Effects of Essential Oil Inhalation on the Enhancement of Plasma and Liver Lipid Metabolism in Mice

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Article

Effects of Essential Oil Inhalation on the Enhancement of Plasma and Liver Lipid Metabolism in Mice

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Abstract: Background/Objectives: The purpose of this study was to determine the effects of essential oil inhalation on body weight, blood lipid profile, liver, and adipose tissue in mice. **Methods**: Middle-aged male mice (C57BL/6J) were exposed to *Lavandula angustifolia* (LO) and *Citrus aurantium* (CAO) essential oils for 7 weeks and compared to mice that did not receive essential oil inhalation treatment. Liver, white adipose tissue, and brown adipose tissue were sampled, kept at −80°C. **Results**: Although essential oil inhalation increased feed intake and body weight compared to control group, the amount of weight gain per feed intake was lower in the *C. aurantium* essential oil group. Moreover, relative weight of fat to body weight, liver fat amount, and blood cholesterol was lower, and triglyceride levels were significantly reduced. Reverse transcription polymerase chain reaction (RT-PCR) expression profiling of genes related to lipid metabolism confirmed changes in the regulation of thermogenesis-related gene *Ucp1* and the cholesterol synthesis-related genes *Hmgcs1* and *Hmgcr*. **Conclusions**: The inhalation of *C. aurantium* essential oil did not reduce the feed intake in mice however, its effectiveness in suppressing the increases in body weight and fat mass was demonstrated.

Keywords: aromatherapy; essential oil inhalation; *Citrus aurantium*; *Lavandula angustifolia* gene expression; lipid metabolism; anti-obesity

1. Introduction

The effects of essential oils through aromatherapy are used as a complementary therapy to promote and maintain human psychological and physiological health. Essential oils are administered by drinking, dermal, and intranasal means. Although most of the uses of essential oils are in the field of cosmetics and perfumes, essential oils have been known since ancient times to have bactericidal and antibacterial properties and have been widely used as antiseptics and infection preventatives. Furthermore, essential oils have anti-inflammatory and antioxidant activity, suggesting the possibility of dietary essential oil supplementation in the prevention of several diseases, including brain dysfunction, cancer, heart disease, and impaired immunity.

The physiological and pharmacological effects of aromatherapy using essential oils have been shown to include antibacterial, anti-stress, anti-depressant, anti-inflammatory, antioxidant, and relaxing effects [1]. The oil is volatile, easily crosses the blood-brain barrier, and also acts directly on brain neurons [2]. Essential oil odor exposure has demonstrated neuropharmacological effects in animal models by affecting multiple neurotransmitter systems [3]. but much of the research on the

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pharmacological effects of essential oils due to their olfactory stimulation has focused on their relaxing and relaxing effects. In other words, essential oils are promoted for their anti-anxiety effects [4,5].

Citrus aurantium (bitter orange) is native to East Asia, and is widely cultivated in the warmer regions of the planet such as the Mediterranean, African continent, South America, and California. The bitter orange essential oil obtained by pressing the peel is used in the food industry, especially in liqueurs and soft drinks. It is widely used as a fragrance. Bitter orange essential oil is extracted from the rind, but sweet orange, neroli for flowers and petitgrain for leaves are produced from the fruit [6] and are widely used in aromatherapy for relaxation, sleep promotion and digestion. Inhalation of *C. aurantium* essential oil has been reported to have sedative and relaxing effects in rats and mice [7–9], to relieve anxiety and pain specific to women [10–12], and to improve sleep quality [13,14].

Recent studies have demonstrated that essential oils and aromatic compounds regulate food intake and energy expenditure. Patchouli essential oil [15], citronella oil [16], fennel essential oil [17] and grapefruit oil [18] have all been shown to reduce food intake and body weight and improve lipid metabolism by inhalation. On the other hand, olfactory stimulation from the smell of lavender essential oil suppressed lipolysis and increased appetite and weight [19]. These reports suggest that the scent of essential oils can be used to help people lose weight in obese people and stimulate appetite in people with anorexia, and that aromatherapy can contribute to the prevention and treatment of eating disorders.

The active ingredient synephrine, which is abundant in the extract of the immature fruit of *C. aurantium*, has been reported in studies to act on fat cells and promotes the burning of body fat [20–22] and is used as a dietary supplement [23,24], and is widely used as a sports performance enhancer [25–27]. However, oral administration has been the most common method of administration in these studies. The National Collegiate Athletic Association (NCAA) has listed synephrine (bitter orange) as a stimulant on its most recent list of banned substances [28]. Although synephrine is not currently a prohibited substance, it is listed as a stimulant in the World Anti-Doping Code Monitoring Program, the international standard for the 2025 Prohibited List.

Improving lipid metabolism by inhalation, which is safer, is therefore attractive. However, there are less studies showing that essential oil inhalation acts on weight loss and heat production in adipose tissue. The present study therefore aimed to determine the potential of aromatherapy to improve lipid metabolism by investigating the effects of inhalation of *C. aurantium* peel essential oil (CAO) and *Lavandula angustifolia* flower buds essential oil (LO) on body weight, blood lipid profile, liver and adipose tissue in the middle-aged mice.

2. Results

2.1. Effects of Essential Oil Inhalation on Mouse (C57BL/6J) Food Intake, Body Weight and Fat Mass

Total food intake (g), body weight gain (g), white fat cell mass (g) and relative fat organ mass (%) of mice (C57BL/6J) after 7 weeks of essential oil (*Lavandula angustifolia*) (*Citrus aurantium*) inhalation are shown in Figure 1. As shown in Figure 1A (next page), the total food intake for 7 weeks was 167 g (DW, distilled water), 160 g (LO, *L. angustifolia* flower buds essential oil) and 206 g (CAO, *C. aurantium* peel essential oil). And, as shown in Figure 1B, the weight gain and percentage gain of mice after 7 weeks of essential oil inhalation was 1.04 g (3.38%, p value = 3.E-03) for DW, 1.82 g (5.96%, p value = 4.E-06) for LO and 1.17 g (3.53%, p value = 1.E-03) for CAO. The increase in white fat cell mass (g) of mice after 7 weeks of inhalation showed a significant trend for LO compared with DW (p value = 0.08) (Figure 1C). Furthermore, relative fat organ mass (%) showed a significant trend for an increase in LO (p value = 0.078) and a decrease in CAO compared to DW (p value = 0.095) (Figure 1D).

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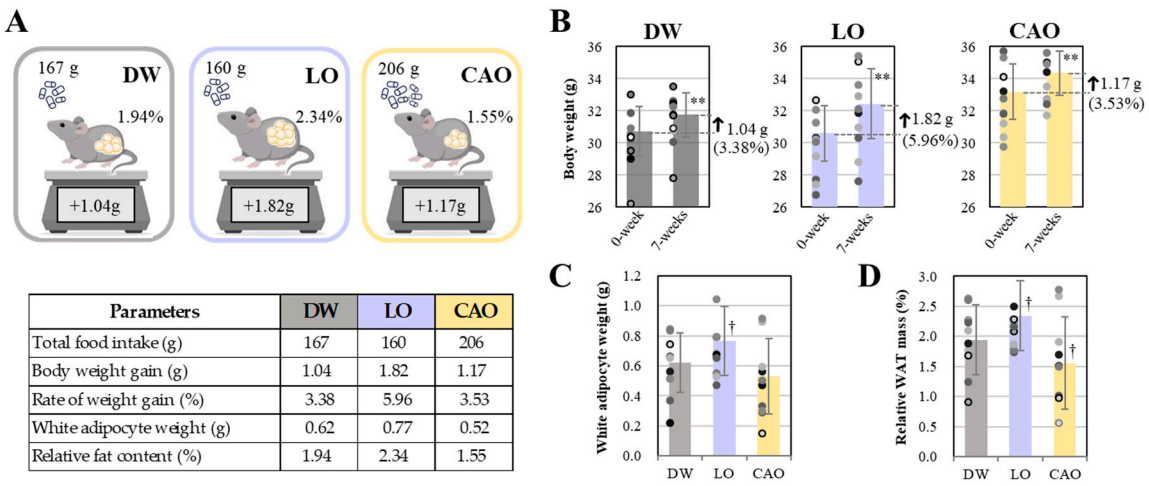

Parameters	DW	LO	CAO
Total food intake (g)	167	160	206
Body weight gain (g)	1.04	1.82	1.17
Rate of weight gain (%)	3.38	5.96	3.53
White adipocyte weight (g)	0.62	0.77	0.52
Relative fat content (%)	1.94	2.34	1.55

Figure 1. Total food intake (g), body weight gain (g) and relative white adipocyte tissue (WAT) mass (%) (A) of mice (C57BL/6J) inhaling essential oils for 7 weeks. Body weight gain (g) and percentage gain (%) (B) of mice after 7 weeks of inhalation of essential oils compared to body weight at the start of the experiment. White adipocyte weight (g) and relative WAT mass (%) (C, D) of mice after inhalation of essential oils for 7 weeks. DW (distilled water), LO (*Lavandula angustifolia)*, and CAO (*Citrus aurantium)* are shown as results for groups of 10 mice (n=10). Error bars represent SE (mean ± standard error). Significant: ** $p < 0.01$, * $p < 0.05$ vs. DW, Significant trend: †$0.05 \leq p < 0.1$ vs DW, Tukey method (n=10).

These results confirm that LO resulted in lower total food intake but higher rate of weight gain and relative fat organ mass compared to DW, whereas CAO inhalation resulted in higher total food intake but lower rate of weight gain and relative fat organ mass.

2.2. Effect of Essential Oil Inhalation on Liver Lipid Accumulation in Mice

To examine the effect of essential oil inhalation on liver lipid accumulation, liver sections from the DW, LO and CAO groups were stained with Oil Red O. The results are shown in Figure 2A (next page) and the graphical representation of Oil Red O staining area in Figure 2B. At 7 weeks after essential oil inhalation, liver lipid accumulation was significantly increased in LO compared with DW (p value = 1.E-03). CAO showed more lipid accumulation in staining compared to DW, but no significant difference was identified.

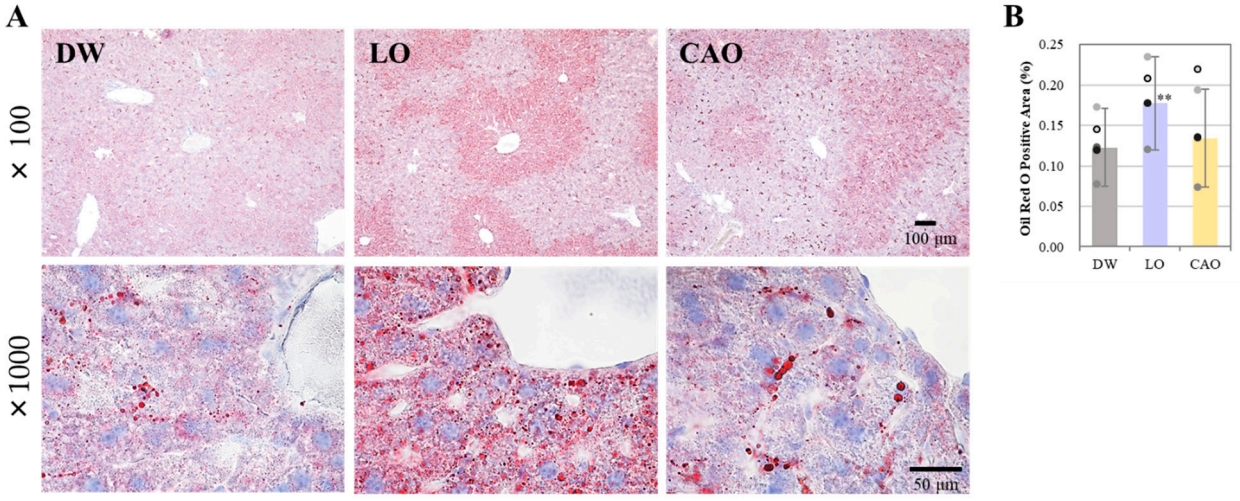

Figure 2. Testing the effect of essential oil inhalation on hepatic lipid accumulation in mice (C57BL/6J). Liver sections from the DW (distilled water) and LO (*Lavandula angustifolia*), and CAO (*Citrus aurantium*) groups were stained with Oil Red O. (A) 100x magnification, scale bar 100 μm, and 1000x magnification, scale bar 50 μm.

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Quantification of Oil Red O staining was performed using imageJ (B). Error bars represent SE (mean ± standard error). ** $p < 0.01$ vs. DW, Tukey method (n=5).

2.3. Effect of Essential Oil Inhalation on Blood Lipid Profiles of Mice

The results of the blood lipid profile of mice inhaling essential oil for 7 weeks are shown in Figure 3. Total cholesterol (TM Cholesterol), LDL and HDL cholesterol were significantly reduced in CAO compared to DW (LDL: p value = 0.014, HDL: p value = 2.E-03). Triglycerides were significantly reduced in LO and CAO (LO: p value = 7.E-04, CAO: p value = 3.E-05), but CAO was found to be more reduced compared to LO; NEFA (free fatty acids) were unchanged and phospholipids were found to be significantly reduced in CAO (p value = 0.051).

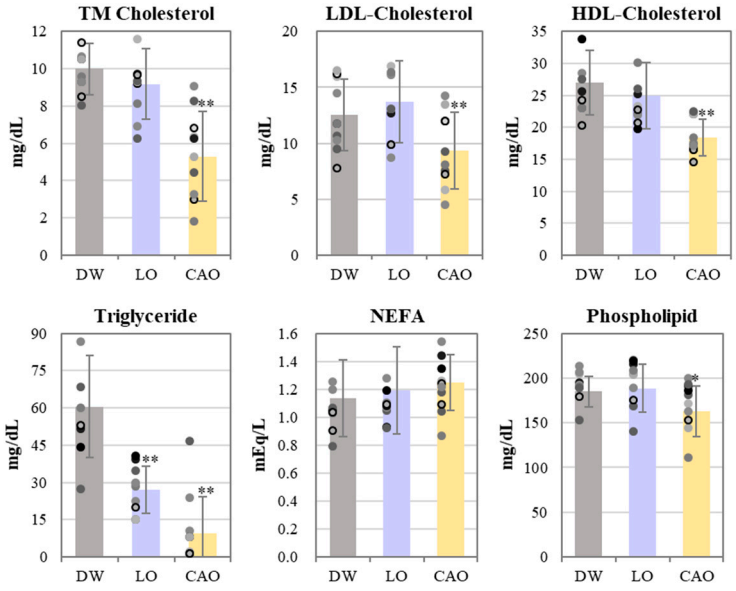

Figure 3. The blood lipid profile of mice (C57BL/6J) inhaling essential oil for 7 weeks. Values of total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, free fatty acids and phospholipids in blood absorbing DW (distilled water), LO (*Lavandula angustifolia)*, and CAO (*Citrus aurantium)* for 7 weeks. Error bars represent SE (mean ± standard error). ** $p < 0.01$, * $p < 0.05$ vs. DW, Tukey method (n=10).

2.4. Effects of Essential Oil Inhalation on Genes Associated with Adipogenesis

The mRNA expression levels of adipogenesis genes (*Fasn*, *Scd1*, *Insig2*), LDL transport protein genes (*Apob*), HDL biosynthesis genes (*Apoa1*), fat burning (*Ucp1*), cholesterol biosynthesis genes (*Hmgcr*, *Hmgcs1*) and bile acid biosynthesis (*Cyp7a1*) were assessed in WAT, BAT and liver to determine whether essential oil inhalation affects the expression of genes associated with adipogenesis (Figure 4). Compared to DW, the *Fasn*, *Scd1*, *Insig2*, *Hmgcr*, *Hmgcs1* and *Cyp7a1* gene expression was significantly down-regulated in CAO compared to DW in liver (p value = *Fasn* : 0.005, *Scd1* : 0.004, *Insig2*: 0.036, *Hmgcr* : 0.018, *Hmgcs1* : 0.007, *Cyp7a1* : 0.014) and WAT (p value = *Fasn* : 0.009, *Scd1* : 0.026, *Insig2*: 0.016, *Hmgcr* : 0.042) , and the *Apob*, *Apoa1* and *Ucp1* genes were significantly up-regulated in BAT (p value = *Apob* : 0.005, *Apoa1* : 0.012) and WAT (p value = *Apob* : 0.058, *Apoa1* : 0.034, *Ucp1* : 0.001). On the other hand, compared to DW, *Fasn* and *Cyp7a1* in LO were significantly down-regulated in liver (p value = *Fasn* : 0.006, *Cyp7a1* : 0.042) and WAT (p value = *Fasn* : 0.004), and the *Apoa1* in WAT (p value = *Apoa1* : 3.E-04) and *Hmgcs1* genes in BAT (*Hmgcs1* : 0.024). Furthermore, compared to DW, the *Fasn*, *Apob* and *Ucp1* in LO WAT (p value = *Apob* : 0.009, *Ucp1* : 0.006) and *Scd1* (p value = 0.038) in BAT were significantly up-regulated.

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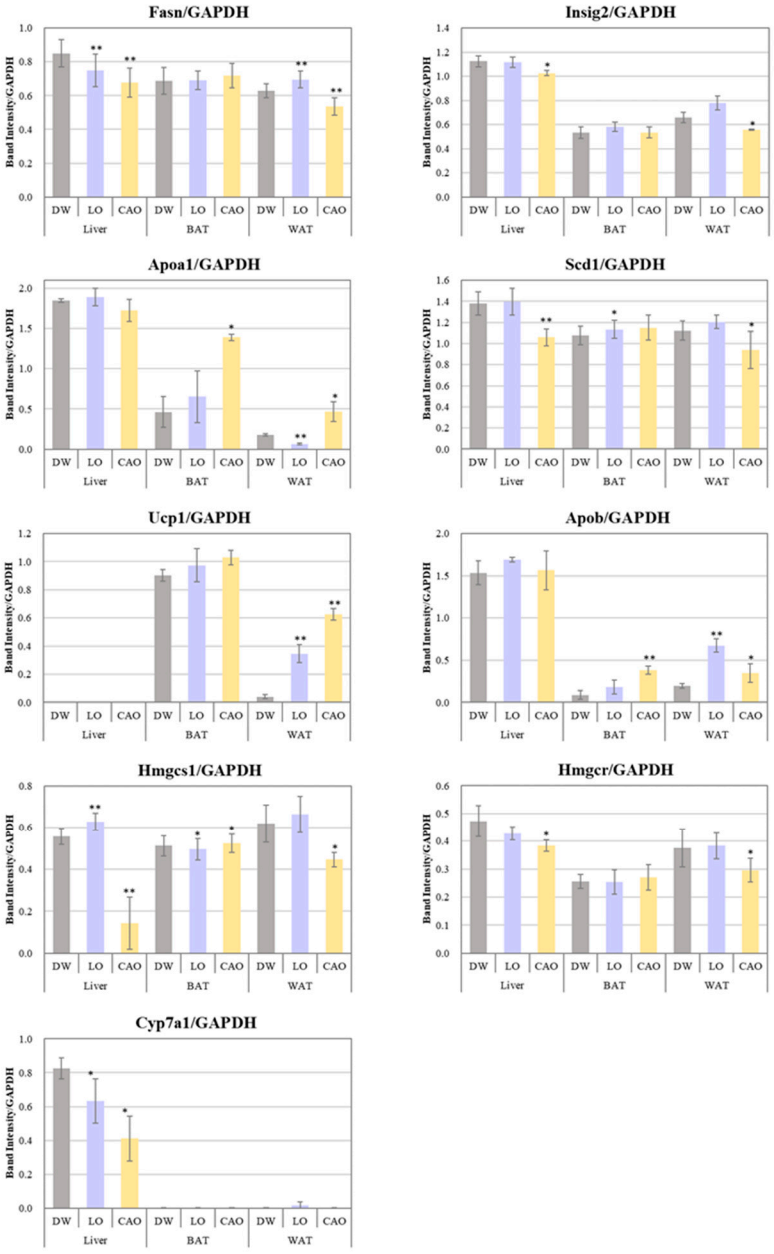

Figure 4. Effect of 7 weeks of essential oil inhalation on gene expression in the mice (C57BL/6J) liver, WAT (white adipose tissue) and BAT (brown adipose tissue). Band intensities of RT-PCR products after electrophoresis were corrected for GAPDH and displayed graphically. Error bars represent SE (mean ± standard error). ** $p < 0.01$, * $p < 0.05$ vs. DW (distilled water), Tukey method (mouse tissue – n=10, PCR technical replicate – n=3).

3. Discussion

Inhalation of the *C. aurantium* peel essential oil not only reduces weight gain by not reducing the food intake, but also lowers the serum cholesterol level. While it is a natural consequence of reduced food intake that both weight gain and serum cholesterol level decreases, the present study confirms that CAO inhalation reduces weight and fat gain without reducing the food intake, and also lowers serum cholesterol and triglyceride levels. The anti-obesity effect of CAO can be explained by the decreased expression of the liposynthesis genes *Fasn*, *Scd1* and *Insig2* and the cholesterol synthesis genes *Hmgcr* and *Hmgcs1* in the liver and WAT, as well as the fat burning effect due to increased expression of the *Ucp1* gene in the WAT (Figure 5A). In contrast, LO showed an increased expression of *Ucp1* genes in WAT, but no suppression in the case of the *Fasn*, *Scd1*, *Insig2*, *Hmgcr* and *Hmgcs1* genes. Hmgcs1 in particular is an important enzyme in the mevalonic acid pathway of cholesterol

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synthesis. Recent studies have shown that dyslipidaemia can be treated by either reducing the expression of *Hmgcs1* or directly inhibiting its activity [25,26]. Statins used as standard treatment for patients with high cholesterol are *Hmgcs1* inhibitors. Therefore, the increased *Hmgcs1* expression in the liver and WAT with LO inhalation compared to DW, and decreased Hmgcs1 expression with CAO inhalation, was correlated with the results of increased relative fat organ mass (%) and liver fat mass with LO and that decreased with CAO (Figure 5A).

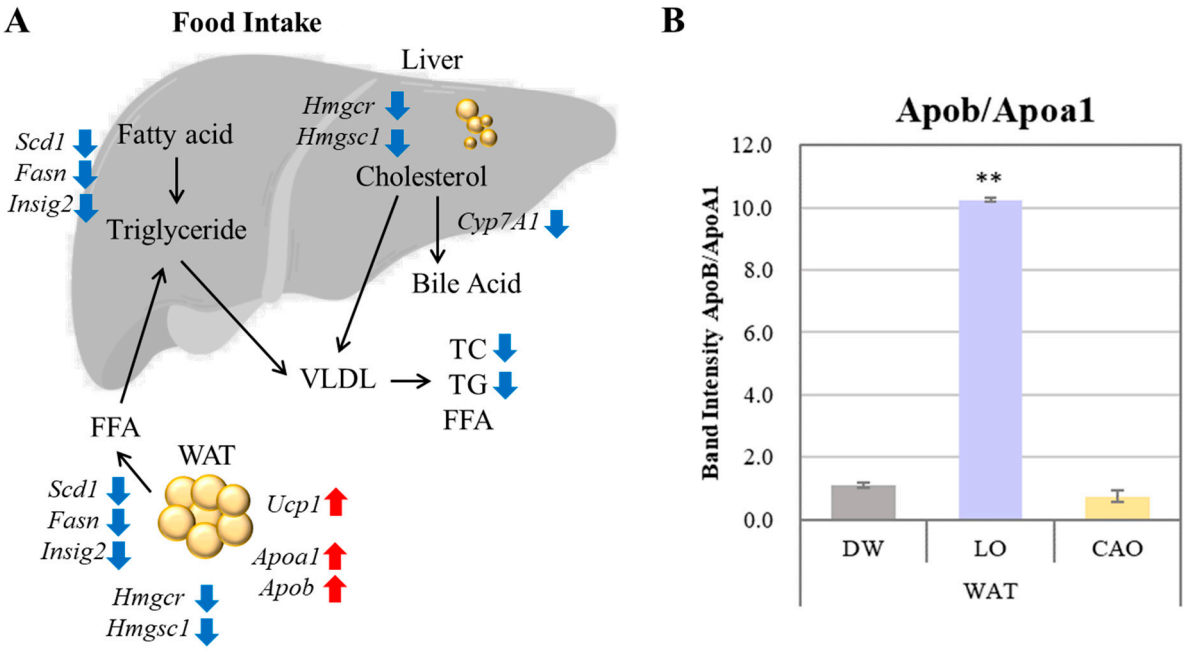

Figure 5. Gene expression changes in mouse (C57BL/6J) liver and WAT (white adipose tissue) after 7 weeks of LO (*L. angustifolia*), and CAO (*C. aurantium*) inhalation. (A), Apob/Apoa1 gene expression ratio in WAT after 7 weeks of essential oil inhalation (B), Error bars represent SE. Tukey method (mouse tissue n=10, PCR technical replicate n=3). VLDL: very low density lipoprotein, TC: total cholesterol, TG: triglyceride, FFA: free fatty acids.

Apoa1 and *Apob* gene expression was up-regulated in adipose tissue by LO and CAO essential oil inhalation (Figure 5A). ApoA1 constitutes the major protein component of HDL and promotes the reverse transport of cholesterol from peripheral tissues to the liver, exerting an anti-arteriosclerotic effect. ApoB is a major protein component of triglyceride-rich lipoproteins and is involved in the transport of cholesterol from liver cells to peripheral cells, promoting cholesterol deposition in arteries and is a known indicator of acute myocardial infarction risk. The results of *Apoa1* and *Apob* gene expression in WAT by inhalation of LO and CAO essential oil in the present study are shown graphically as the ApoB/ApoA1 ratio (Figure 5B). The results showed a higher ApoB/ApoA1 ratio in LO compared to DW and CAO; the ApoB/ApoA1 ratio is considered a strong predictor of atherosclerotic cardiovascular disease [29]. Therefore, although *Apoa1* and *Apob* gene expression was enhanced by CAO essential oil inhalation, the ApoB/ApoA1 ratio was not high, which may be related to the lower relative fat organ mass (%) in CAO than in LO.

Cyp7a1 (cholesterol 7α-hydroxylase) is the rate-limiting enzyme that regulates the biosynthesis of bile acids. Many studies have reported that activating Cyp7a1 to promote bile acid synthesis can reduce high-fat diet-induced hypercholesterolaemia [27]. In the present study, CAO was found to down-regulate the *Cyp7a1* gene, which may be partly due to the fact that all reported studies of the anti-obesity effects of Cyp7a1 activation are under conditions of a high-fat diet rather than a normal diet. Increased cholesterol in the liver increases *Cyp7a1* expression, which increases cholesterol extravasation [28,30,31], and since Cyp7a1 promotes bile acid synthesis reactions using cholesterol as a substrate, the down-regulation of *Cyp7a1* by CAO inhalation in the present study may be a possible

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mechanism to increase hepatic lipid and cholesterol synthesis-related genes *Fasn*, *Scd1*, *Insig2*, *Hmgcr* and *Hmgcs1*, suggesting that it is associated with the suppression of these genes.

About 96% of the components of CAO are the monoterpene hydrocarbon limonene. Inhalation of limonene has been reported to have anti-inflammatory [32,33], anti-stress [34] and analgesic effects [35] in addition to antioxidant effects, but there are no reports of improved lipid metabolism. β-myrcene, which has the next highest content (1.59%) after limonene, has been reported to have anxiolytic and sedative effects by inhalation [36], but like limonene, there are no reports on improving lipid metabolism. Therefore, in future studies, it would be desirable to conduct more detailed research on which components of CAOs improve lipid metabolism in order to elucidate the anti-obesity effects of CAO inhalation.

4. Materials and Methods

4.1. Mouse Maintenance and Essential Oil Inhalation

The essential oil inhalation method in mice is shown in Figure 6 (next page). Briefly, twenty-six-week-old C57BL/6J mice were randomly allocated to groups of 10 (n=10) and exposed to each essential oil (LO (*Lavandula angustifolia*), CAO (*Citrus aurantium*)) or DW (distilled water) continuously on a daily basis after 1 week of acclimatization. After 7 weeks, animals were weighed and dissected to obtain the samples for analyses (Figure 6).

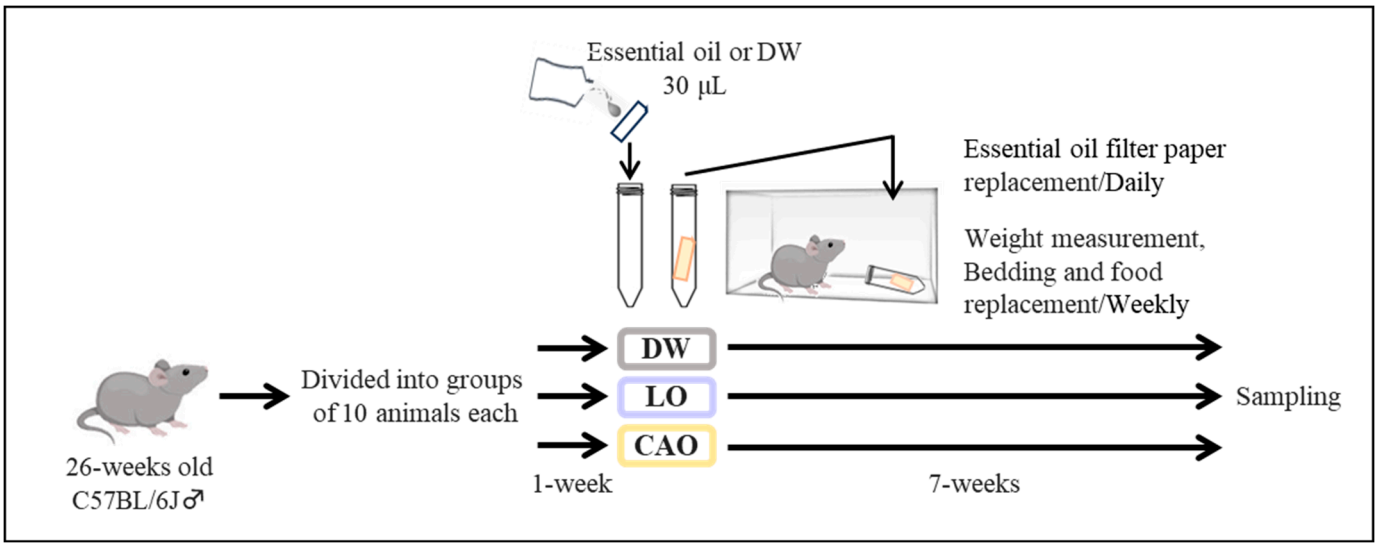

Figure 6. The study experimental design and essential oil exposure methodology for the research.

The 26-week-old C57BL/6J mice were randomly divided into groups of 10 each (n=10) into DW (distilled water), LO (*Lavandula angustifolia*, P-98: PRANAROM), and CAO (*Citrus aurantium*, P-37: PRANAROM) for 1 week and acclimatized. The essential oils used were purchased as commercially available oils from PRANAROM (Hainaut, Belgium). The table of the compositional analysis (by GC-MS; Gas Chromatography Mass Spectrometry) of PRANAROM's essential Oils, CAO and LO used in this study are presented in the (Supplementary Data).

Mice were housed ad libitum at 22±2°C from 6:00 to 18:00 with a 12 h/12 h light/dark cycle. After acclimatization, mice were continuously exposed to each essential oil (LO, CAO) or DW every day. For essential oil exposure, a filter paper with 30 μL of essential oil was dropped into a 1.5 mL microfuge tube that was placed in the cage with the tube cap open, and the tube was replaced with a new tube at 10 a.m. the next day. Body weight was measured weekly, and bedding and food were changed on the same day. After measuring body weight and diet at 7 weeks, the animals were dissected for sample collection. No animals were excluded from the sample/present study.

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4.2. Sampling

Seven weeks after the start of essential oil inhalation, triple anesthesia (Medetomidine (Kyoritsu Seiyaku Corporation, Tokyo, Japan), Midazolam (Nichi-Iko Pharmaceutical Co., Ltd., Toyama, Japan) and Putorphanol (Meiji Seika Pharma Co., Ltd., Tokyo, Japan)) was administered, and cardiac blood was collected. Thereafter, the liver, white adipose tissue (WAT), and brown adipose tissue (BAT) were sampled. The collected blood was passed through blood heparin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), centrifuged at 4°C, 11,000 rpm for 10 minutes, and the supernatant was stored at -80°C. A portion of the liver was saved for frozen sectioning. Liver, WAT, and BAT were stored at −80°C until ribonucleic acid (RNA) extraction.

4.3. Oil Red O Staining

Livers were fixed in 4% paraformaldehyde/PB solution overnight at 4°C. Fixed tissues were replaced with PBS for 24 hours, 20% sucrose for 24 hours, 30% sucrose for 48 hours, and frozen embedded. Frozen sections were prepared. Oil red O (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). Oil red O was prepared by mixing oil red and DW (Oil red O: DW=6:4) before use and filtered to obtain a staining solution. Frozen section slides were air-dried for 5 minutes and soaked in 60% isopropanol (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). The staining solution was added dropwise and allowed to stand at 37°C for 15 minutes. The cells were quickly passed through 60% isopropanol, nuclear staining was performed with hematoxylin (Fujifilm Wako), and the cells were mounted. Section images were taken using a BZ-X 710 bright field system (Keyence Corp, Osaka, Japan), and images were analyzed using ImageJ software, version 1.54. (National Institute of Health, Bethesda, MD, USA).

4.4. LabAssay

Plasma triglyceride, NEFA (Non-esterified Fatty Acid), Cholesterol, LDL-Cholesterol and high-density lipoprotein (HDL)-Cholesterol were detected using LabAssay (Fujifilm Wako) according to the manufacturer's protocol.

4.5. Total RNA Extraction for Gene Expression Analysis

Liver, WAT, and brown adipose tissue stored at -80°C was ground in a mortar in liquid nitrogen, mixed with QIAzol Lysis Reagent (Qiagen, Hilden, Germany), and then processed using the RNeasy Mini Kit (Qiagen). Total RNA was extracted according to the manufacturer's protocol. The RNA concentration of the extracted RNA was measured using a microvolume spectrophotometer (DS-11, DeNovix, Wilmington, DE, USA), and the 260/280 and 260/230 ratios were confirmed to be 1.8 or higher.

4.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The cDNA was synthesized from extracted RNA using Affinity Script QPCR cDNA synthesis kit (Agilent, Santa Clara, CA, USA). PCR reactions were performed using Emerald Amp PCR Master (Takara, Kusatsu, Japan) using primers specific to the gene of interest (Table 1). The PCR reaction consisted of initial denaturation at 97 °C for 5 min, heat denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, 27–36 cycles, and extension at 72 °C for 10 min. After the PCR reaction, the PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide under UV light [37]. The expression level of the visualized target gene was corrected by the expression level of the GAPDH gene, which is a known housekeeping gene, and was graphed.

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Table 1. The primer sequences and each primer set (gene-specific primers) used for the gene expression analyses experiment in this study.

	Forward Primer	Reverse Primer	
Accession	**Nucleotide sequence (5'-3')**	**Nucleotide sequence (5'-3')**	**Gene name**
NM_007988	AATCCATCATCAACATCATCCA	CCACTGACTCTTCACAGACCAG	*Fasn*
NM_009692	ACGAATTCCAGAAGAAATGGAA	GTGGTACTCGTTCAAGGTAGGG	*Apoa1*
NM_009127	CACCTTCTTGCGATACACTCTG	CTCCCGTCTCCAGTTCTCTTAA	*Scd1*
NM_009463	AACTCTCTGCCAGGACAGTACC	AACGGAGCTGTTCATTTGATTT	*Ucp1*
NM_009693	GTTGGTGAGTCCACAAGATTGA	GCTTGGTTGCAGGTATAGTTCC	*Apob*
NM_145942	TGGTATCTGGTCAGAGTGGATG	GACCACAACAGGAAGCATGTTA	*Hmgcs1*
NM_001360166	TCACATGGTT CACAACAGAT CA	GCAC AGAGACTCCT CAGATGTG	*Hmgcr*
NM_153526	ACCACGTCTGGAACTATCCAAG	CTCCCAGGTGACTGTCAATACA	*Insig1*
NM_007824	AAATACGACCGGTACCTTGATG	TAACGCTCAGCAGTCGTTACAT	*Cyp7a1*
NM_001001303	GCTACACTGAGGACCAGGTTGT	CTCCTGTTATTATGGGGGTCTG	*GAPDH*

4.7. Statistical Analysis

Data are expressed as mean ± standard error (SE). Statistical comparisons were made using the Tukey test for multiple comparisons. The p-values less than 0.05 were considered significant, and p-values between 0.05 and 0.1 were considered a significant trend. Significant: **p<0.01, *p<0.05, significant trend: †0.05 ≤ p < 0.1.

5. Conclusions

Aromatherapy may be a safe and efficient intervention and a simple and applicable tool for an actionable affect towards improving eating disorders and lipid metabolism. The identification of the beneficial effects of the diverse bioactive substances in citrus peels used for essential oils could also be aimed at valorizing waste products and improving the quality of by-products. In this study, CAO inhalation was found to improve lipid metabolism in the blood and liver. However, the mechanism underlying the increased food intake due to CAO and LO inhalation is not clear.

Our laboratory has been experimenting with essential oil exposure to regulate feeding in mice for quite a few years. Leite and co-workers [7] describes essential oil exposure methods and, like numerous references, examines many conditions such as dilution of essential oil, time, and high fat diet. However, based on our (groups) experience to date, it has been understood that most of the conditions do not yield clear results. So, we reviewed all the related literature and combined with our experience (previous research), the experimental design of this study was prepared. Here, we use middle-aged mice maintained on a normal diet, and the essential oil was simply changed daily with 30 microliters of undiluted solution for 7 weeks, and we do not believe that or have identified there are any reports of experiments with a similar design.

It is known that the scent of essential oils affects physiological functions. It was confirmed that neurotransmission by the scent of osmanthus decreased the mRNA expression of appetite-promoting neuropeptides such as agouti-related protein, neuropeptide Y, melanin-concentrating hormone, and prepro-orexin, and further increased anorexia neuropeptides, which suppressed feeding [38]. Furthermore, our laboratory has confirmed that the scent of ginger essential oil may promote feeding via hypothalamic MCH [39]. However, further research is needed to understand the potential benefits of essential oil inhalation, including whether odor stimulation affects neuropeptide-containing neurons in the hypothalamus that control appetite.

Because there are few reports of experiments on lipid metabolism by inhalation of essential oil, and the purpose of this experiment was to obtain basic data, and the C57BL/6J mice, which have high genetic uniformity and good experimental reproducibility, were used as models for many human

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diseases, including obesity and diabetes. Thus, the C57BL/6J mice were used for this study. Moreover, the experimental methods and conditions of this study are likely to generalize to other species. The essential oils used in this study are commonly used (e.g., at home) and nasal inhalation is a simple method. Also, the evaluation of blood samples has a very high potential for use in human clinical experiments.

Although the effects of nasal inhalation of essential oil on lipid metabolism and body weight in mice were confirmed, the essential oil aromatic components that affect lipid metabolism and body weight remain to be determined. This experimental design presented in this study is characterized by its ease of application to humans. We would like to conduct the human clinical trials in the future to identify essential oil components that affect lipid metabolism and body weight, and to apply the results not only for weight loss, but also for the elderly and others who need to gain weight in a healthy manner.

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Institutional Review Board Statement: This study was performed under the strict guidelines of the Hoshi University of Pharmacy and Life Sciences Animal Ethics Review Board. All the experimental procedures involving animals (mice) in this study were approved by the Institutional Animal Care and Use Committee of Hoshi University. Specifically, the “Effects of essential oil aromatic components on feeding center" has been approved for ethical review of animal experiments since 2019. Additionally, the researchers handling the mice undertook animal experimentation training and obtained permission to use the animal center (Number: 15-A404 (M.Y.)) since 2015 to date.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article. The raw data are available upon reasonable request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Dhifi, W.; Bellili, S.; Jazi, S.; Bahloul, N.; Mnif, W. Essential oils' chemical characterization and investigation of some biological activities: A critical review. Medicines (Basel, Switzerland) **2016**, 3(4), 25.
2. Spisni, E.; Valerii, M.C.; Massimino, M.L. Essential Oil Molecules Can Break the Loop of Oxidative Stress in Neurodegenerative Diseases. Biology (Basel) **2023**, 12(12), 1504.
3. Lizarraga-Valderrama, L.R. Effects of essential oils on the central nervous system: focus on mental health. Phytother. Res. **2021**, 35(2), 657-679.
4. Zhu, M.Y.; Dong, W.Y.; Guo, J.R.; Huang, J.Y.; Cheng, P.K.; Yang, Y.; Liu, A.; Yang, X.L.; Zhu, X.; Zhang, Z.; et al. A Neural Circuit For Bergamot Essential Oil-Induced Anxiolytic Effects. Adv. Sci. (Weinh) **2025**, 12(1), e2406766.
5. Zhang, Y.; Long, Y.; Yu, S.; Li, D.; Yang, M.; Guan, Y.; Zhang, D.; Wan, J.; Liu, S.; Shi, A.; et al. Natural volatile oils derived from herbal medicines: a promising therapeutic way for treating depressive disorder Pharmacol. Res. **2021**, 164, 105376.
6. Suntar, I.; Khan, H.; Patel, S.; Celano, R.; Rastrelli, L. An Overview on Citrus aurantium L.: Its Functions as Food Ingredient and Therapeutic Agent. Oxid. Med. Cell Longev. **2018**, 2018, 7864269.
7. Leite, M.P.; Fassin, Jr. J.; Baziloni, E.M.F.; Almeida, R.N.; Mattei, R.; Leite, J.R. Behavioral effects of essential oil of Citrus aurantium L. inhalation in rats. Braz. J. Pharmacogn. **2008**, 18, 661-666.

 doi:10.20944/preprints202505.1059.v1

8. de Moraes Pultrini, A.; Almeida Galindo, L.; Costa, M. Effects of the essential oil from Citrus aurantium L. in experimental anxiety models in mice. Life Sci. **2006**, 78(15), 1720-1725.
9. Tan, L.; Liao, F.F.; Long, L.Z.; Ma, X.C.; Peng, Y.X.; Lu, J.M.; Qu, H.; Fu, C.G. Essential oils for treating anxiety: a systematic review of randomized controlled trials and network meta-analysis. Front. Public Health **2023**, 11, 1144404.
10. Scandurra, C.; Mezzalira, S.; Cutillo, S.; Zapparella, R.; Statti, G.; Maldonato, N.M.; Locci, M.; Bochicchio, V. The effectiveness of neroli essential oil in relieving anxiety and perceived pain in women during labor: a Randomized controlled trial. Healthcare (Basel) **2022**, 10(2), 366.
11. Nascimento, J.C.; Gonçalves, V.S.D.S.; Souza, B.R.S.; Nascimento, L.C.; Carvalho, B.M.R.; Nogueira, P.C.L.; Santos, J.P.S.; Borges, L.P.; Goes, T.C.; Souza, J.B.; et al. Effectiveness of aromatherapy with sweet orange oil (Citrus sinensis L.) in relieving pain and anxiety during labor. Explore (NY) **2024**, 21(1), 103081.
12. Bargi, S.; Bahraminejad, N.; Jafari, S.; Fallah, R. The Effect of Aromatherapy with Citrus aurantium Aroma on Pain after Orthopedic Surgery: A Randomized Clinical Trial. J. Caring Sci. **2023**, 12(2), 116-122.
13. Fang, C.S.; Tu, Y.K.; Chou, F.H.; Fang, C.J.; Chang, S.L. Effect of inhaled aromatherapy on sleep quality in critically ill patients: A systematic review and network meta-analysis. J. Clin. Nurs. **2024**, 34(3), 1000-1012.
14. Mohammadi, F.; Moradi, M.; Niazi, A.; Jamali, J. The Impact of Aromatherapy with Citrus Aurantium Essential Oil on Sleep Quality in Pregnant Women with Sleep Disorders: A Randomized Controlled Clinical Trial. Int. J. Community Based Nurs. Midwifery. **2022**, 10(3), 160-171.
15. Hong, S.J.; Cho, J.; Boo, C.G.; Youn, M.Y.; Pan, J.H.; Kim, J.K.; Shin, E.-C. Inhalation of Patchouli (Pogostemon Cablin Benth.) essential oil improved metabolic parameters in obesity-induced Sprague Dawley rats. Nutrients **2020**, 12, 2077.
16. Batubara, I.; Suparto, I.H.; Sa'diah, S.; Matsuoka, R.; Mitsunaga, T. Effects of inhaled citronella oil and related compounds on rat body weight and brown adipose tissue sympathetic nerve. Nutrients **2015**, 7(3), 1859-1870.
17. Hong, S.J.; Yoon, S.; Jo, S.M.; Jeong, H.; Youn, M.Y.; Kim, Y.J.; Kim, J.K.; Shin, E.C. Olfactory Stimulation by Fennel (Foeniculum vulgare Mill.) Essential Oil Improves Lipid Metabolism and Metabolic Disorders in High Fat-Induced Obese Rats. Nutrients **2022**, 14(4), 741.
18. Shen, J.; Niijima, A.; Tanida, M.; Horii, Y.; Maeda, K.; Nagai, K. Olfactory stimulation with scent of grapefruit oil affects autonomic nerves, lipolysis and appetite in rats. Neurosci. Lett. **2005**, 380, 289-294.
19. Shen, J.; Niijima, A.; Tanida, M.; Horii, Y.; Maeda, K.; Nagai, K. Olfactory stimulation with scent of lavender oil affects autonomic nerves, lipolysis and appetite in rats. Neurosci. Lett. **2005**, 383(1-2), 188-193.
20. Guimarães, A.C.; de Moura, E.G.; Silva, S.G.; Lopes, B.P.; Bertasso, I.M.; Pietrobon, C.B.; Quitete, F.T.; de Oliveira Malafaia, T.; Souza, É.P.G.; Lisboa, P.C.; et al. Citrus aurantium L. and synephrine improve brown adipose tissue function in adolescent mice programmed by early postnatal overfeeding. Front. Nutr. **2024**, 10, 1278121.
21. Dodonova, S.A.; Zhidkova, E.M.; Kryukov, A.A.; Valiev, T.T.; Kirsanov, K.I.; Kulikov, E.P.; Budunova, I.V.; Yakubovskaya, M.G.; Lesovaya, E.A. Synephrine and Its Derivative Compound A: Common and Specific Biological Effects. Int. J. Mol. Sci. **2023**, 24(24), 17537.
22. Gougeon, R.; Harrigan, K.; Tremblay, J.F.; Hedrei, P.; Lamarche, M.; Morais, J.A. Increase in the thermic effect of food in women by adrenergic amines extracted from Citrus aurantium. Obesity Res. **2006**, 13, 1187-1194.
23. Sharpe Patricia, A.; Granner Michelle, L.; Conway Joan, M.; Ainsworth Barbara, E.; Dobre, M. Availability of weight-loss supplements: Results of an audit of retail outlets in a southeastern city. J. American Dietetic Assoc. **2006**, 106(12): 2045–2051.
24. "Bitter Orange". National Center for Complementary and Integrative Health. April 2008. Retrieved 2008-11-03.
25. Colker, C.M.; Kaiman, D.S.; Torina, G.C.; Perlis, T.; Street, C. Effects of Citrus aurantium extract, caffeine, and St. John's wort on body fat loss, lipid levels, and mood states in overweight healthy adults. Curr. Therapeutic Res. **1999**, 60(3), 145-153.

 doi:10.20944/preprints202505.1059.v1

26. Stohs, S.J.; Preuss, H.G.; Shara, M. Issues regarding a FACT review paper on the efficacy of herbal supplements containing Citrus aurantium and synephrine alkaloids for the management of overweight and obesity. Focus on Alternative and Complementary Therapies **2013**, 18(1), 45-47.
27. Haaz, S.; Fontaine, K.R.; Cutter, G.; Limdi, N.; Perumean-Chaney, S.; Allison, D.B. Citrus aurantium and synephrine alkaloids in the treatment of overweight and obesity: an update. Obes. Rev. **2006**, 7(1), 79-88.
28. Arbo, M.D.; Schmitt, G.C.; Limberger, M.F.; Charão, M.F.; Moro, A.M.; Ribeiro, G.L.; Dallegrave, E.; Garcia, S.C.; Leal, M.B.; Limberger, R.P. Subchronic toxicity of Citrus aurantium L. (Rutaceae) extract and p-synephrine in mice. Regul. Toxicol. Pharmacol. **2009**, 54(2), 114-117.
29. Deng, F.; Li, D.; Lei, L.; Yang, Q.; Li, Q.; Wang, H.; Deng, J.; Zheng, Q.; Jiang, W. Association between apolipoprotein B/A1 ratio and coronary plaque vulnerability in patients with atherosclerotic cardiovascular disease: An intravascular optical coherence tomography study. Cardiovasc. Diabetol. **2021**, 20, 188.
30. Song, T.; Wang, P.; Li, C.; Jia, L.; Liang, Q.; Cao, Y.; Dong, P.; Shi, H.; Jiang, M. Salidroside simultaneously reduces de novo lipogenesis and cholesterol biosynthesis to attenuate atherosclerosis in mice. Biomed. Pharmacother. **2021**, 134, 111137.
31. Ma, X.; Bai, Y.; Liu, K.; Han, Y.; Zhang, J.; Liu, Y.; Hou, X.; Hao, E.; Hou, Y.; Bai, G. Ursolic acid inhibits the cholesterol biosynthesis and alleviates high fat diet-induced hypercholesterolemia via irreversible inhibition of HMGCS1 in vivo. Phytomedicine **2022**, 103, 154233.
32. Chambers, K.F.; Day, P.E.; Aboufarrag, H.T.; Kroon, P.A. Polyphenol Effects on Cholesterol Metabolism via Bile Acid Biosynthesis, CYP7A1: A Review. Nutrients **2019**, 11(11), 2588.
33. Peet, D.J.; Turley, S.D.; Ma, W.; Janowski, B.A.; Lobaccaro, J.M.; Hammer, R.E.; Mangelsdorf, D.J. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell **1998**, 93, 693-704.
34. Jelinek, D.F.; Andersson, S.; Slaughter, C.A.; Russell, D.W. Cloning and regulation of cholesterol 7 alpha-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis. J. Biol. Chem. **1990**, 265, 8190-8197.
35. Attia, H.G.; El-Morshedy, S.M.; Nagy, A.M.; Ibrahim, A.M.; Aleraky, M.; Abdelrahman, S.S.; Osman, S.M.; Alasmari, S.M.; El Raey, M.A.; Abdelhameed, M.F. Citrus clementine Peel Essential Oil Ameliorates Potassium Dichromate-Induced Lung Injury: Insights into the PI3K/AKT Pathway. Metabolites **2024**, 14(1), 68.
36. Patel, M.; Narke, D.; Kurade, M.; Frey, K.M.; Rajalingam, S.; Siddiquee, A.; Mustafa, S.J.; Ledent, C.; Ponnoth, D.S. Limonene-induced activation of A(2A) adenosine receptors reduces airway inflammation and reactivity in a mouse model of asthma. Purinergic Signal. **2020**, 16(3), 415-426.
37. Wittmeier, P.; Hummel, S. Agarose gel electrophoresis to assess PCR product yield: comparison with spectrophotometry, fluorometry and qPCR. Biotechniques. **2022**, 72(4), 155-158.
38. Yamamoto, T.; Inui, T.; Tsuji, T. The odour of Osmanthus fragrans attenuates food intake. Sci. Rep. **2013**, 3, 1518.
39. Takenoya, F.; Yamashita, M.; Shibato, J.; Kimura, A.; Chiba, Y., Hirabayashi, T., Shioda, S. Histological observation of rat hypothalamic feeding-regulated neurons in peppermint and ginger essential oil exposure. J. Japanese Society Aromatherapy **2020**, 21(1), 40-48.