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ORIGINAL ARTICLE

Nutrition, Metabolism, and Prevention of NCDs

Ameliorative Effects of *Citrus aurantium* Juice on Metabolic Disorders Induced by Trans Fatty Acid-Rich Amalgam in Wistar rats

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ABSTRACT

Background: Consumption of Trans Fatty acids (TFA) has been correlated with an increased risk of obesity and the development of type 2 diabetes mellitus (T2DM).

Aims: This study aimed to assess the anti-obesogenic and anti-diabetic effect of *Citrus aurantium (CA)* juice in Wistar rats following the administration of a Trans fatty acids rich diet.

Methods: Male Wistar rats were administered CA juice after induction of hyperlipidemia using escalating concentrations of a fatty product. The fatty acid methyl ester profile of the fat amalgam was determined using gas chromatography-mass spectrometry (GC-MS). Phytochemical analysis of the CA juice was conducted employing ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS). Furthermore, the antioxidant activity of the juice was assessed through the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. TFA-induced metabolic disorders were assessed by measuring various anthropometric parameters (Body Mass Index BMI, Lee Index, Ponderal Index PI, Waist to Length Ratio WLR, Adiposity Index AI). Glycated hemoglobin HbA1c by nephelometry. Plasma pancreatic lipase activity, aspartate aminotransferase (GOT), and alanine aminotransferase (GPT) levels using biochemical kits. Additionally, liver tissues were microscopically examined after staining with hematoxylin and eosin (H&E).

Results: Obesogenic and diabetogenic effects were observed in rats receiving the high-fat amalgam. GC-MS analysis of the fatty product revealed the predominant presence of the trans fatty acid octadecadienoic acid. Qualitative UPLC-MS-MS identification of the CA juice highlighted the presence of 13 bioactive compounds, with epicatechin and quercetin being the most abundant. The juice exhibited significant antioxidant activity in the DPPH assay with an IC_{50} value of 1.125 µg/mL. Analysis of anthropometric parameters demonstrated a significant anti-obesogenic effect in animals treated with CA juice. Conversely, the administration of the fatty amalgam resulted in significant disturbances in the tested biochemical parameters. Notably, the administration of CA juice significantly contributed to the re-establishment of lipase expression, HbA1c levels, total cholesterol levels, GOT, and GPT. Histological examination of liver sections stained with Hematoxylin and Eosin revealed that the high-fat amalgam induced hepatic steatosis and pancreatic beta-cell disappearance. However, CA juice was able to decrease the extent of hepatic steatosis.

Conclusions: The administration of *Citrus aurantium* juice to rats demonstrated significant effectiveness in counteracting TFA- induced metabolic disorders.

Keywords: Trans Fatty Acids (TFA); *Citrus aurantium*; Metabolic Disorders; Obesity; Type 2 Diabetes Mellitus (T2DM).

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1 INTRODUCTION

Obesity is recognized as a metabolic condition, characterized by the excessive accumulation of adipose tissues. This pathological condition is frequently associated with Type 2 Diabetes Mellitus (T2DM) and a range of cardiovascular diseases (Stohs *et al.*, 2012). Abdominal obesity has been identified as significant precursor to the onset of T2DM with the accumulation of visceral adipose tissue being associated with both glucose intolerance and hyperinsulinemia (Girard, 2003).

In the context of managing body weight and glucose metabolism, *Citrus aurantium* (CA) commonly recognized as sour orange, bitter orange, Seville orange, or bigarade (Maksoud *et al.*, 2021; Mannucci *et al.*, 2018) has garnered attention as a dietary supplement (Stohs *et al.*, 2012). Originally from Southeast Asia, CA is now cultivated in Mediterranean regions, the Americas and Africa (Li *et al.*, 2017). While traditionally considered an ornamental, recent research has increasingly focused on the diverse biological effects of CA and its derivatives including antioxidant (Divya *et al.*, 2016), antimicrobial (Benzaid *et al.*, 2021; Gopal *et al.*, 2012; Kacániová *et al.*, 2020), anticancer (Jayaprakasha *et al.*, 2008), anxiolytic (Pimenta *et al.*, 2016), antidiabetic (Aslan *et al.*, 2023; Gandhi *et al.*, 2020), and anti-obesity effects (Aslan *et al.*, 2023; Gandhi *et al.*, 2020; Stohs *et al.*, 2011; Stohs *et*

al., 2017). Notably, several studies have demonstrated that CA consumption can enhance energy expenditure and fat oxidation, both at rest and physical activity, while also reducing appetite and promoting satiety (Fugh-Berman & Myers, 2004).

According to literature, mechanisms by which trans fatty acids rich amalgam in diets are able to promote many metabolic disorders by disturbing lipid metabolism, atherosclerosis, insulin resistance, oxidative stress, chronic inflammatory process, metabolic syndrome development, T2DM, and non-alcoholic liver steatosis (Mozzafarian et *al.*, 2009; Oteng & Kersten, 2020).

The interplay between fatty acids and glucose metabolism is pertinent to the development of T2DM. Elevated concentrations of free fatty acids (FFAs) have been observed diurnally in patients with T2DM (Reaven *et al.*, 1988), and the existence of a « glucose-fatty acids cycle » highlights their interrelation, with FFAs influencing insulin secretion (Girard, 2003). Chronic excessive FFA exposure can dysregulate gene expression and contribute to lipotoxicity (Unger, 1995; Unger, 2003). Mechanisms of this lipotoxicity are implicated in the deterioration of pancreatic beta-cells, a key feature of T2DM, which is characterized by a loss of the first phase of insulin secretion, diminished secretory oscillations, and a delayed and reduced postprandial insulin response, alongside increased pro-insulin secretion (Girard, 2003).

Trans fatty acids, formed during the industrial processing of vegetable oils and through ruminal biohydrogenation of animal fats, involve modifications of "cis" and "trans" double bonds (Zhang *et al.*, 2025). A substantial body of research has demonstrated the detrimental health effect of TFAs, particularly concerning cardiovascular diseases and the development of insulin resistance (Mozaffarian et *al.*, 2009; de Souza *et al.*, 2015).

The accumulation of TFAs is hypothesized to be a source of lipotoxicity, contributing to the dysfunction of pancreatic beta-cells and the pathogenesis of T2DM, a condition marked by a compromised early-phase insulin secretion, loss of pulsatile insulin release, impaired postprandial insulin secretion, and elevated pro-insulin levels (Boden *et al.*, 2003; Bowman *et al.*, 2018; Girard, 2003). Animal studies have revealed significant lipid accumulation in the islets of Langerhans, leading to beta-cells apoptosis (Girard, 2003; Postic *et al.*, 2008; Unger, 2003). This lipid accumulation is associated with several biochemical abnormalities, including decreased expression of the GLUT2 glucose transporter and impaired insulin secretion, as well as increased nitric oxide (NO) production and stimulation of apoptosis (Girard, 2003).

Given the established roles of both obesity and TFAs in the pathogenesis of T2DM, and the potential of *Citrus aurantium* as a modulator of metabolic processes, the present study investigates the biological activities of *Citrus aurantium* juice in a male Wistar rat model following exposure to a fatty product containing trans fatty acids (TFAs).

In the future, additional research could involve human studies with obesity and a long-term history of consuming an unbalanced high-fat diet. A key inclusion criterion should be elevated blood levels of trans-fatty acids. Alternatively, research could explore therapeutic interventions to mitigate the harmful effects of TFAs.

2 Material and Methods

2.1 Sample Collection and Botanical Identification

Citrus aurantium fruits were collected from an ornamental domestic tree located within a domestic setting in Saida, Algeria. The botanical identity of the collected sample was verified against the reference sample by the faculty's designated expert, and an herbarium voucher specimen (number TM-CA-015-2023) was deposited. We collected 10 kg of fully ripened fruits in May 2023. These fruits were immediately transported and subsequently stored at 4°C at the laboratory of Water Resources and Environment, University of Saida, Algeria.

2.2 Origin and Preparation of TFAs

To investigate the deleterious effect of TFAs, a domestic fatty amalgam was prepared utilizing transformed animal and vegetable fats. The animal fats employed are derived from Algerian "*Mechoui*" preparation. Mechoui is an Algerian traditional meal which refers to lamb or mutton meat oven-baked until the complete separation of muscle from the bones. According to Davidson *et al.* (2014) "Mechoui is the name given in North Africa, especially in Algeria and Morocco, to a whole lamb spit-roasted or cooked in a pit. The term comes from the Arabic root *shawa* (to grill or roast). While the vegetable fats consisted of vegetal oils resulting from an over frying. Thus, this fat mixture underwent a heat treatment process. The resulting product was filtered and then subjected to analysis utilizing GC-MS technique.

2.3 Determination of the Chemical Nature of Fatty Acids in the Fatty Product by GC-MS

The analysis of the prepared fatty product was carried out employing a Hewlett Packard Agilent 6890 Plus gas chromatograph coupled with Hewlett Packard Agilent 5973 mass spectrometer detector.

2.3.1 Operational Conditions

- A 1 µL aliquot of the sample was injected in splitless mode at an injector temperature of 250°C.
- The chromatographic separation was performed using an HP-5MS column with the following dimensions: 30 m length x 0.25 mm internal diameter and a 0.25 µm film thickness. The stationary consisted of 5% phenyl and 95% dimethylpolysiloxane.
- The GC oven temperature program was as follows: initial temperature 70°C, ramped to 130°C, at a rate of 3°C/min, then to 220°C at 3°C/min, and finally to 280°C.
- Helium gas (purity N6), was used as the carrier gas at a flow rate 0.5 mL/min.
- Mass detection was performed in Scan Total Ion Current (TIC) mode, with a mass range of 30 to 550 m/z. The solvent delay was set at 3.5 minutes. The interphase temperature was maintained at 280 °C. Electron impact ionization was employed with a filament energy of 70 eV. A quadrupole mass analyzer was used, and the ion source temperature was maintained at 230 °C.

2.4 Composition of Diets

- Standard diet.
- TFAs diet: The standard diet was supplemented with the prepared fatty product containing high concentrations of the trans fatty acids 9,12-octadecadienoic acid, methyl ester (E,E)- and 9,11-octadecadienoic acid, methyl ester (E,E)- (Table 02).

2.5 Preparation of *Citrus aurantium* juice

Fresh juice was extracted from the entire *Citrus aurantium* fruit using an electric juice extractor immediately prior to administration. The obtained juice was subsequently filtered to remove any residual fibers and excess seeds (Ezeigwe *et al.*, 2022; Haraoui *et al.*, 2019; Jabri Karoui & Marzouk, 2013; Tounsi, 2010).

2.5.1 Chemical Composition of CA Juice

The analysis of the *Citrus aurantium* juice was performed utilizing a Shimadzu 8040 Ultra-High Sensitivity UPLC-ESI-MS-MS system, equipped with UFMS technology and a binary bump Nexera XR LC-20AD.

Electrospray Ionization (ESI) conditions

- The electrospray ionization source was operated under the following conditions: collision-induced dissociation (CID) gas pressure, 230 kPa; conversion dynode voltage, –6.00 kV; desolvation line (DL) temperature, 250 °C; nebulizing gas flow rate, 3.00 L/min; heat block, 400 °C; drying gas flow rate, 10 L/min.

The mobile phase consisted of:

- The mobile phase A: 2 mmol ammonium formate + 0.002% formic acid in water
- The mobile phase B: 2 mmol ammonium formate + 0.002% formic acid in methanol
- The flow rate was maintained at 0.4 mL/min and the injection volume was 10 µL. Chromatographic separation was achieved using a Shim-pack C8 column (2.2 µm particle size, 150 mm x 2.0mm internal diameter).

The gradient program was assessed according to the data displayed in Table 1.

Table 1. Gradient Program of UPLC-MS-MS analysis

Step	Time (min)	Mobile phase A %
0	0.1	98
1	0.2	98
2	2.5	45
3	4	5
4	7	5
5	7.1	98
6	12	Stop

2.5.2 Evaluation of Antioxidant Activity

a. Juice Extract Preparation

Citrus aurantium juice was centrifuged twice at 1500 revolutions per minute (rpm) for 10 minutes using Hettich EBA 20 centrifuge apparatus. The clear supernatant solution obtained was used for UV spectrophotometric identification (Cherif *et al.*, 2006).

b. Preparation of Ascorbic Acid and DPPH Solutions

Ascorbic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) solutions were prepared in methanol at concentrations of 8.10^{-4} mol/L and 10^{-4} mol/L respectively. The DPPH solution was stored at 4°C in darkness. Ascorbic acid solution was used as a standard for comparison (Cherif *et al.*, 2006).

c. Antioxidant Activity Measurement

The antioxidant activity was assessed by quantifying the scavenging activity of the stable free radical DPPH. Two milliliters of the purple DPPH methanolic solution were added to varying volumes of the juice extract (2–20 µL) and ascorbic acid solutions of different concentrations. The resulting mixture agitated until a yellow color was obtained, after which the absorbance was measured at 515 nm using a

spectrophotometer. The results were expressed by inhibitory percentage (I%). Whereas, the IC_{50} value was determined graphically (Cherif *et al.*, 2006; Jabri Karoui & Marzouk, 2013; Santos & Gonçalves, 2016). The percentage of Inhibitory concentration was calculated using the following equation:

$$I\% = \frac{(Abs\ control - Abs\ sample)}{Abs\ control} x\ 100$$

Where:

I%: Inhibitory percentage

IC_{50}: Half maximal inhibitory concentration.

2.6 *In Vivo* Study Design

Male adult Wistar albino rats were initially allocated into two primary groups:

- Rats maintained on a standard diet.
- Rats administered increasing concentrations of a fatty product via the oral route, the composition of which is detailed in Table 2.

These two primary rat groups were further subdivided as follows; each group contains 8 rats:

- **Control group TMM:** Rats maintained on a standard diet for 180 days.
- **Intoxicated group TXM:** Rats rendered hyperlipidemic and potentially diabetic through the oral administration of increasing concentrations of the fatty product, characterized by a predominance of trans fatty acids.
- **The 1ST phase**: This phase is considered as preliminary stage on which rats received the trans fatty acid rich amalgam through gavage using increased concentrations ranging from 0.1 g/kg/day of body weight up to 6 g/kg/day ((0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7……6 g/kg/day) for 60 days.
- **The 2nd phase**: After the acclimatization of rats with the dose escalation of the trans fatty acids rich amalgam, the latter was directly introduced to the normal diet via heat maceration in order to ensure that the amalgam is totally incorporated to the usual diet (50g of amalgam vs 100g of diet) after that, the mixture should be subjected to freeze ambiance to maturate the new diet. Prior to administration, this special diet has to be brought from storage at low temperature to animal care unit ambient temperature, in order to prevent animals from thermal chock or stress. During a period of 120 days for the TXM group and 90 days for the TXMT group.
- **Intoxicated treated group TXMT:** Rats initially belonging to the TXM group, treated orally by *Citrus aurantium* juice at a dosage of 8 mL/kg body weight (Ezeigwe *et al.*, 2022) for a period of 30 days.
- **Treated group TMMT:** Rats belonging to the TMM group, treated orally with *Citrus aurantium* juice at a dosage of 8 mL/kg body weight (Ezeigwe *et al.*, 2022) for a period of 30 days.

The employed protocol was approved by the Institutional Review Board (IRB) of the LAB-NUPABS under the reference IRB-A003/03.2025

2.7 Anthropometric Parameters

2.7.1 Body Mass Index

Body Mass Index was determined according to the methodology described by Novelli *et al.* (2007) and Bouderbala *et al.* (2016), involving the measurement of body weight, abdominal circumference, thoracic circumference, and body length (naso-anal length). The BMI was calculated using the following formula:

$$BMI = \frac{\text{Body Weight (g)}}{\text{Length}^{2}\ (\text{cm})^{2}}$$

2.7.2 Adiposity Index (AI)

The Adiposity index (AI) was calculated using the formula provided by Suarèz *et al.* (2021):

$$AI = \frac{Total\ Adipose\ Tissue\ (g)}{\text{Body weight (g)}}$$

2.7.3 Lee Index Body

Lee index, an indirect and rapid indicator of adiposity in rats, was determined according to the formula proposed by Bernardis (1970):

$$Lee\ Index = \frac{Naso - anal\ length(mm)}{\text{Body weight}^{1/3}\ (\text{g}) \times 1000}$$

2.7.4 Mass Gain

Body mass gain was calculated as the difference between the final and initial body weights of the rats during the experimental period, according to the method outlined by Novelli *et al.* (2007):

$$Weight\ Gain(g) = Final\ Weight(g) - Initial\ Weight(g)$$

2.7.5 Ponderal Index (PI)

The Ponderal Index (PI), an anthropometric parameter used to assess rat corpulence and provide a relationship between weight and length as an indicator of growth, was calculated using the formula described by Wlodek *et al.* (2005) and Zohdi *et al.* (2015):

$$PI = \frac{Body\ weight\ (g)}{Body\ Length^{3}\ (cm^{3})}$$

Table 2. GC-MS DATA of Fatty acids Methyl esters (FAMEs) profiling

Pk#	RT	Area%	Fatty Acid Methyl Esters	Ref#	CAS#	Qual
1	8.445	0.08	Nonanoic acid, methyl ester	37238	001731–84-6	64
			Tridecanoic acid, methyl ester	75098	001731–88-0	64
			Decanoic acid, methyl ester	46652	000110–42-9	64
2	12.063	0.09	Decanoic acid, methyl ester	46660	000110–42-9	87
			Undecanoic acid, methyl ester	56087	001731–86-8	64
			Decanoic acid, methyl ester	46661	000110–42-9	64
3	17.024	0.13	Tridecanoic acid, methyl ester	75098	001731–88-0	80
			Hexadecenoic acid, 15-methyl-, methyl ester	108886	006929–04-0	74
			Octadecanoic acid, methyl ester	116666	000112–61-8	74
4	22.734	0.17	Methyl Z-11-tetradecenoate	82435	1000130–82-8	86
			9-Octadecenoic acid (Z)-, methyl ester	115452	000112–62-9	76
			9-Hexadecenoic acid, methyl ester, (Z)-	99355	001120–25-8	64
5	23.168	1.78	Tridecanoic acid, 12-methyl-, methyl ester	83718	005129–58-8	95
			Undecanoic acid, methyl ester	56085	001731–86-8	76
			Methyl tetra decanoate	83691	000124–10-7	72
6	26.334	0.51	Pentadecanoic acid, methyl ester	92263	007132–64-1	97
			Pentadecanoic acid, methyl ester	92264	007132–64-1	95
			Pentadecanoic acid, methyl ester	92265	007132–64-1	93
7	28.832	3.87	7-Hexadecenoic acid, methyl ester, (Z)-	99348	056875–67-3	99
			9-Hexadecenoic acid, methyl ester, (Z)-	99349	001120–25-8	99
			9-Hexadecenoic acid, methyl ester, (Z)-	99355	001120–25-8	93
8	29.678	19.54	Hexadecenoic acid, methyl ester	100704	000112–39-0	97
			Pentadecanoic acid, 14-methyl-, methyl ester	100727	005129–60-2	95
			Hexadecenoic acid, methyl ester	100711	000112–39-0	93
9	32.587	1.43	Heptadecanoic acid, methyl ester	99348	056875–67-3	99
			Heptadecanoic acid, methyl ester	99349	001120–25-8	99
			Heptadecanoic acid, methyl ester	99355	001120–25-8	93
10	34.850	32.96	9,12-Octadecadienoic acid, methyl ester, (E,E)-	114393	001731–92-6	97
			8,11-Octadecadienoic acid, methyl	114376	001731–92-6	97
			9,11-Octadecadienoic acid, methyl ester, (E,E)-	114396	013038–47-6	97
11	35.079	28.46	9-Octadecenoic acid (Z)-, methyl ester	115454	000112–62-9	99
			10-Octadecenoic acid, methyl ester	11543	013481–95-3	99
			8-Octadecenoic acid, methyl ester	11549	002345–29-1	99
12	35.702	9.52	Octadecanoic acid, methyl ester	11666	000112–61-8	99
			Heptadecanoic acid, 16-methyl-, methyl ester	116689	005129–61-3	98
			Octadecanoic acid, methyl ester	116665	000112–61-8	97
13	40.440	0.29	11-Eicosenoic acid, methyl ester	129957	003946–08-5	99
			9-Octadecenoic acid (Z)-, methyl ester	115452	000112–62-9	70
			7-Hexadecenoic acid, methyl ester, (Z)-	99348	056875–67-3	46
14	41.206	0.37	Eicosanoic acid, methyl ester	130939	001120–28-1	98
			Eicosanoic acid, methyl ester	130937	001120–28-1	98
			Eicosanoic acid, methyl ester	130935	001120–28-1	95
15	47.173	0.42	Docosanoic acid, methyl ester	142871	000929–77-1	99
			Docosanoic acid, methyl ester	142872	000929–77-1	99
			Docosanoic acid, methyl ester	142869	000929–77-1	98
16	51.225	0.18	Tetracosanoic acid, methyl ester	15218	002442–49-1	95
			Tetracosanoic acid, methyl ester	152019	002442–49-1	94
			Tetracosanoic acid, methyl ester	152017	002442–49-1	91
17	54.797	0.14	Cholest-2-ene, 2-methyl-, (5. alpha.)-	152625	022599–90-2	48
			Pentacyclo [9.1.0.0(2,4).0(5,7).0(8,10)] dodecan3,3,6,6,9,9,12,12-octaethyl	152629	1000150–21-4	47
			Cholest-4-en-6-one	152564	013095-36-8	45

Note: Pk #: Peak number; RT: Retention Time Ref#: Reference Number; CAS#: Chemical Abstracts Service Number; Qual: Quality.

2.7.6 Waist-to-Length Ratio (WLR)

The Waist-to-Length Ratio (WLR), an anthropometric index used to evaluate abdominal fat distribution by relating abdominal circumference (AC) to body length, was calculated using the formula provided by Agbaje (2024):

$$WLR = \frac{Waist\ Circumference(cm)}{Body\ Length(cm)}$$

2.8 Blood and Tissues Sampling

At the termination of the experiment (180th day), euthanasia had been assessed according to the Guide for the Care and Use of Laboratory Animals, 8e 2d (2011) and GUIDLINES for the Euthanasia of Animals, (2020). Animals were anesthetized via intraperitoneal injection IP using ketamin/xylidin combination with 80mg/kg of ketamin and 8mg/kg of xylazine. After the confirmation of death, blood had been performed by cardiac puncture and collected into appropriate tubes for further analysis. Liver tissues were rapidly excised, washed with physiological saline, and weighed.

2.9 Biochemical Parameters

2.9.1 Determination of Total Cholesterol, Aspartate Aminotransferase (GOT), and Alanine Aminotransferase (GPT)

Total cholesterol, GOT and GTP levels were determined using commercially available test kits from BIOLABO, France, following the manufacturer's instructions.

2.9.2 Determination of lipase activity

Lipase activity was measured using enzymatic colorimetric methods employing a commercially available diagnostic kit from Cypress Diagnostics (Belgium), according to the provided protocol.

2.9.3 Determination of Glycated Hemoglobin (HbA1c)

The estimation of HbA1c was performed using a nephelometric method with a commercially available GENRUI kit (Genrui Biotech Inc.), following the manufacturer's instructions.

2.10 Histopathological Examination

Portions of liver were carefully sampled, rinsed with tap water, weighed, and fixed in 10% buffered formalin. The specimens were then dehydrated using a graded series of absolute ethanol solutions, cleared in xylene, and embedded in paraffin wax. Paraffin beeswax blocks were sectioned at a thickness of 3–5 mm using a Leica RM2235 microtome. The obtained tissue sections were placed in a water bath, collected onto glass slides, deparaffinized, and stained with Hematoxylin and Eosin (HE) stains for histopathological examination using a Leica light microscope (Bancroft *et al.*, 2019; Li *et al.*, 2021).

2.11 Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) and analyzed using one-way, two-way and three-way analysis of variance (ANOVA) followed by Tukey's or Sidak's multiple comparisons post-hoc tests, as appropriate for the experimental design. Statistical analyses were performed using GraphPad PRISM version 8.0.2 (263) for Windows and Microsoft Excel version 2501, (Microsoft 365). A significance level of $p < 0.05$ was adopted for all statistical tests.

3 Results

3.1 Characterization of the fatty amalgam with GC-MS

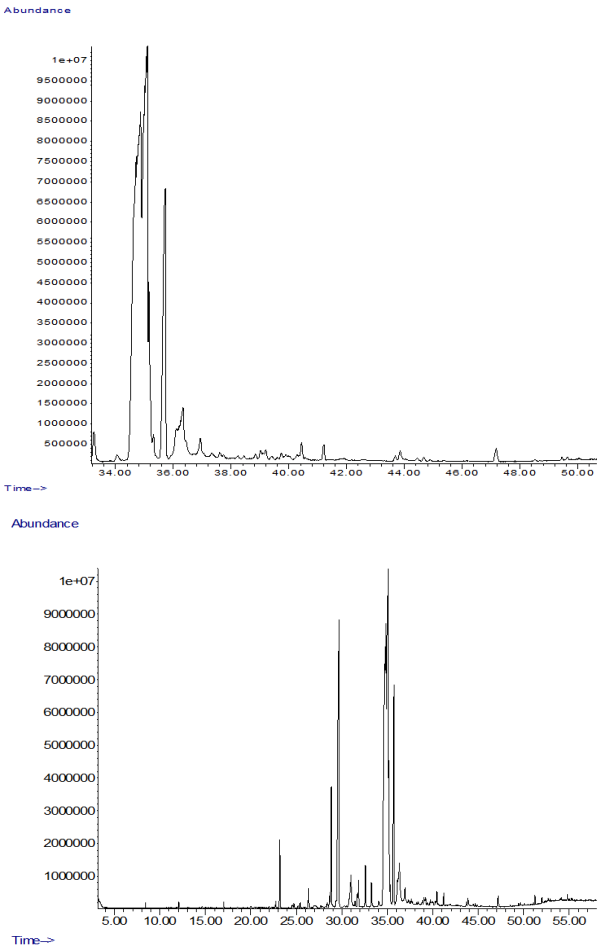
GC-MS calibration range analysis of the fatty amalgam revealed the presence of 17 peaks within the calibration range, representing various fatty acid methyl ester fractions. These fractions were identified based on their respective retention times and peak area abundance, which corresponds to the relative concentration of each fatty acid within the studied substance. Each identified peak comprised up to three derivatives. Notably, the predominant peak (retention time = 34.850 minutes, relative abundance = 32.96%) contained two trans fatty acid methyl esters: 9,12-octadecadienoic acid methyl ester (E,E) and 9,11-octadecadienoic acid, methyl ester (E,E)-, indicating the presence of the corresponding trans fatty acids, 9,12-octadecadienoic acid (E,E) (Figure 1 and Table 2) and 9,11-octadecadienoic acid (E,E)-, at high concentrations within the tested fatty product.

3.2 UPLC-MS-MS Identification of Phytochemicals Compounds of the Juice

UPLC-MS-MS analysis of the *Citrus aurantium* juice identified 18 distinct peaks (Figure 2). The collected data, relative to maximal absorbance, masses of fragmented ions, and retention time (RT) (Figure 2 and Table 3), facilitated the identification of extractable compounds. These compounds are classified according to their relative abundance.

1. **Quercetin**: RT=4.289 min, area=1,591,132, height of 3,908,919.
2. **Rutin:** Retention Time: 6.194 min) with an area of 1,594,052 and a height of 72,125
3. **Naringenin:** (Retention Time: 4.827 min) with an area of 2,573,918 and a height of 937,017.
4. **Epicatechin:** (Retention Time: 6.592 min, Area: 15,186, Height: 6,305).
5. **Resveratrol:** (Retention Time: 5.793 min, Area: 19,560, Height: 8,494).
6. **Luteolin:** (Retention Time: 4.622 min, Area: 682,870, Height: 224,707).

7. **Oleuropein:** (Retention Time: 6.037 min, Area: 112,033, Height: 12,333).
8. **Riboflavin:** (Retention Time: 6.473 min, Area: 1,790,057, Height: 3,581,513).
9. **Curcumin:** (Retention Time: 3.296 min, Area: 421,450, Height: 30,717).
10. **Beta-carotene:** (Retention Time: 7.369 min, Area: 14,342, Height: 7,218).
11. **Oleanolic acid:** (Retention Time: 7.279 min, Area: 46,940, Height: 16,015).
12. **Sinapic acid:** (Retention Time: 3.907 min, Area: 437,897, Height: 35,839).
13. **Caffeic acid:** (Retention Time: 2.687 min, Area: 23,355, Height: 9,728).
14. **Gallic Acid**: (Retention Time:3.198 min): Area 19,876, Height 8,210).
15. **Coumaric Acid:** (Retention Time: 4.112 min): Area 28,945, Height 11,567).
16. **Vanillic Acid:** (Retention Time: 2.978 min: Area 14,032, Height 6,542).
17. **2-Methoxybenzoic Acid:** (Retention Time: 4.735 min: Area 7,582, Height 2,719).
18. **Kojic Acid:** (Retention Time: 5.612 min, Area 8,423, Height 3,118).



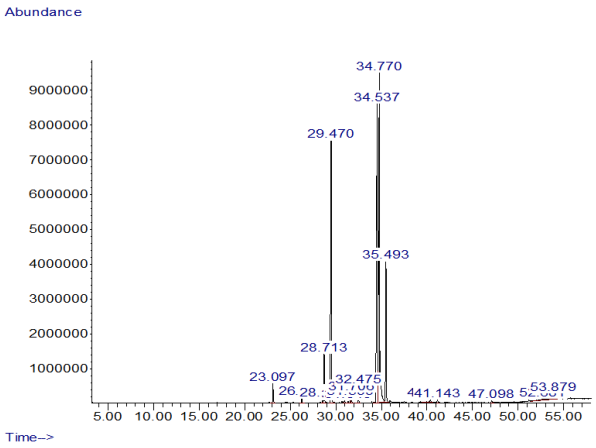


Figure 1. GC-MS Chromatograms of Free Fatty acids Methyl Esters (FAMEs)

3.3 DPPH Scavenging Activity

The antioxidant activity of the methanolic extract of *Citrus aurantium* against the stable free radical DPPH• was assessed by spectrophotometer by monitoring the reduction of DPPH•, indicated by a color change from purple to yellow (DPPH-H), measurable at 515 nm. The obtained results are graphically plotted on the curves (Figure 3).

- Non-linear statistical analysis of the obtained data revealed thar IC_{50} values of 1.125 µg/mL and 1.059 µg/mL for the *Citrus aurantium* extract and ascorbic acid methanolic solution, respectively.

- Two-way ANOVA comparison analysis states indicated a highly significant difference ($p < 0.001$) in the percentage of inhibition across the tested concentrations (Figure 4). Furthermore, very significant ($p < 0.01$) and significant ($p < 0.05$) differences were observed between the Inhibitory percentages of the *Citrus aurantium* methanolic solution and ascorbic acids at the tested concentrations.

3.4 Anthropometric Parameters

Initial and final body weights of the experimental rat group are presented in Table 4.

At the commencement of the study, no statistically significant differences in body weight were observed among the groups. However, the body weight gains exhibited significant statistical differences ($p < 0.0001$) across the experimental groups. Rats maintained on the high-fat diet (TXM) demonstrated the most substantial weight gain, exhibiting the highest final body weight (353.875 ± 11.05 g) followed by the *Citrus aurantium* juice-treated high-fat diet

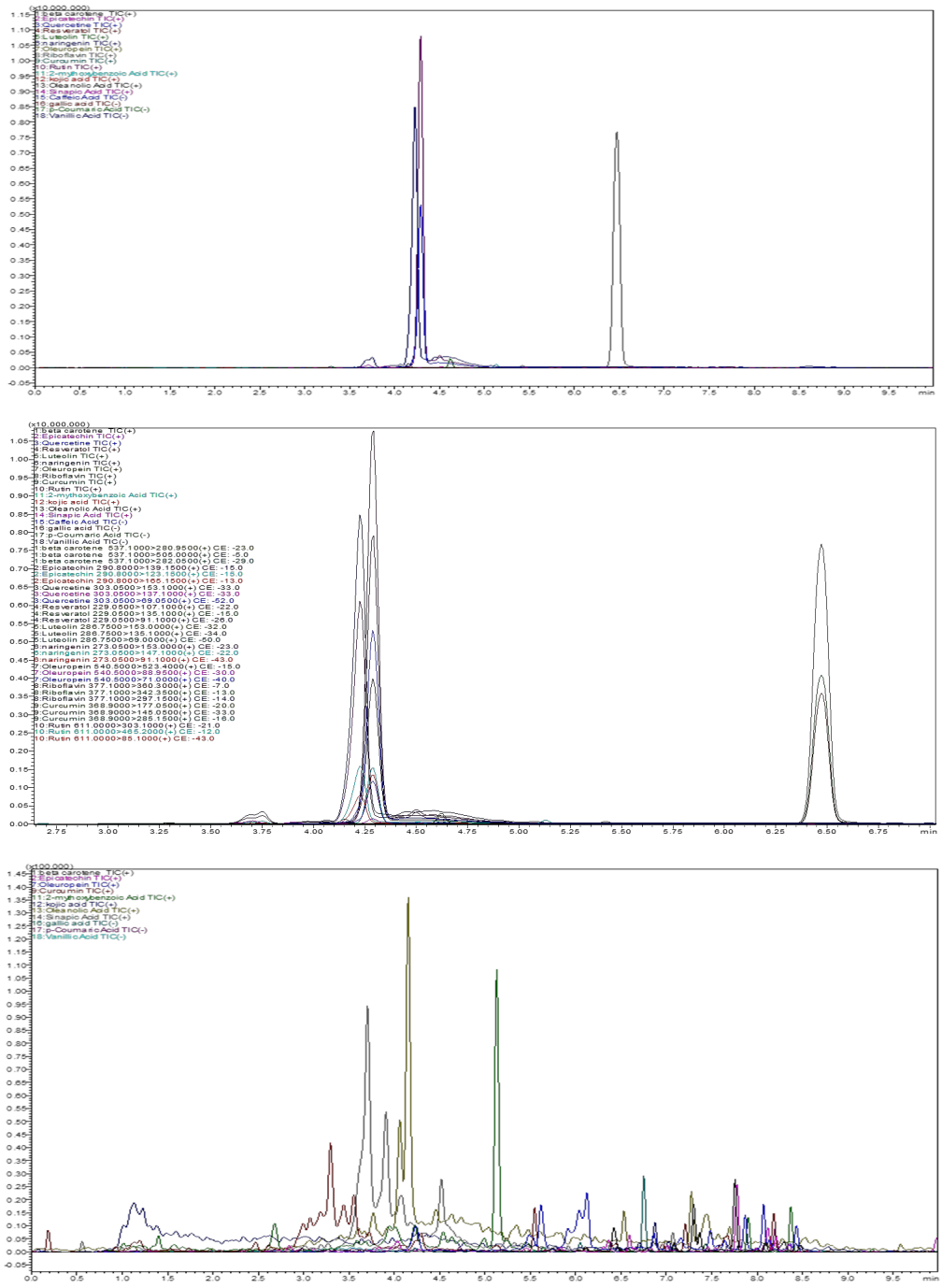


Figure 2. UPLC-MS-MS Chemical Profiling Chromatograms of Secondary Metabolites of *Citrus aurantium* Juice

group (TXMT) (244.75 ± 19.88 g), the control group (TMM) (262 ± 5.44 g), and the group receiving only *Citrus aurantium* juice (TMMT) (233.5 ± 2.54 g).

At the commencement of the study, no statistically significant differences in body weight were observed among the groups. However, the body weight gains exhibited significant statistical differences (p < 0.0001) across the experimental groups. Rats maintained on the high-fat diet (TXM) demonstrated the most substantial weight gain, exhibiting the highest final body weight (353.875 ± 11.05 g) followed by the *Citrus aurantium* juice-treated high-fat diet group (TXMT) (244.75 ± 19.88 g), the control group (TMM) (262 ± 5.44 g), and the group receiving only *Citrus aurantium* juice (TMMT) (233.5 ± 2.54 g).

Table 3. UPLC-MS-MS Data of Chemical Profiling of *Citrus aurantium* Juice

ID#	Name	Molecular Formula	Molecular Weight	ESI Charge (+/-)	transition m/z	Ret. Time	Area	Height
1	Epicatechin	$C_{15}H_{14}O6$	290.27	(+)	290.8000 > 139.1500	6.592	15186	6305
2	Quercetin	$C_{15}H_{10}O7$	302.23	(+)	303.0500 > 153.1000	4.289	15911322	3909819
3	Resveratrol	$C_{14}H_{12}O3$	228.24	(+)	229.0500 > 107.1000	5.793	19560	8494
4	Luteolin	$C_{15}H_{10}O6$	286.24	(+)	286.7500 > 153.0000	4.622	682870	224707
5	naringenin	$C_{15}H_{12}O5$	272.25	(+)	273.0500 > 153.0000	4.227	25739813	5970778
6	Oleuropein	$C_{30}H_{48}O3$	540.5	(+)	540.5000 > 88.9500	6.037	112033	12333
7	Riboflavin	$C_{17}H_{20}N_4O_6$	376.4	(+)	377.1000 > 360.3000	6.473	17900757	3581513
8	Curcumin	$C_{21}H_{20}O_6$	368.4	(+)	368.9000 > 177.0500	3.296	421450	30717
9	Rutin	$C_{27}H_{30}O_{16}$	610.5	(+)	611.0000 > 303.1000	4.289	29873891	7794231
10	Beta carotene	$C_{40}H_{56}$	536.87	(+)	537.1000 > 280.9500	7.369	14342	7218
11	2-mythoxybenzoic Acid	C_8H8O_3	152.15	(+)	No peak is found	0	0	0
12	Kojic acid	C_6H6O_4	142.11	(+)	No peak is found	0	0	0
13	Oleanolic Acid	$C_{30}H_{48}O3$	456.7	(+)	457.3000 > 411.4000	7.279	46940	16015

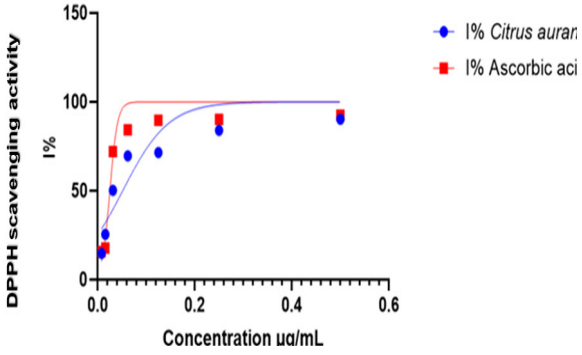


Figure 3. DPPH Radical Scavenging Activity of *Citrus aurantium* Juice Compared to Ascorbic Acid (referenced antioxidant)

Data represent mean ± standard deviation SD of triplicate measurements (n=3). Concentration-response curves were fitted according to non-linear regression test. Values of IC_{50} were calculated as 1.125 µg/mL and 1.059 µg/mL for *Citrus aurantium* juice and ascorbic acid respectively. Statistical analysis was assessed using Two-way ANOVA followed by Tukey's post-hoc test. Differences were considered as significant ($p < 0.05$).

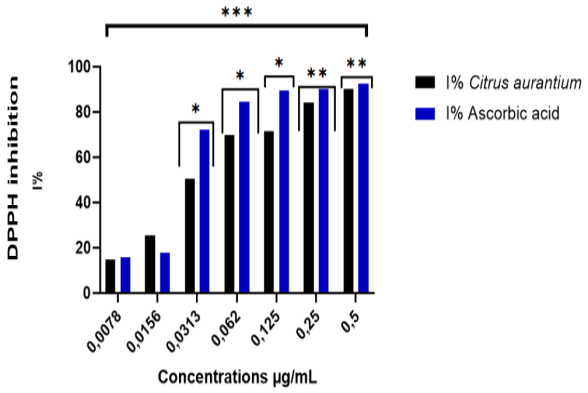


Figure 4. DPPH Radical Inhibition Percentage of *Citrus aurantium* Juice and Ascorbic Acid at Various Concentrations

Data are expressed as mean± standard error of mean SEM (n=8). Statistical analysis was performed using Two-way ANOVA followed by Tukey's multiple comparison test. Significance difference levels: * p<0.05 (significant), ** p<0.01 (very significant), ***p<0.001 (highly significant).

No statistically significant differences ($p > 0.05$) were observed in the initial and final measured lengths of the rats across the experimental period.

The final BMI of the TXM group (0.78 ± 0.016 g/cm2) was significantly higher ($p < 0.0001$) than that of the other groups, indicating a pronounced increase in BMI in the high-fat diet group. Conversely, the *Citrus aurantium* juice-treated control group (TMMT) group exhibited the lowest BMI value (0.48 ± 0.012 g/cm2).

Lee Index calculations revealed the highest value in the TXM group (0.294 ± 0.002), with extreme statistical significance ($p < 0.0001$) among the tested groups.

The Ponderal Index (PI) demonstrated a highly significant increase ($p < 0.0001$) in TXM group (0.036 ± 0.0008 g/cm^2) compared to the other groups, especially the TMMT group, which exhibited the lowest PI value (0.022 ± 0.001 g/cm^2).

Abdominal Circumference (AC) exhibited a highly significant increase ($p < 0.0001$) in the TXM group (23.75 ± 0.16 cm) followed by the treated high-fat diet group (TXMT) (20.25 ± 0.31 cm), then the control group (TMM) (16 ± 0.26 cm), and the *Citrus aurantium* juice-treated control group (TMMT) (15.75 ± 0.31 cm). Notably, the TXMT group exhibited a significant decrease ($p < 0.0001$) in AC compared to the TXM group, indicating a mitigating effect of *Citrus*

Table 4. Anthropometric Parameters and Organ Weights

		TMM	TXM	TXMT	TMMT
Body weight (g)	Initial	123.25 ± 4.30	129.5 ± 6.82	131.75 ± 5.53	156.25 ± 2.44
	Final	262 ± 5.44	353.875 ± 11.05	244.75 ± 19.88	223.5 ± 2.54
Length (cm)	Initial	17.750 ± 0.31	17.25 ± 0.31	18 ± 0.46	18.75 ± 0.16
	Final	21.75 ± 0.16****	21.25 ± 0.25****	21.75 ± 0.16****	21.5 ± 0.18****
Weight Gain%		1.38 ± 0.092****	2.244 ± 0.115****	1.130 ± 0.158****	0.67 ± 0.034****
BMI (g/cm²)	Initial	0.390 ± 0.005	0.43 ± 0.01	0.40 ± 0.01	0.44 ± 0.009
	Final	0.554 ± 0.013****	0.78 ± 0.016****	0.51 ± 0.036****	0.48 ± 0.012****
Lee index	Initial	0.280 ± 0.002	0.29 ± 0.002	0.28 ± 0.004	0.28 ± 0.002
	Final	0.294 ± 0.002****	0.33 ± 0.002****	0.28 ± 0.006****	0.28 ± 0.0031****
PI (g/cm³)	Initial	0.022 ± 0.0005	0.025 ± 0.0005	0.022 ± 0.001	0.023 ± 0.0007
	Final	0.025 ± 0.0007****	0.036 ± 0.0008****	0.023 ± 0.001****	0.022 ± 0.0007****
AC (cm)	Initial	13 ± 0.26	13 ± 0.26	12.5 ± 0.42	13.5 ± 0.42
	Final	16 ± 0.26****	23.75 ± 0.16****	20.25 ± 0.31****	15.75 ± 0.31****
THC (cm)	Initial	12.5 ± 0.18	12.75 ± 0.16	12.75 ± 0.16	12.75 ± 0.31
	Final	14.25 ± 0.16****	19.75 ± 0.16****	16.75 ± 0.16****	14.75 ± 0.49****
WLR	Initial	0.705 ± 0.013	0.74 ± 0.02	0.71 ± 0.01	0.68 ± 0.022
	Final	0.655 ± 0.007****	0.93 ± 0.014****	0.77 ± 0.001****	0.68 ± 0.022****
Adiposity Index%		0	23.250 ± 1.634	12 ± 0.463	0
MAT (gr)		0	6.633 ± 0.554	5.15 ± 0.457	0
Liver weight (gr)		10.250 ± 0.313	12.750 ± 0.559	10 ± 0.463	7.5 ± 0.627
Kidney's weight (gr)		2.625 ± 0.157	0.950 ± 0.033	1.43 ± 0.215	0.8 ± 0.026
Testis weight (gr)		3.12 ± 0.20	5.750 ± 0.164	1.25 ± 0.164	3.75 ± 0.619

Note: **BMI:** Body Mass Index. **PI:** Ponderal Index. **AC:** Abdominal Circumference. **THC:** Thoracic Circumference. **WLR:** Waist to Length Ratio **MAT:** Mesenteric adipose tissue; Values are mean ± standard deviation of the mean, N=8. Means with different superscripts differ significantly, $p < 0.0001$.

aurantium juice on abdominal fat accumulation in the high-fat diet group.

Thoracic Circumference THC was significantly greater (19.75 ± 0.16 cm) in the TXM group compared to the other groups: TXMT (16.75 ± 0.16 cm), TMMT (14.75 ± 0.49 cm), and TMM (14.25 ± 0.16 cm), with a high level of statistical significance ($p < 0.0001$).

The Waist-to-Length Ratio (WLR), an indicator of abdominal fat accumulation was markedly elevated and statistically significant p < 0.0001 in the TXM group (0.93 ± 0.014%), followed by the treated group TXMT (0.77 ± 0.001%), TMMT (0.68 ± 0.022%), and the TMM group (0.655 ± 0.007%).

The Adiposity Index (AI) and the mass of the Mesenteric Adipose Tissue (MAT), two closely related anthropometric factors, were only quantifiable in the TXM and TXMT groups, wherein all rats exhibited a considerable amount of free adipose tissue in the abdominal cavity (AI: 6.633 ± 0.554%; MAT: 5.15 ± 0.457%) (Figure 5).

The statistical significance for these parameters was extremely high ($p < 0.0001$), as no free adipose tissue was detected in the abdominal cavities of rats belonging to the control group (TMM) and the treated group TMMT.

The mean liver weight of the TXM group (12.750 ± 0.007 g) was the highest among the experimented groups, although no statistically significance ($p > 0.05$) were observed among the other groups. Nevertheless, this mean liver weight exhibited an extremely significant difference ($p < 0.0001$) when compared to the mean final body weight of the same group (355.875 ± 11.05 g).

Kidneys weights across the four experimental groups revealed a considerable decrease in the rats belonging to groups receiving different dietary substances (TXM: 0.950 ± 0.033 g, TXMT: 1.43 ± 0.215 g, TMMT: 0.8 ± 0.026 g) compared to the control group (TMM: 2.625 ± 0.157 g), although this difference was not statistically significant ($p > 0.05$). Conversely, an extremely significant difference ($p < 0.0001$) was observed between final body weights and kidney weights across all groups.

Testis weights also presented a range of values similar to the other organs previously described.

3.5 Biochemical Parameters

The results of the various biochemical parameters assessed are presented in the Table 5.

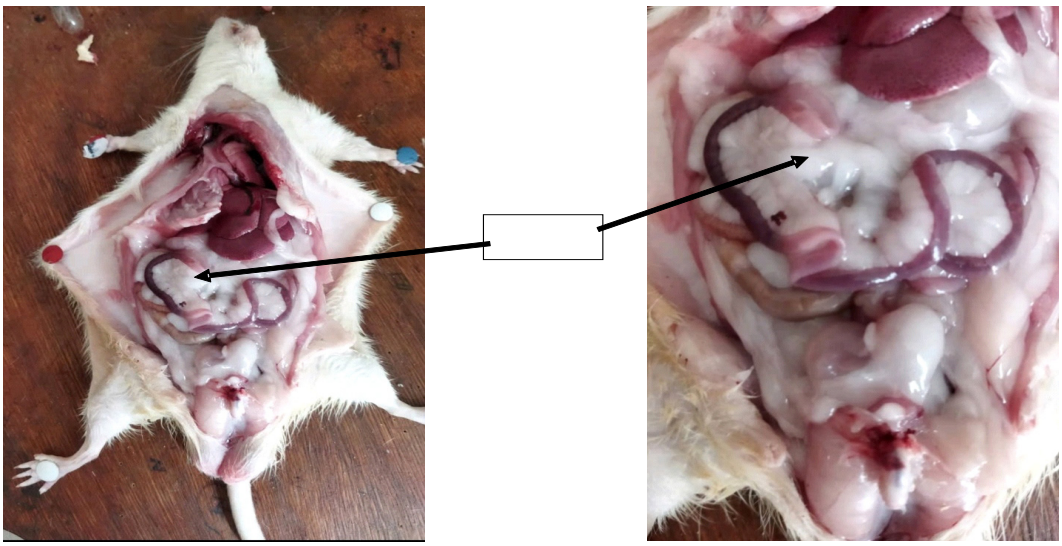


Figure 5. Representative Images of Rat Abdominal Dissection with Trans-Fatty-Acid-Rich Amalgam
MAT: Mesenteric Adipose Tissue of an adult male Wistar rat following chronic administration of a Trans fatty acid rich amalgam for 180 days

Table 5. Blood Biochemical Parameters

	TMM	TXM	TXMT	TMMT
Total Cholesterol g/L	0.63 ± 0.058	1.423 ± 0.105	0.978 ± 0.087	0.683 ± 0.032
HbA1c %	2.125 ± 0.048	4.32 ± 0.316	3.523 ± 0.247	1.96 ± 0.070
Lipase (UI/L)	20.485 ± 0.218	19.465 ± 2.181	28.58 ± 1.801	31.878 ± 3.607
GOT (UI/L)	202.71 ± 1.055	38.07 ± 3.982	85.54 ± 2.550	174.145 ± 12.309
GPT (UI/L)	38.195 ± 3.422	17.523 ± 1.589	43.078 ± 5.303	57.278 ± 17.168

Values are mean ± standard deviation of the mean. N=8.

3.5.1 Total Cholesterol

The administration of the fatty product resulted in a considerable and highly significant ($p < 0.0001$) increase in the serum total cholesterol level (1.423 ± 0.105 g/L). Statistical analysis revealed a significant difference ($p \leq 0.05$) between rats belonging to the control group (TMM) and the treated group (TMMT), and a very significant difference ($p \leq 0.01$) between the TXM and the TXMT groups.

3.5.2 Glycated Hemoglobin (HbA1c)

The percentage values of HbA1c, a direct indicator of glycemic control, were highest in the TXM group (4.32 ± 0.316%), exhibiting an extremely significant difference ($p < 0.0001$) compared to the control group (TMM) (2.125 ± 0.048%). Furthermore, a very significant difference ($p \leq 0.01$) was observed between the TXM and TXMT groups, and a significant difference ($p \leq 0.05$) between the TMM and TXMT groups. Notably, the *Citrus aurantium*-treated groups, TXMT and TMMT, showed the lowest HbA1c values: 3.523 ± 0.247% and 1.96 ± 0.070%, respectively.

3.5.3 Lipase

Analysis of pancreatic lipase enzyme activity revealed the lowest lipase level (19.465 ± 2.181 UI/L) in the high-fat diet administered group (TXM). Conversely, the highest level was recorded in the treated control group (TMMT) (31.878 ± 3.607 UI/L). Statistical analysis indicated no significant difference ($p > 0.05$) between the control group (TMM) and the high-fat diet administered group (TXM). However, a significant difference ($p \leq 0.05$) was observed between the TXM and TXMT groups, and a very significant difference between the TXM and TMMT groups.

3.5.4 Aspartate Aminotransferase (GOT) Activity

This biochemical parameter, indicative of liver functions, showed the highest GOT level (202.71 ± 1.055 UI/L) in the control group (TMM). In contrast, the TXM group exhibited the lowest value (38.07 ± 3.982 UI/L). Statistical evaluation revealed extremely significant differences ($p < 0.0001$) among the experimental groups, with the exception of the comparison between the control group (TMM) and the treated control group (TMMT), which showed a significant difference ($p \leq 0.05$).

3.5.5 Alanine Aminotransferase (GPT) Activity

The results indicated that the lowest GPT level (17.523 ± 1.589 UI/L) was observed in the TXM group, compared to the treated control group (TMMT) (57.278 ± 17.168 UI/L). Statistic assessment highlighted a significant difference ($p \leq 0.01$) between the TXM and TXMT, a very significant difference between TXM and TMMT groups, and no significant difference ($p > 0.05$) among the other groups.

3.6 Histological Evaluation

Histological examination of liver and pancreas tissue sections stained with Hematoxylin and Eosin (HE) revealed the following microscopic features:

- The hepatic parenchyma of the control (TMM) presented a normal architecture, characterized by the Glisson's capsule extending connective tissue septa that delineated lobules.
- These lobules were composed of hepatocytes separated by sinusoidal spaces and bordered by portal triads containing a vein, artery, and bile canaliculi.

In contrast, the pancreatic parenchyma of the Trans Fatty Acid-treated group (TXM) displayed steatonecrosis, characterized by necrosis and inflammation of the peri-pancreatic adipose tissue (Figure 6 TXM1 pancreas) and intense to moderate, macro- and microvesicular, and diffuse steatosis lesions characterized by the presence of intracellular lipid vacuoles and inflammatory cell infiltration (Figure 6 TXM, TXM1, TXM2). Hepatocytes also exhibited ballooning degeneration and significant vascular congestion (Figure 6 TXM1, TXM2).

However, the consumption of *Citrus aurantium* juice in the treated high-fat diet group (TXMT) was associated with a discernible decrease in hepatic steatosis, and no cellular ballooning was observed (Figure 6 TXMT).

4 Discussion

Trans fatty acids (TFAs), unsaturated fats generated through industrial hydrogenation processes, are well-established for their deleterious effects on health, particularly concerning cardiovascular diseases, obesity, and diabetes. Notably, TFA consumption has been associated with dysregulated cholesterol levels, contributing to an elevated risk of CVDs and metabolic dysfunction, including insulin resistance and non-alcoholic fatty liver disease (NAFLD) (Micha *et al.*, 2014; Restrepo et *al.*, 2016). Consistent with these findings, the present study utilized a Wistar rat model, to investigate the adverse effects of orally administered fat amalgam over a period of 180 days. GC-MS profiling of the fatty acid methyl esters identified the presence of 9,12-octadecadienoic acid, methyl ester (E,E)- and 9,11-Octadecadienoic acid, methyl ester (E,E)- both of which are TFAs.

Citrus aurantium (bitter orange), a member of the Rutaceae family, possesses a rich phytochemical profile and has demonstrated beneficial health effects (Gandhi *et al.*, 2020). Whereas, *Citrus aurantium* juice has garnered attention for its significant therapeutic potential.

The antioxidant potential of *Citrus aurantium* juice is attributed to its ability to scavenge free radicals. In this study, the antioxidant potential of *Citrus aurantium* juice in scavenging the DPPH radical was evaluated. The results indicated that the juice exhibited substantial antioxidant activity, with an inhibitory concentration (IC_{50} = 1.125 µg/mL), comparable to the ascorbic acid standard (IC_{50} = 1.059 µg/mL). The absence of a significant difference between the juice and ascorbic acid, a known potent antioxidant, was noted.

Comparative studies, have reported varying IC_{50} values for *Citrus aurantium* extracts, with a Tunisian variety showing an IC_{50} of approximately 17.49 µg/mL (Cherif *et al.*, 2006), and an aqueous fruit extract presenting an IC_{50} of 2.87 ± 0.42 µg/mL (EL Yazouli *et al.*, 2024), and 97.05 ± 0.38 µg/mL for a fresh fruit juice (Jabri Karoui & Marzouk, 2013). A commercial extract reported by Gonçalves *et al.* (2019) of *Citrus aurantium* presented a higher IC_{50} of 0.404 mg/mL, suggesting potential degradation of antioxidant components due to commercial processing, including food additives, packaging, and thermal treatment. While, *Citrus aurantium essential* oils have been reported to possess an IC_{50} equal to 67.1147 ± 0.353 µg/mL (Ouguelmane & Houichiti, 2020). These results underscore the high antioxidant capacity of the *Citrus aurantium* juice utilized in the present study. This power is highly variable from a study to another. This may be due to the diversity of the fruit active phytochemicals including Vitamin C and polyphenols (citroflavonoids) (Cherif *et al.*, 2006).

Analysis of anthropometric parameters revealed that the consumption of TFAs led to a significant increase in body weight, BMI, and weight gain, indicative of excessive abdominal adipose tissue accumulation. These results support the association of TFA intake with obesity-related metabolic disorders, consistent with the findings of Chajès *et al.* (2015) and Hansen *et al.* (2014), who highlighted the role of TFAs in rapid fat tissue accumulation as a key point in metabolic dysfunction.

BMI values in the TFA-administered group indicated obesity (0.78 ± 0.016 g/cm^2) at the end of the experiment, exceeding the range of 0.45 to 0.68 g/cm^2 established for non-obese rats (Novelli *et al.*, 2007). The Lee Index, a typical obesity indicator of obesity in rats, was also significantly elevated in the TFA group (0.33 ± 0.002), exceeding the threshold of 0.3 indicative of obesity in rodents (Yustisia *et al.*, 2022). While the literature extensively documents the association of high-fat diets with elevated Lee Index, this study provides novel evidence linking TFA intake specifically to a significant increase in this parameter.

The Adiposity Index (AI), a widely used measure of adiposity in rat obesity studies that increases proportionally with obesity development (Taylor & Phillips, 1996), was assessed in the TFA-administered groups based on the palpable mesenteric adipose tissue (MAT) (Figure 5). In this context, AI% specifically reflects

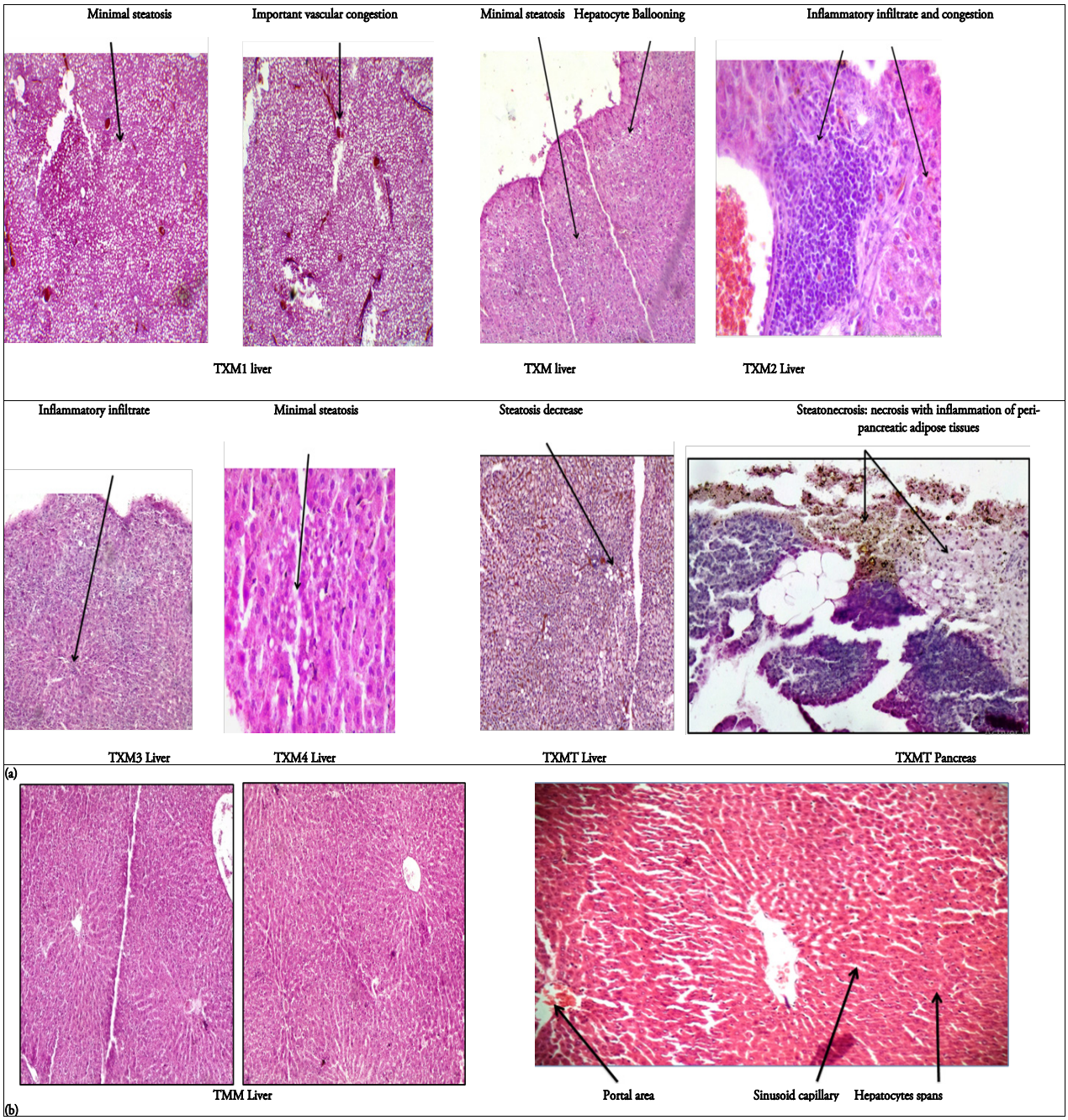


Figure 6. Histopathological Sections in Rat Liver and Pancreas of Experimental Rats Stained with Hematoxylin and Eosin (H&E)

(a) Representative histological changes in liver and pancreas of experimental rats and pancreas:

TXM1: liver showing minimal steatosis and marked vascular congestion.
TXM: liver showing minimal steatosis and ballooning hepatocytes.
TXM2: liver with inflammatory infiltrates and vascular congestion.
TXM3: liver showing inflammatory infiltrates.
TXM4: liver with minimal steatosis.
TXMT: liver displaying decreased steatosis.
TXM1: Pancreas showing steatonecrosis with inflammation of peripancreatic adipose tissue.s

(b) Representative hepatic architecture in normal (control) rat displaying hepatocytes spans, sinusoid capillary and portal area.

abdominal obesity, registering at 0% in the non-treated groups due to the absence of detectable free abdominal adipose tissue. These findings align with Arika *et al.* (2019), who identified obesity index, abdominal circumference, and thoracic circumference as strong predictors of intra-abdominal fat thickness and central obesity in rats.

Furthermore, the observed decrease in organ weights in the treated rats at the end of the experiment may be attributed to the regulation of hunger and appetite signals, which play a crucial role in maintaining homeostatic balance (Diepvens *et al.*, 2007 and Hovarth, 2012). Leptin and melanocyte-stimulating hormones promote the suppression of food intake, while peptide YY (PYY cholecystokinin (CCK), and glucagon-like peptide 1 (GLP-1) contribute to satiety by slowing gastric emptying and intestinal transit.

The mechanism by which TFAs induce metabolic disorder involves the downregulation of Adipose Triglycerides Lipase (ATGL) expression by suppressing Insulin Receptor Substrate 1 (IRS-1)-dependent insulin receptor signaling and phosphorylated Protein Kinase C (PKC) in liver tissues (Zhao *et al.*, 2016).

The Ponderal Index (PI) was measured to assess rat corpulence**.** Changes in PI values from the commencement to the end of the experiment provided insight into the effectiveness of TFAs-induced adiposity. While Mohajan and Mohajan (2023) suggested that PI offers more accurate results than BMI but is less frequently utilized, Beck *et al.* (1999) proposed a classification of human nutritional status based on PI values. Given the paucity of correlating rat PI with TFA consumption, we extrapolated from our findings to suggest that the TFA-exposed group exhibited obesity, while the other groups were within the normal healthy range. Furthermore, Crusell *et al.* (2017) indicated that elevated PI is a significant risk factor for obesity and its related diseases, such as the development of diabetes observed in our study. T2DM onset in TFA treated rats were verified through dosing HBA1c parameter after sacrifice of animals. And during the experimental period these rats exhibited significant polyphagia, polyurea and polydipsia, elevation of the rate of, total Cholesterol, GOT, GPT, HBA1C, with a decrease in the expression of Lipase and liver steatosis.

The Waist-to-Length Ratio (WLR) was highest in the TFA-administered group, with a slight decrease observed post-treatment. Due to the limited prior research on this parameter in rodents, comparisons were drawn with human studies. Interestedly, Agbaje (2024), Baioumi *et al.* (2019), and Lo *et al.* (2021), have demonstrated that human waist-to-height ratio (WHtR), identical to WLR, and WC are more potent indicators of cardiovascular and metabolic diseases, especially diabetes, than BMI.

Several studies have indicated that TFA consumption contributes to weight gain and the development of obesity (Axen *et al.*, 2003; Hua *et al.*, 2020; Smit *et al.*, 2010), although Chajès *et al.* (2015) did not notice a significant obesogenic effect.

Our findings revealed significantly elevated levels of total cholesterol, GOT, GPT, HbA1c in the experimental TFA-exposed groups. Guerra *et al.* (2021) and Akinlade & Oladokun (2024) have associated elevated serum aminotransferase levels with hepatic steatosis. Given the presence of liver steatosis in our TFA-treated rats, we posit the accumulation of substantial amounts of free fatty acids (FFAs) in their livers. According to Zámbó *et al.* (2013) these FFAs are re-esterified into triglycerides within hepatocytes, incorporated into VLDL, and released into the bloodstream, a key mechanism in lipotoxicity development. Our results regarding cholesterol levels are consistent with previous studies by Uauy *et al.* (2009), Mozzaffarian *et al.* (2009), and Hua *et al.* (2020), and Mazidi *et al.* (2018), who associated TFAs to hepatic fat accumulation due to plasma accumulation, leading to triglyceride accumulation in hepatocytes resulting from an imbalance between lipid acquisition and catabolism. Furthermore, animal studies have suggested that TFAs enhance hepatic de novo lipogenesis and suppress lipolysis, promoting hepatosteatosis. In contrast, our findings are inconsistent with those of Ibrahim *et al.* (2005) who observed a decrease in cholesterol levels as a result of TFA consumption.

Albright *et al.* (1994) emphasized the greater importance of HbA1c in classifying rodent glycemic status compared to blood glucose assessments. Our evaluation of HbA1c showed the highest levels in the TFA-administered group, with a subsequent decrease following *Citrus aurantium* juice administration. While the HbA1c value in the TFA group (4.32 ± 0.316 %) was below the diabetic threshold of 5.67% reported by Blanc *et al.* (1980), for rats, it is crucial to note the different analytical techniques employed, such as nephelometry in our study vs. chromatography in cited studies (Albright *et al.*, 1994; Benaicheta *et al.*, 2015). Our baseline HbA1c levels in control rats (2.125 ± 0.048%) were consistent with those reported by Nagisa *et al.* (2003) using chromatography (2.3 ± 0.3%). Our results showed a glycation occurrence, as evidenced by the differences in values across the groups: Normal rats: 2.125 ± 0.048%; TFAs rats: 4.32 ± 0.316%; *Citrus aurantium* juice-treated group: 3.523 ± 0.247% and 1.96 ± 0.070%. Compared to our initial finding of 2.125 ± 0.048%, these results demonstrate that the treatment effectively reduced the HbA1c levels in the respective groups.

Camara (2014), attributed the decrease in HbA1c with phytochemical treatments to the slow and irreversible fixation of glucose on the N-terminal β chain of hemoglobin A1.

Hemp *et al.* (2012) correlated decreased HbA1c with microvascular complications similar to human diabetes, such as nephropathy and retinopathy. Microvascular congestion was observed in the hepatic tissues of our TFA-treated rats (Figure 6), and retinopathy was evident in one rat (Figure 7). Significant hepatic steatosis (Figure 6) was also noted, consistent with Bertola *et al.* (2018), who highlighted liver steatosis as a crucial indicator of diabetes in specific rat models. Microscopic examination of the pancreas, in our study revealed a loss of β-cells, the key regulators of blood glucose. Based on these findings, we propose that a male Wistar rat with an HbA1c level of 4.32% can be considered indicative of developing type 2 diabetes mellitus in this experimental context.

The increased liver weight in the TFA-treated group may be attributed to liver steatosis (Caldwell *et al.*, 2004). Microscopic evaluation of the liver also revealed inflammatory cells, consistent with Odegaard & Pereira (2006), who suggested that TFAs enhance inflammatory processes by increasing the level of TNF-

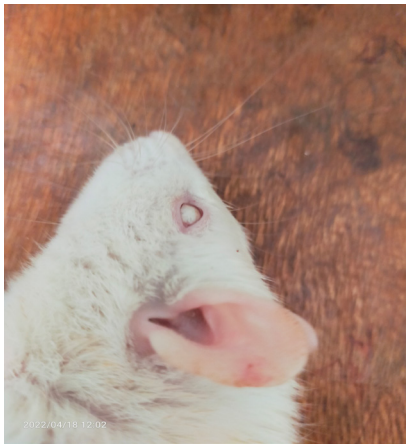


Figure 7. Rat With Retinopathy
Macroscopic image of an experimental rat showing an opaque eye with whitish color, relative to retinopathy.

α, IL-6, and CRP. Odegaard & Pereira (2006) also highlighted that diabetes, high TFA intake, and obesity are key factors in the development of insulin resistance. Zhao *et al.* (2016) proposed that TFAs promote liver steatosis by suppressing pancreatic lipase expression. Pancreatic lipase plays a crucial role in hydrolyzing dietary fats into absorbable molecules (Sanneur *et al.*, 2023). The reported downregulation of pancreatic lipase expression by TFAs (Saravanan *et al.*, 2005) and the decrease in its blood levels under TFA action (Privett *et al.*, 1977) are consistent with our findings of a significant increase in lipase activity in rats consuming TFAs. A strong correlation between Lipoprotein Lipase (LPL) and insulin resistance has been reported (Saravanan *et al.*, 2005), suggesting that insulin sensitivity may be enhanced by fatty acid oxidation during triglycerides clearance and reduced hepatic glucose production. In our study, pancreatic lipase likely facilitated the release of TFAs from the fatty amalgam into the bloodstream. However, chronic intake of TFAs led to their unmetabolized fractions on the tissues. resulting in an imbalanced enzymatic status due to LPL gene regulation and expression, impaired insulin sensitivity and the subsequent development of diabetes.

Our histological investigation revealed the disappearance of pancreatic β-cells as displayed in Figure 6 associated with steatonecrosis, consistent with Oh *et al.* (2018), who stated that TFAs enhance the release of toxic metabolites (fatty acyl CoA, triacylglycerol, and ceramide), leading to lipotoxicity, β-cell dysfunction, insulin resistance, and the development of type 2 diabetes.

Conversely, the therapeutic potential of *Citrus aurantium* juice was assessed. UPLC-MS-MS profiling revealed the presence of several bioactive compounds with quercetin, rutin, and naringenin being predominant. Quercetin and rutin are reputable for their antioxidant and anti-inflammatory effects, which can improve insulin sensitivity and reduce of fat accumulation (Gandhi *et al.*, 2020). Rutin, specifically, has been shown to improve insulin sensitivity, reduce fat accumulation, and significantly decrease biochemical parameters such as glucose, cholesterol, triglycerides, LDL, and FFAs (Gandhi *et al.*, 2020; Gao *et al.*, 2013). Naringenin enhances lipid metabolism by increasing fatty acid oxidation and decreasing lipogenesis (Gandhi *et al.*, 2020; Mulvihill *et al.*, 2010). Epicatechin another identified flavanol, plays a crucial role in enhancing mitochondrial function, improving muscle cell glucose uptake by translocating GLUT 4 transporter (Taub *et al.*, 2012; Ueda-Wakagi *et al.*, 2005), and has been suggested to improve palmitate-induced insulin resistance in muscles (Song *et al.*, 2024). Resveratrol, also potentially present, is known to improve insulin sensitivity and reduce oxidative stress (Brasnyó *et al.*, 2012).

Curcumin, luteolin, and riboflavin have the potential to modulate key signaling pathways and enhance both glucose and lipid metabolism (Gandhi *et al.*, 2020; Ghorbani, 2024). Curcumin, in particular, plays a significant role in modulating inflammatory mechanisms and enhancing the function of pancreatic β-cells, thereby influencing glucose and lipid metabolism (Ghorbani, 2024). Luteolin, a flavonoid commonly found in various fruits and vegetables, has been shown to have a positive impact on metabolic health by targeting key signaling pathways involved in glucose and lipid homeostasis (Kwon *et al.*, 2018).

Oleuropein and epicatechin have been shown to decrease fat accumulation and improve vascular function (Bullota *et al.*, 2014; Sotoudeheian *et al.*, 2023). Studies have indicated that oleuropein can modulate lipid metabolism, leading to decreased fat accumulation. In animal models, oleuropein administration resulted in reduced body weight gain, lower fat mass, and improved blood lipid profiles. These effects are partly attributed to the activation of AMP-activated protein kinase (AMPK) in white adipose tissue, a key regulator of lipid metabolism and energy homeostasis (Sotoudeheian *et al.*, 2023).

Beta-carotene and oleanolic acid have demonstrated effectiveness in preventing oxidative stress, decreasing fat accumulation in adipocytes, and stimulating lipolysis (Kang *et al.*, 2021; Li *et al.*, 2021; Mounien *et al.*, 2019).

Caffeic acid, gallic acid, sinapic acid, coumaric acid and vanillic acid have the ability to neutralize free radicals and struggle against oxidative stress associated with obesity, regulate inflammatory process, decrease blood glucose levels, and modulate insulin levels (Salau *et al.*, 2022). Acting synergically, these phytochemicals can reduce inflammatory processing and improve glucose management, contributing to the prevention of obesity and type 2 Diabetes (Kumar *et al.*, 2019). Sinapic acid, a hydroxycinnamic acid derivative commonly found in cereals, fruits, and vegetables, exhibits significant antioxidant and anti-inflammatory activities, suggesting its potential in managing conditions such as oxidative stress, inflammation, and type 2 diabetes (Mathew *et al.*, 2015). P-Coumaric acid, another hydroxycinnamic acid, is recognized for its antioxidant properties, reducing oxidative stress and inflammation, and reinforcing metabolic health pathways. Vanillic acid, a hydroxybenzoic acid

derivative, is renowned for its antioxidant capacity, promoting a decrease in oxidative stress and inflammation (Mathew *et al.*, 2015).

The observed anti-obesogenic effect of *Citrus aurantium* juice may be attributed to the singular, additive and/or synergistic effects of these phytochemicals. Despite these beneficial effects, it is imperative to acknowledge the potential for overconsumption of *Citrus aurantium* sub-products to negatively impact the cardiovascular system by elevating arterial pressure and cardiac frequency (Benjamim *et al.*, 2022; Bui *et al.*, 2006; Stohs, 2017).

The study has some limitations that should be considered. First, it remains difficult to identify the specific compound of the juice which is responsible for the protective observed effects. Second, the obtained results cannot be directly extrapolated to humans, because further studies and deep research are crucially required and should be made with caution before the consideration of clinic applications. Finally, the lack of comparison with reference pharmaceutical remedy reduces the ability to introduce this juice as a therapeutic issue.

5 Conclusions

The present study has demonstrated that *Citrus aurantium* juice contains a spectrum of phytochemicals that may exert ameliorative effects on pancreatic lipase activity through their bioactive properties. Our findings suggest that *Citrus aurantium* juice, or specific isolated constituents thereof, possesses the potential to modulate pancreatic enzyme expression, glycated hemoglobin levels, cholesterol concentrations, transaminases activities, and anthropometric parameters in a rat model exposed to trans fatty acids. Further investigation involving the isolation and individual and synergistic testing of the identified phytochemicals may contribute to the development of food supplements or nutraceuticals to complement pharmaceutical therapies for individuals with metabolic disorders. These findings indicate that *Citrus aurantium* extracts hold promise as a natural ingredient in functional foods aimed at the prevention and management of postprandial blood glucose levels and diseases associated with hyperlipidemia. Furthermore, the isolation of the identified compounds may contribute to the discovery of novel, safe, and effective pharmaceutical agents.

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