP induction is a slow regulatory process and more time is needed to reach the steady state enzyme levels resulting from a new balance between rate of biosynthesis and degradation, unlike CYP inhibition which could be an almost immediate response [85]. In this study, the *C. elegans* were treated with different samples at one concentration for 24 hours before RNA extraction. Therefore, the insignificant *cyps* upregulation could be due to the amount of CYP enzymes captured right after the 24-hour treatment. We hypothesised that the *cyps* expression could be more significant if RNA were extracted after a long period of treatment e.g. 3 days, as *cyps* induction and upregulation take time. Strikingly, other RT-PCR experiments indicated that *cyp-35A2* mRNA was upregulated in response to treatment with xenobiotics such as PCBs (polychlorinated biphenyls) or PAHs

(polycyclic aromatic hydrocarbons). KEGG pathway mapping is used to map the molecular data sets in genomics with related signalling pathways extracted by the pathway mining tool [86].

To gain more insights about the underlying molecular pathways associated with CYP1A2, CYP2C9, and CYP2C19, IPA was used to generate integrated pathways, networks and upstream regulators, and related genes, respectively. IPA analysis revealed that KEE, cathinone, cathine and sibutramine treatment upregulated CYP1A2, CYP2C9 and CYP2C19 human homologs to *C. elegans cyp-14A3*, *cyp-34A9* and *cyp-35A2* (Figure 7) and highest -log(p-value) fell under the drug metabolism category which corroborated the notion that khat, cathinone, cathine and amphetamine-like compounds e.g., sibutramine metabolism upregulated the *cyp* genes expression. The IPA tox list analyses revealed a significant enrichment of CYP450 enzymes, xenobiotic metabolism, and fatty acid metabolism. Table 1 displays the most significant pathways and upstream regulators associated with CYP1A2, CYP2C9 and CYP2C19 from IPA. Transcription factors involved in regulating CYP mRNA levels include nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), retinoid X receptor (RXR), aryl hydrocarbon receptor (AhR), and human nuclear factors (HNFs), are responsible for downregulation of DMEs [87]. Distinguishing the role of nuclear transcription factors in CYPs expression and drug or inflammation-mediated expression is therefore recommended. The top upstream regulator involved, based on our IPA data (Table 1), was PXR which is a drug or xenobiotic-activated transcription factor crucial for major CYP metabolising expression and regulation in the liver and intestine [88].

PXR is involved in the regulation of drug metabolism and excretion, metabolic, immunological functions and cancer pathogenesis [89]. PXR binds to retinoid-X-receptor α (RXRα, *NR2B1*) to response elements of nucleotides in the proximal promoters, distal and far enhancers region of its target genes for instances, the far enhancer region involved in regulation of CYP3A4 gene [89] or binding of PXR and PGC1α to the promoter of CYP2A6 [90] or HNF4α has been reported to increase the PXR-mediated induction of CYP2C9 promoter in HepG2 cells [91]. Referring to the KEGG analysis in Figure 8 (https://www.kegg.jp/kegg-bin/show_pathway?cel00982) for *C. elegans cyps* drug metabolism, i) tamoxifen is metabolised by CYP2C9 (*C. elegans cyp-35A2*) and CYP2D6, ii) cyclophosphamide and ifosfamide are metabolised by CYP2C (*C. elegans cyp-34A9* and *cyp-35A2*), CYP2B6 and CYP3A4, iii) citalopram is metabolised by CYP2C19 (*C. elegans cyp-34A9*), CYP2D6 and CYP3A4, iv) lidocaine is metabolised by CYP1A2 (*C. elegans cyp-14A3)* and CYP3A4, v) carbamazepine is metabolised by CYP2C8 (*C. elegans cyp-34A9*) and CYP3A4, and vi) valproic acid is metabolised by CYP2C9 (*C. elegans cyp-35A2)*,

CYP2B6 and CYP2A6 (*C. elegans cyp-34A9* and *cyp-35A2)* respectively. The activation of *C. elegans cyp* genes in response to drugs involves several CYP isoforms and regulators for drug metabolism and excretion, inflammation [92], and/or pathogenesis. Although these preliminary results require further validation, CYP1A2, CYP2C9, and CYP2C19 (*cyp-14A3*, *cyp-34A9,* and *cyp-35A2* in *C. elegans*) might have a significant clinical impact on the management of drug-mediated expression, better assessment of drugs interactions, efficient therapies with minimal side effects and application of personalized treatment regimens.

Data have shown the potential for phytochemicals in khat to activate *cyp-14A3*, *cyp-34A9,* and *cyp-35A2* in *C. elegans*, suggesting that these *cyp* genes mediate drug/xenobiotic metabolism in the worms and may stimulate antioxidant effects and increased survival rate. However, the exact mechanism of how khat and its active compounds regulate these effects in the *C. elegans*, and other genes, effectors, and/or transcription factors involved are unmapped. Despite *C. elegans*' features as a powerful model organism, its limitations include not having lungs, blood transport system, blood-brain barrier, first-pass metabolism processes in the liver, and blood filtration in kidneys, as they lack these organs, which equals to inability to accurately predict specific signalling pathways or epigenetic effects [93]. With that said, biological processes in these organs in humans cannot be impersonated by *C. elegans* [93]. The worms are covered with an almost impermeable cuticle layer and eggshell before hatching, and possess robust mechanisms for excreting toxic chemical substances, which are features that can complicate or alter apparent sensitivity to chemicals, especially in toxicology studies [93]. The routes of exposure of *C. elegans* are also poorly understood, although the heightened sensitivity to chemicals in cuticle mutants suggests that exposure is likely transdermal or via ingestions [93]. Additionally, bacterial food sources can present confounding effects as bacterial-by products can create genetic responses in the *C. elegans* and these responses are often overlooked [94]. *C. elegans* also has a larger complement of CYP genes that do not completely overlap with the human complement, and seemingly excludes specific toxicologically important CYPs but it can be argued that human genes with no direct sequence homology in *C. elegans* may have uncharacterised functional orthologues for these genes that can be informative [93]. Another caveat of this study is we used only one reference or housekeeping gene, pan actin, to compare C_T values of three *cyp* genes namely *cyp-14A3*, *cyp-34A9,* and *cyp-35A2*, and hence future studies should use other recommended housekeeping genes such as *act-1, ama-1, cdc-42, csq-1, eif-3.C, mdh-1, gpd-2, pmp-3, tba-1, Y45F10D.4, rgs-6,* and *unc-16* to accurately normalise using stably expressed reference genes [43].

5. Conclusion

The current study demonstrates the relevant appetite suppressing, anti-aging (extended lifespan) properties, reproduction (egg-laying) impairment in *C. elegans* under the exposures of khat extracts, cathinone, cathine, and sibutramine, which is in agreement with khat effects observed *in vivo* human and animal studies. Interestingly, *cyp-14A3, cyp-34A9,* and *cyp-35A2* expression in khat-, cathinone-, cathine- and sibutramine-treated *C. elegans* were all upregulated despite insignificant, from 1 to 3-fold as compared to control. The outcome of this study answers previous questions but also raises new unexplored avenues for *C. elegans cyp* genes and whether its outcome is indicative of how khat, cathinone, and cathine would exert their effects via similar pathways or genetic routes in humans. The drug- or xenobiotic-inducible gene expression of *C. elegans* proved to be a valuable means to disclose drug metabolising genes or signalling pathways in humans that are encoded by genes homologous to *C. elegans* genes. These results suggest that khat extract and its active compounds, cathinone and cathine, despite their inhibitory effects on CYP enzyme activities, could be a potential supplement and alternative medicine with appetite-suppressing, antioxidant, and anti-aging benefits, of course with additional studies using other *in vivo* models to corroborate our findings in *C. elegans*. Future venture is recommended to explore whether *cyp14*, *cyp34,* and *cyp35* gene alterations with regards to their human homologs that were expressed in different organs or tissues and yet share close homology, could extend to human diseases as well as toxicological activities as an overture to find cures for many age-old and *cyps* gene-related diseases.

Supplementary Materials: The files are available online at the journal website.

Author Contributions: Conceptualization, S.Y.M.L., M.A.A., M.A. and Y.P.; methodology, M.A.A., M.A., C.K., C.M.F. and Y.P.; software, M.A.A.; validation, M.A.A., M.A., C.K., C.M.F. and Y.P.; formal analysis, S.Y.M.L.; investigation, S.Y.M.L.; resources, M.A.A., M.A., C.M.F. and Y.P.; data curation, S.Y.M.L.; writing – original draft preparation, S.Y.M.L.; writing – review and editing, S.Y.M.L., M.A. and C.K.

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