2024

MEDICAL UNIVERSITY "PROF. DR. PARASKEV STOYANOV"-VARNA

FACULTY OF MEDICINE

DEPARTMENT OF PHARMACOLOGY AND CLINICAL PHARMACOLOGY AND THERAPY

Elis Rafailova Gasanzadeeva

PHARMACOLOGICAL INVESTIGATION WITH ANETHOLE IN AN EXPERIMENTAL MODEL OF OBESITY

THESIS SUMMARY

Research Supervisor: Prof. Stefka Vasileva Valcheva-Kuzmanova, MD, PhD, DSc

ABBREVIATIONS USED

COX-2 – cyclooxygenase-2

MAO-A – monoamine oxidase-A

HbA1c- glycated hemoglobin

hASC – human adipose-derived stem cells

NO - nitric oxide

PGE2- prostaglandin E2

PI3K/Akt/mTOR- phosphatidylinositol 3/ serine-threonine protein B (PKB; or AKT)

/mammalian target of rapamycin

BMI –body mass index

LDL – low-density lipoprotein)

HDL –high-density lipoprotein)

FDA – Food and Drug Administration

CK-MB – MB fraction of creatine kinase enzyme

PC1 – prohormone convertase 1

POMC – proopiomelanocortin

AST – aspartate aminotransferase

ALT – alanine aminotransferase

AP – alkaline forfatase

HbA1c − glycated hemoglobin

SOD – superoxide dismutase

TBARS –substances reacting with thiobarbituric acid

GTT - glucose-tolerance test

WHO - World Health Organization

VSG – vertical sleeve gastrectomy

I. INTRODUCTION

Obesity is a global problem with epidemic proportions and increasing incidence nowadays. Genetic, socioeconomic and endocrine factors, as well as underlying disease or drug therapy are seen as underlying causes. The condition is not only aesthetically unattractive but is also a serious medical problem, increasing the risk of developing cardiovascular disease, diabetes, infertility, neurodegenerative and cognitive diseases, cancer, and others (Britannica. 2024). Obesity and its co-morbidities lead to significant health care costs due to the morbidity, mortality and reduced productivity it causes. It affects different age groups, including children, adolescents, and adults of both sexes (World Health Organization. Obesity). Managing obesity requires a combination of lifestyle changes and medication treatment. A 5-10% reduction in body weight provides numerous benefits in terms of cardiovascular and metabolic health. Although rarely appropriate, bariatric surgery can also be used. Weight gain after stopping treatment is not uncommon. Medicinal plant extracts are gaining great popularity as a weight loss therapy.

Phytochemical products have been used for a long time for prophylaxisand treatment of chronic diseases. However, further research is needed on their efficacy, long-term safety, appropriate dose and side effects (Esteghamati et al., 2015).

According to the World Health Organization, 80% of people get their primary medical care from traditional medicine by using plant extracts or their active components. Aromatic plants are used in traditional medicine for a multitude of ailments. A large number of active phytochemicals have been found in them, including flavonoids, terpenoids, lignans, polyphenols, coumarins, and others. In fact, many medicines are of plant origin - reserpine, salicylic acid, senna, digitalis glycosides, paclitaxel, and others (Craig. 1999).

The food industry has prioritized the search for natural substances as a replacement for synthetic dietary supplements. For this reason, aromatic plants with their health benefits are finding increasing use in food production. The food industry is taking advantage of the significant nutritional content and strong antioxidant capacity of these substances. Various extracts of medicinal and aromatic plants that have been scientifically proven to provide health benefits are being incorporated into food compositions. The high presence of bioactive substances in them and their economic yield as sources make them conveniently usable in minimally processed food

products (Dikme, 2023). So far, only a single study has examined the effects of the monoterpene anethole in obesity (Noreen et al., 2023). This necessitates further studies on the effects of aromatic co-addition on obesity-induced health outcomes.

II. AIM AND OBJECTIVES

1.1. III.THE OBJECTIVE of the present dissertation was to investigate and summarize the pharmacological effects of anethole administered orally in increasing doses to rats in a model of obesity induced by a high calorie diet (HCD).

1.2. To accomplish this goal, we set the following OBJECTIVES:

1.3. 1.To induce obesity in healthy experimental animals by administering a high calorie diet (HCD) for 10 weeks.

1.4. 2.In the obesity model thus obtained to study the effects of anethole on:

1.5.Body weight

1.6.Calorie and fluid intake

1.7.Behaviour, analysing data on:

1.7.1.Total physical activity

1.7.2.Anxiety

1.7.3.Depressive-like behaviour

1.7.4.Spatial memory

1.8.Biochemical and clinical laboratory indices:

1.8.1.Blood glucose

1.8.2.Serum triglycerides

1.8.3.Serum cholesterol

1.8.4.Liver enzymes - aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP)

1.8.5.Blood clotting time

1.9.Indicators of antioxidant protection and oxidative stress:

1.9.1.Superoxide dismutase (SOD) in serum

1.9.2.Thiobarbituric acid reactive substances (TBARS) in serum and tissue homogenates from brain, liver and heart

1.10.Tissue indices:

1.10.1.Adipose tissue indices (of retroperitoneal, mesenteric, perigonadal, paranephric and total adipose tissue)

1.10.2.Liver index

1.11.Histology of liver, retroperitoneal adipose tissue, myocardium and coronary vessels

2.8. Immunohistochemical markers of apoptosis/antiapoptosis and inflammation in liver and retroperitoneal adipose tissue

2.9. Carrageenan-induced acute inflammation of the hind paw.

III.MATERIALS AND METHODS

1. Materials

1.1.Experimental animals

The experiments were conducted on 50 male Wistar rats kept in the Vivarium of the Medical University of Varna under standard laboratory conditions, in ventilated rooms with a room temperature of 20-25℃ and a 12-h light-dark cycle, housed in standard plastic cages. Experiments were conducted in accordance with national and international laws and policies based on the European Directive for the Protection of Animals in Science (2010/63/EU) and ethically approved by the Bulgarian Food Safety Authority (Document No. 177/07.07.2017).

1.2. Anethole

In the current experiment, the animals were treated with the experimental substance: Anethole (Sigma-Aldrich, Germany), which is a clear, colorless to amber liquid with a specific aniseed-like sweet aroma and taste. The density of anethole was 0.99 g/cm3 at 20 ºC and the molecular weight was 148.21 g/mol. The experimental substance is highly soluble in ethanol and sparingly soluble in water. Sunflower oil was used as solvent in the present experiment.

2. Methods

1.1.Obesity induction and treatment

Animals were allocated into 5 groups of 10 members each: control, HCD, HCD+62.5A, HCD+125A and HCD+250A. For the duration of 10 weeks, animals in the control group (C) received standard laboratory rat chow pellet and plain drinking water, and the HCD groups received a high-calorie diet. HCD was rich in animal fat (17%) and simple sugars (17%) and was obtained by enriching standard pellets with lard and fructose. Moreover, drinking water was replaced with a 10% fructose solution in animals of the HCD groups (Gancheva et al., 2015b). The energy content of the standard pelleted diet was 279 kcal/100 g and that of the high calorie diet was 405 kcal/100 g, and the animals also get additional 40 kcal for every 100 ml of fructose solution consumed. The HCD+62.5A, HCD+125A and HCD+250A groups were treated daily

with anethole at doses of 62.5 mg/kg, 125 mg/kg and 250 mg/kg, respectively. Anethole was administered orally via a soft probe, dissolved in sunflower oil, in a volume of 0.3 ml/100 g body weight at the same time in the morning at 8 hours. Control and HCD groups were probed daily with the diluent administered in the same volume. Experimental animals were euthanized under general ether anesthesia 24 hours after the last treatment.

Summary data on diet and treatment of experimental animals are presented in **Table 1.**

Table 1. Experimental protocol; High calorie diet- lard 17% and fructose 17% to standard pellets; 10% fructose solution instead of drinking water.

Group	Diet	Oral treatment (10 weeks)				
	Standard	HCD	Anethole 62.5 mg/kg	Anethole 125 mg/kg	Anethole 250 mg/kg	Sunflower oil
Control	√					√
HCD		√				√
HCD+62.5A		√	√			
HCD +125A		√		√		
HCD +250A		√			√	

2.2. Measurement of caloric and fluid intake, and body weight gain

A known amount of food (measured in grams) was given to the rats *ad libidum* and the remaining amount of food was weighed every 3 days. Calculating the food intake in grams per rat for each 3 days and finally is used to calculate the average food intake per rat for the entire treatment period. Caloric intake is calculated by converting food and fructose fluid intake into calories, based on our known carolability data. Fluid intake was also monitored every 3 days and calculated similarly to food intake. The body weight of the animals was measured weekly. Body weight gain in grams was calculated using the following formula : final body weight - initial

body weight. The percentage body weight gain was calculated using the following formula : [(final body weight - initial body weight)/ initial body weight] * 100.

2.3. *Behavioural methods*

2.3.1. Open field test (OFT)

The open field test is widely used to investigate exploratory behaviour and general activity in rodents (Crusio et al., 2001). The open field consisted of an enclosed wooden arena measuring 100×100×40 cm. The floor was graphed into equal sized squares with a blue line measuring 20×20 cm. The rat is placed in the center of the arena and allowed to explore it for 5 minutes. This was done for each animal, and the results were reported manually. The number of horizontal movements (squares the animal traverses with its four paws) and vertical movements (number of hind paw lifts, including propping up the field walls with the front paws) are recorded as a measure of locomotor activity (Belovicova et al., 2017).

2.3.2. Social interaction test (SIT)

The social interaction test is conducted in the same square arena used for the open field test. It is used to determine the level of anxiety in rodents (File et al., 1978). Each rat is tested with an unknown partner from another cage with whom they do not differ in treatment and body weight by more than 10%. The paired rats were placed in opposite corners of the arena and observed for 5 min. Time spent in active social interaction (sniffing, straightening fur, chasing, climbing on or crawling under the partner) was recorded, which was inversely proportional to the level of anxiety. Time spent in passive interaction (sitting or lying down in contact with the partner) is not considered social interaction.

2.3.3. Forced swim test (FST)

This is a commonly used test to determine the presence of depressive behavior. The test consists of two sessions, a training session and a test session, conducted on two consecutive days over 24 hours. The rats are placed in a transparent glass cylinder (17 cm in diameter and 50 cm in high) filled with water (temperature 21±1 °C) to a level of 30 cm, which prevents the animals from supporting themselves with tail or paw to the bottom. The training and test sessions were 5 min each. The presence/absence of movement in the cylinder was recorded manually, and only the

results of the test session were analysed. The amount of time in which the animals are immobile and do not attempt to escape the water is considered an indicator of depressive behavior (Slattery et al., 2012). A reduction in immobility time by an experimental substance is interpreted as a manifestation of an antidepressant effect (Slattery et al., 2012).

2.3.4. Object location recognition test (OLRT)

This test is a modification of the place recognition test for novel objects and aims to assess spatial memory (Vogel-Ciernia et al., 2014). The test was conducted in a rectangular wooden arena, which was part of an open field test arena, measuring 65 x 45 cm, surrounded by 40-cm walls. Thus, the animals were already familiar with the arena, making a special habituation session unnecessary. The test consisted of 2 sessions, a training session and a test session, each of them lasting 3 min. The training session was conducted with two identical objects (parallelepipeds attached to the floor with no possibility of moving) placed symmetrically. The time spent orienting the animal's nose to the object from a distance of 1 cm or less, actively approaching and sniffing the object, climbing on the object (but not sitting on it), was considered as exploration. After each animals, the arena and the objects were cleaned with 70% ethanol. The test session was conducted 30 min after the training one. During the test session, one of the objects was moved to a location different from that in the training session. The length of time spent exploring the object with the new location (B) and the object with the known location (A) was recorded. The recognition index B/(A + B) was calculated as the ratio of the time B to the total exploration time (A + B) during the test session and was used as a measure of spatial memory.

2.4. Fractionation of tissues and organs and preparation of organ homogenates

2.4.1.Preparation of blood serum

At the end of the experiment, after anaesthetizing the experimental animals with diethyl ether, blood was drawn from the lingual veins, collected in tubes and centrifuged at 2000 x g rpm for 10 min. The separated serum was stored at minus 20 ºC until biochemical analyses.

2.4.2. Isolation of organs

After decapitation of the experimental animals, retroperitoneal adipose tissue - mesenteric, paranephric, perigonadal, retroperitoneal - was dissected and the individual fractions were weighed. Total adipose tissue was calculated. The liver, heart and brain were dissected. The liver was weighed. Samples of liver, heart and retroperitoneal adipose tissue were fixed for histological examination, and liver and retroperitoneal adipose tissue for immunohistochemical examination. Brain, heart and liver tissue samples were frozen for tissue homogenates.

2.4.3. Preparation of tissue homogenates

Homogenates were prepared from brain, heart and liver tissue for biochemical studies. Tissues were homogenized using ice-cold Tris/HCl, 50 mM, pH 7.4 at a ratio of 1:5 for brain and 1:10 for liver and heart. Homogenates were centrifuged (2000 rpm, 10 min, 4 °C). Supernatants were used.

2.5. Biochemical and clinical laboratory tests

2.5.2. Glucose tolerance test (GTT) and determination of blood serum glucose levels

The glucose tolerance test was performed by intraperitoneal injection of the experimental animals with 40% glucose solution at a dose of 2 g/kg. Blood glucose was tested with ACCU-CHEK Performa glucometer and ACCU-CHEK Performa test strips. Blood samples were collected by incision at the distal end of the tail according to Fluttert's method (Fluttert et al., 2000) immediately before glucose injection and 30, 60 and 90 minutes afterwards**.**

2.5.2. Triglyceride levels measurement

Triglyceride levels were measured in blood serum using standard kits (BioMaxima S.A., Poland) according to the manufacturer's instructions. In this method triglycerides are hydrolysed by lipoprotein lipase to glycerol and fatty acids. Glycerol is phosphorylated with ATP by glycerol kinase to yield glycerol-3-phosphate and adenosine diphosphate. Glycerol-3-phosphate is oxidized by glycerol kinase to dihydroxyacetone phosphate and hydrogen peroxide. The latter binds 4-chlorophenol and 4-aminoantipyrine, resulting in a coloured complex. The colour intensity of the resulting complex is measured photometrically at 500 nm and is proportional to the triglyceride concentration. An AURIUS 2021 spectrophotometer (Cecil Instruments Ltd., UK) was used.

2.5.3. Cholesterol levels measurement

Cholesterol levels were measured in blood serum with standard kits (BioMaxima S.A., Poland) according to the manufacturer's instructions. In this method cholesterol esters are hydrolysed by cholesterol esterase to cholesterol and free fatty acids. The free cholesterol is oxidized by cholesterol oxidase releasing hydrogen peroxide. It binds to 10-phenol and 4-aminoantipyrine, producing a coloured complex. The colour intensity of the resulting complex is measured photometrically at 500 nm and is proportional to the cholesterol concentration. An AURIUS 2021 spectrophotometer (Cecil Instruments Ltd., UK) was used.

2.5.4. Serum liver enzymes measurement

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using colorimetric kits Qumica Clinica Aplicada S.A., Poland, and alkaline phosphatase (AP) - Biomaxima S.A., Poland. Liver enzymes were measured spectrophotometrically. The results were expressed as units per liter (U/l).

2.5.5. Serum and tissue homogenate sthiobarbituric acid reactive substances (TBARS) levels determination

They serve as a marker for lipid peroxidation, and are quantified in brain, heart and liver tissue. TBARS were measured colorimetrically (spectrophotometer CE2021, Cecil Instruments Ltd, UK) at a wavelength of 532 nm following the procedure described by Ohkawa et al., 1979. This approach quantified the absorbance of the colour generated by the reaction between thiobarbituric acid and lipid peroxides. TBARS levels were measured in nmol/g tissue. The reference standard is malondialdehyde (MDA), the major product derived from membrane fatty acids during their peroxidation.

2.5.6. Serum superoxide dismutase (SOD) levels measurement

Antioxidant enzyme activity was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Boster Biological Technology Co., Ltd.) and was performed following the manufacturer's instructions using a BioTek 800 TS microplate reader. The kit contains a tetrazolium salt to identify superoxide radicals produced by xanthine oxidase and hypoxanthine.

One unit of SOD is defined as the amount of enzyme required to induce 50% dismutation of the superoxide radical. The activity of SOD is measured in U/ml.

2.5.7. Clotting time measurement

The parameter was measured manually by counting the time from reaction initiation to fibropolymer formation, measured in seconds. It represents the expression of the initiation of the coagulation system, the formation of thrombin and the first fibrinopolymer.

2.6. Ttissue indices determination

2.6.1. Mesenteric, paranephric, perigonadal, retroperitoneal and total adipose tissue indices

After euthanasia of the experimental animals, mesenteric, paranephric, perigonadal and retroperitoneal adipose tissue was dissected, the individual fractions were weighed, and total adipose tissue was calculated. The obesity index, expressed as a percentage of total body weight, was calculated by the total adipose tissue index using the following formula: adipose tissue weight (g)/total body weight (g) × 100. Fat tissue indices were calculated using the formula: fat depot (g) / total body weight (g) × 100.

2.6.2. Liver index measurement

Liver index was calculated using the formula: liver weight (g)/total body weight (g) × 100.

2.7. Histological methods

Tissue pieces of retroperitoneal adipose tissue, liver, myocardium and coronary vessel were used for histological examination and fixed in 10% neutral formalin. The material was processed by alcohol dehydration and paraffin embedding. Staining was with hematoxylin/eosin.

2.8. Immunohistochemical methods

For immunohistochemical study mini KIT high Ph DAKO K8024 was used
Indirect immunoperoxidase technique. To ensure negative controls, paraffin block slices were incubated with normal nonimmune serum instead of the primary antibody. The EnVision FLEX versatile high-sensitivity antibody detection imaging system was used to assess immunohistochemical expression of the proapoptotic marker BAX, antiapoptotic marker BCL-2,

and proinflammatory marker MAC387, using rabbit polyclonal antibodies against BAX (Santa Cruz Biotechnology, sc-6236) and BCL-2 (Santa Cruz Biotechnology, sc-492) and mouse monoclonal antibody against MAC387 (Santa Cruz Biotechnology, sc-66204). All three markers were at a dilution of 1:50.

Immunohistochemical expression of BAX and BCLl-2 and MAC387 was assessed semiquantitatively by determining the expression of a field consisting of a minimum of fifty cells from each sample. For liver, the intensity of cytoplasmic expression was scored as : 0 no response, 1+ weak positivity, 2+ moderate positivity or 3+ strong positivity. Estimation of marker expression in adipose tissue varied due to peripheral location of nuclei and presence of large cytoplasmic lipid inclusions. It is defined as: 0 - no reaction or 1+ positive depending on the absence or presence of intranuclear immune deposits. BAX/BCL-2 ratio was determined for each group and values were compared between groups.

2.9. Hind paw acute inflammation induction

At the end of the treatment period, a dose of 1 mg carrageenan was injected into the plantar surface of the left hind paw of the rats. Carrageenan was administered as a freshly made solution in 0.9% saline to generate an acute inflammatory response. Paw volumes (in milliliters) were measured at different time points: before injection, at the 30th minute, and at the 1st, 2nd, 3rd, 4th, and 5th hour after injection. Measurements were made using a digital plethysmometer LE7500 Panlab, Barcelona. The difference in paw volume before and after injection was directly proportional to the intensity of the inflammation caused. The calculation of paw edema (in milliliters) was performed using the following formula: paw edema was calculated using the formula Vs - V0, where Vs represents the paw volume measured at six time intervals after carrageenan injection and V0 represents the initial paw volume.

2.10. Statistical methods

Data were presented as mean ± standard error (SEM) and analysed using Student's t-test, linear trend test, one-factor analysis of variance (One-way ANOVA) followed by Dunnet's post test. Two-factor analysis of variance Two-way ANOVA followed by Bonferroni post test was used to process the blood glucose data in the course of glucose tolerance test. A level of $p < 0.05$

was considered significant. GraphPad Prism 7.00 statistical software (GraphPad Software, Inc., CA, USA) was used.

IV. OWN RESULTS AND DISCUSSION

1. Effect of anethole on body weight, calorie and fluid intake

1.1. Results

The results are presented in Table 2 and Figures 3 and 4.

In the present experiment, HCD did not significantly alter the final body weight of the experimental rodents, and no change in body weight gain was observed as an absolute value and as a percentage of baseline. The results were against a background of reduced food consumption in grams ($p<0.001$) but increased caloric intake and increased fluid intake ($p<0.001$). Treatment with anethole at all doses did not change final body weight, but the high dose prevented body weight gain relative to the HCD group ($p<0.001$). Anethole treatment increased food consumption significantly ($p<0.001$) relative to the HCD group at all doses: $p<0.05$ for HCD+62.5A and HCD+125A and $p<0.001$ for HCD+250A, with the effect determined to be concentration-dependent by post-test for linear trend ($P<0.0001$). Fluid intake was also increased by the 125 mg/kg dose of anethole in comparison to the HCD group ($p<0.001$). Caloric intake was significantly increased in the anethole-treated group compared to the HCD group: $p<0.05$ for VCD+62.5A, $p<0.01$ for HCD+125A and $p<0.001$ for HCD+250A. The post-test for linear trend found a dose-dependent effect of anethole ($P<0.0001$).

Table 2. Biological parameters related to energy metabolism in rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M.; ***p<0.001, *p<0.05 vs. control group (C); #p<0.05, ##p<0.01 vs. HCD

Index	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
Initial body weight (g)	280,8 ± 7.2	281,0 ± 7.9	281,8 ± 7.7	280,8 ± 7.6	280,8 ± 10.1
Final body weigh (g)	336,4± 12.0	332,2± 13.0	360,0 ± 12.1	339,8 ± 8.6	308,2 ± 12.4
Weight gain (g)	55.6 ± 7.1	59.6 ± 3.9	79.1 ± 9.5	59.0 ± 4.2	21.6 ± 6.1 ***###
Weight gain (% from the initial one)	19,7 ± 2,3	21,2 ± 1.4	28,5 ± 3.7*	21,1 ± 1.6	7,6 ± 2,1 **###
Food consumption (ml/day/rat)	18,70 ± 0.3	12,73 ± 0.3 ***	13,94 ± 0.4 ***#	14,01 ± 0.3***#	15,02 ± 0.4 ***###
Fluid consumption (ml/day/rat)	33,43 ± 0.6	44,21 ± 0.8 ***	46,18 ± 0.9 ***	53,51 ± 1.1 ***###	46,37 ± 0.9 ***
Caloric intake (kcal/day/rat)	52,17 ± 0.9	69,26 ± 1.4 ***	74,93 ± 1.5 ***#	78,17 ± 1.4 ***###	79,38 ± 1.6 ***###

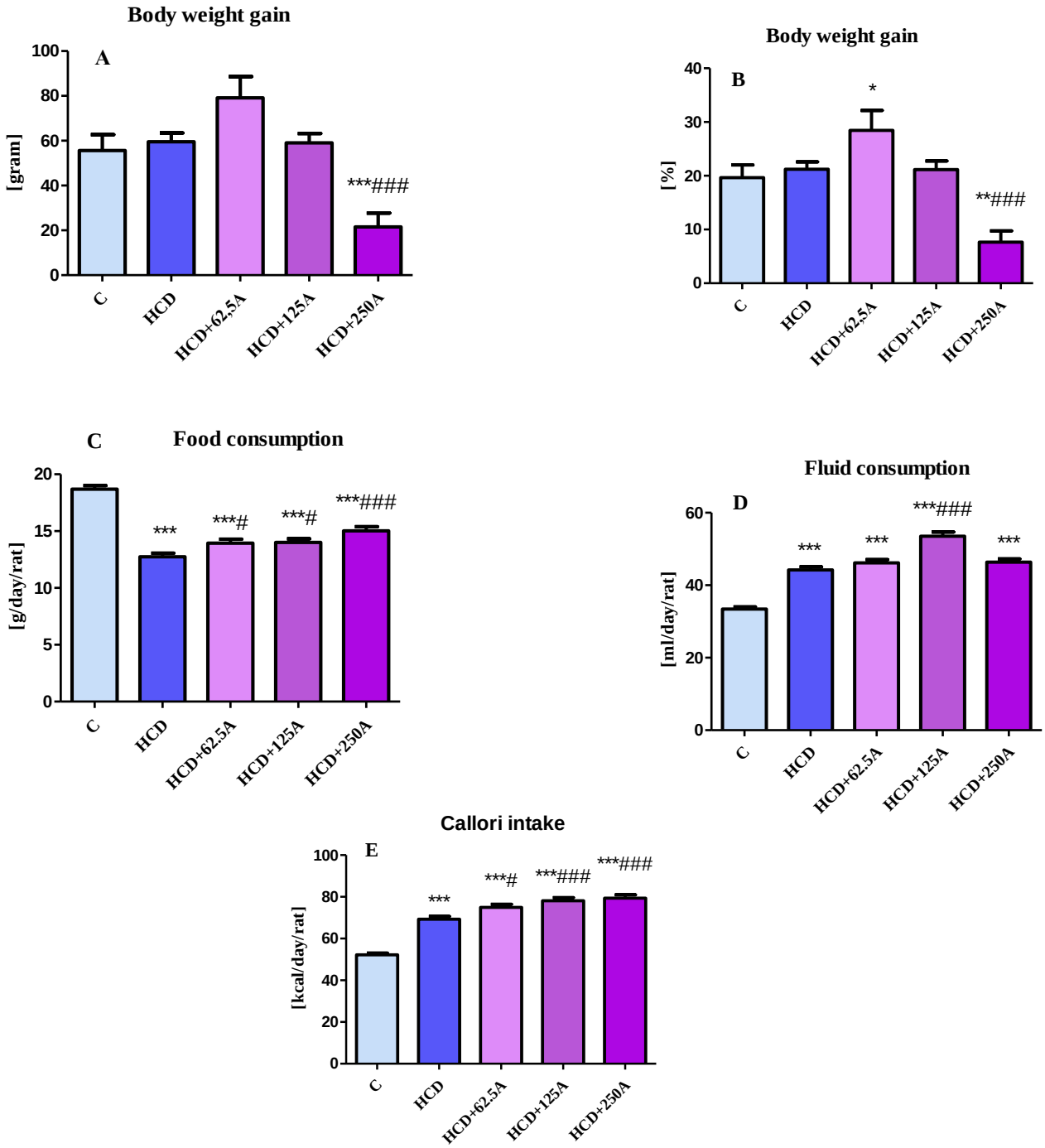

Figure 3. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62. 5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A), on body weight gain (measured in grams and as a percentage (A and B), food consumption (C), fluid consumption (D) and caloric intake (E); ***p<0.001, *p<0.05 vs. control group (C); #p<0.05, ##p<0.01 vs. HCD

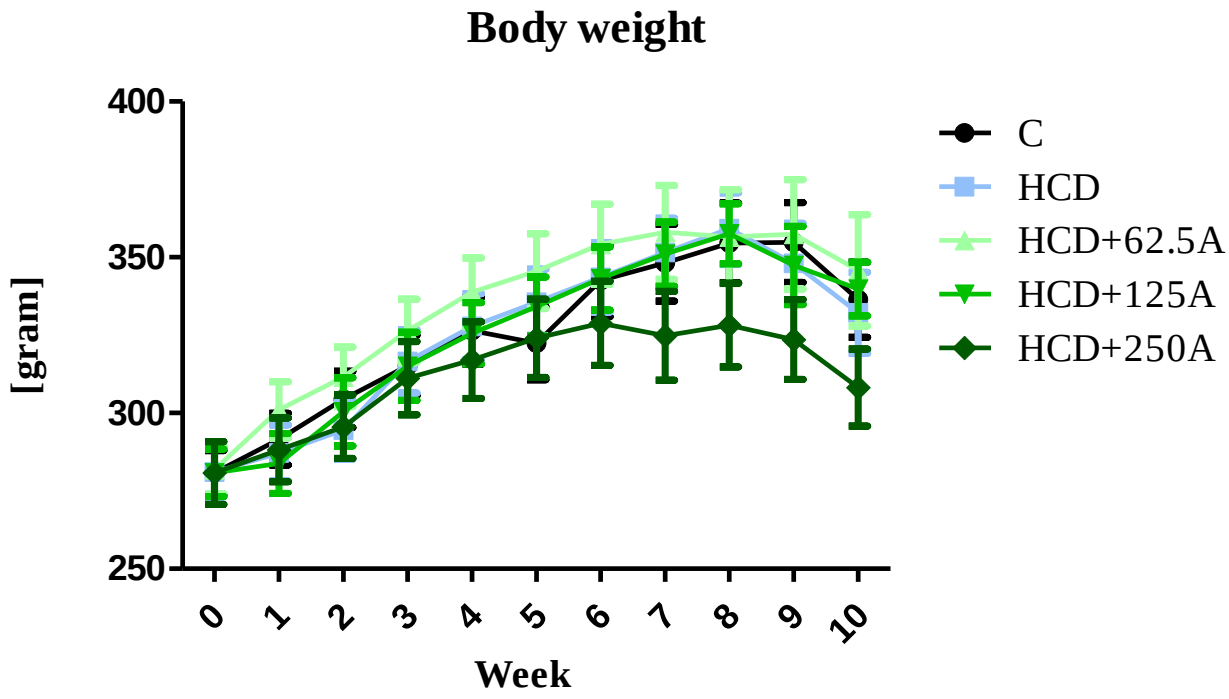

Figure 4. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on change in body mass by week.

1.2. Discussion

During 10 weeks of treatment, the percent fat and fructose rats in the HCD group showed no significant increase in body weight compared to the control. Other studies also concluded that administration of this diet for 16 weeks in juvenile rats did not show a significant increase in body weight (Marija et al., 2023). After 10 weeks, the high requirement diet resulted in reduced growth of the young animals (Even et al., 2021). There was also a significant reduction in average food consumption in the HFD group on the background of increased caloric intake compared to the control group on the usual diet. This leads to the conclusion that even if the diet they consume is more caloric, the total amount of food consumed is not sufficient to cause an increase in body weight. Studies with high-fat and high-fructose diets with 45% fat and 30% or 15% fructose solution had lower average daily intakes. However, increases in caloric intake,

body weight, and fat intake were observed in these animals. On the other hand, a 60-day fructose diet did not result in an increase in body mass in 30-day-old male Wistar rats compared to rats on a normal diet, as reported by De Castro et al. (2013).

Administration of anethole did not affect body weight of the rats, but a reduction in body weight gain was observed from high doses of anethole. Other studies also found similar results, regarding that trans-anethole supplementation did not significantly affect average daily gain, body weight and feed/gain ratio in broilers. Average daily feed consumption was significantly increased (Yu et al., 2021). Both fennel and trans-anethole reduced blood glucose levels and intensity of weight loss in rats with induced diabetes (Samadi-Noshahr et al., 2020). Reductions in food and water intake have also been observed (Samadi-Noshahr et al., 2020). Other studies have shown opposing effects of anethole versus body weight. Administration of anethole prevents weight loss of Parkinson's disease animals during treatment, which is most likely related to its antioxidant properties on the gastrointestinal tract and its ability to improve digestion and absorption (Moradi Vastegani et al., 2023a). Body weight was found to be reduced in animals with diabetic nephropathy. There was an increase in body weight of animals treated with anethole-rich extract (Vellapandian et al., 2022). Administration of trans-anethole at doses ranging from 105 to 550 mg/kg bw per day in various concentrations (0.25 to 1%) resulted in a transient decrease in body weight in rats (Truhaut et al., 1989). Imbabi et al. showed that, compared with the untreated group, supplementation with fennel oil containing 75% anethole increased ($p<0.05$) the body weight of rabbits at 8 and 12 weeks and the mean daily gain (Imbabi et al., 2021). Anethole resulted in a reduction in body weight of rats with high cholesterol after 6-week treatment (Noreen et al., 2023). Subacute treatment with anethole at a dose of 30 mg/kg/day did not affect body weight or behaviour of treated mice (Massimiliano et al. 2007).

Dehydration leads to diseases such as heat stroke but also increases the likelihood of developing kidney stones, chronic kidney disease, urinary tract infections, cardiovascular disorders and metabolic diseases. Moderate dehydration affects both mood and cognitive ability (Nakamura Y, 2020) In the present experiment, anethole in moderate doses increased fluid intake without affecting total body weight. Anethole increased food consumption and the amount of

calories ingested in the current study. The same result was observed with the inhalation of substances containing phenylpropanoid structures. Curry essential oil or its individual constituents (transcinnamaldehyde, eugenol and trans-anethole) increased appetite and the amount of food consumed in mice (Ogawa et al., 2016). According to Hur et al. in rats, inhalation of fennel essential oil did not affect either body weight gain or the amount of food intake (Hur et al, 2006). According to Bae et al. examining the effect of fennel on appetite in overweight women, intake of fennel tea before meals suppressed the subjective feeling of hunger and increased the feeling of satiety, although it did not change the amount of food consumed (Bae et al., 2018).

2. Effect of anethole on behavior in a model of obesity

2.1. Open field test (OFT)

Figure 5 and **Table 3** present the changes in the number of squares crossed in the arena in the OFT used to assess horizontal locomotor activity and the changes in the number of rodent uprights, giving information on verical activity. One-way ANOVA analysis of variance showed no significant treatment effect on horizontal activity: $F(4,43) = 1.342$, $P = 0.2701$. There was also no statistically significant differance in vertical locomotor activity after the anethole treatment of the experimental animals: $F(4,45) = 0.5457$, $P = 0.7030$.

Table 3. Number of horizontal and vertical movements in open field test in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M.

Index / Group	Horizontal locomotor activityт (number of squares crossed)	vertical locomotor activity (number of uprights)
C	72.2 ± 5,48	20,2 ± 5,45
HCD	100,2 ± 8,57	25,4 ± 11,23
HCD+62.5A	90,3 ± 8,55	22,9 ± 8,84
HCD+125A	96,1 ± 12,05	22.1 ± 10,27
HCD+250A	88.0 ± 8.45	20.5 ± 8,03

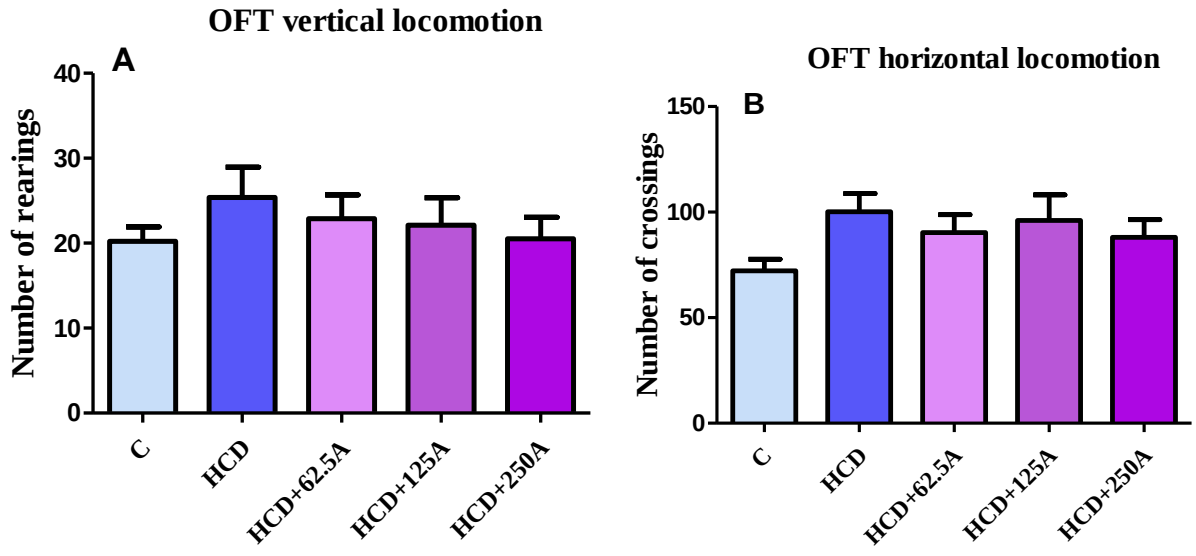

Figure 5. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on vertical (A) and horizontal (B) movements in open field test (OFT).

2.1. Effect of anethole in social interaction test (SIT)

As shown in **Figure 6** and **Table 4**, the high calorie diet reduced the interaction time between rats (19.71 ± 2.15 sec) compared to the control (28.32 ± 2.18 sec) with statistical significance $p<0.05$. The low dose of 62.5 mg/kg anethole prevented this effect, and the interaction time between rats in the HCD+62.5A group was not significantly different from that of the control (23.69 ± 3.22 sec). The interaction time between rats of the HCD+125A group was 19.41 ± 1.57 s, and that of the HCD+250A group was 17.44 ± 1.19 s. Thus, the social interaction time in these groups was not significantly different from that of the HCD group and remained significantly lower than that of the control ($p < 0.05$ for the HCD+125A group and $p < 0.01$ for the HCD+250A group).

Table 4. Time, measured in seconds, for social interaction in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M. *p<0.05 versus control (C); **p<0.01 versus control (C)

Group / **Index**	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
Time for social interaction (seconds) (mean ± SEM)	28,3 ± 2,2	19,7 ± 2,1 *	23,7 ± 3,2	19,4 ± 1,6 *	17,4± 1,2 **

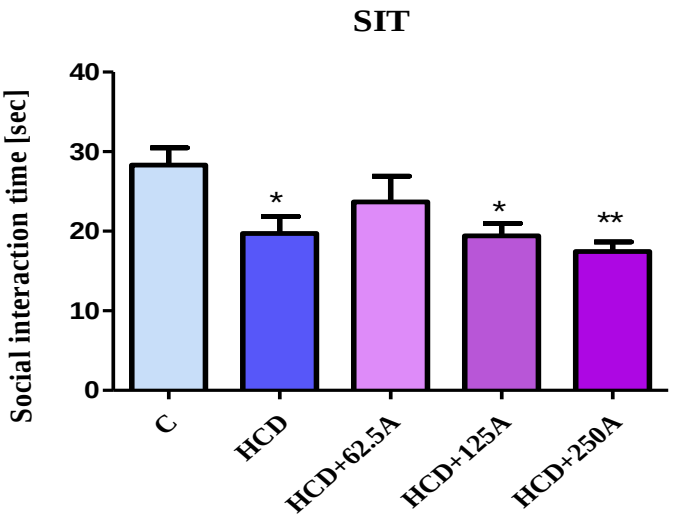

Figure 6. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the interaction time between animals in social interaction test (SIT); *p<0.05 vs. control (C); **p<0.01 vs. control (C)

2.3. Effects of anethole in forced swim test (FST)

The data in Figure 7 and Table 5 for the FST, which were analysed by one-way ANOVA, revealed a trend towards a reduction in the duration of immobility in the anethole-treated groups, with no statistical significance [F(4,42)=0.7794, P=0.5449]. This trend is probably not due to reduced locomotor activity as none was observed in the OFT. Hence, the trend to decrease immobility time could be considered a manifestation of an antidepressant effect.

Table 5. Time, measured in seconds, spent in immobility in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M.

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
Immobility time (seconds)	37.85 ± 4.36	40.6 ± 5.06	30.75 ± 3,62	33,28 ± 2.24	32.85 ± 5.86

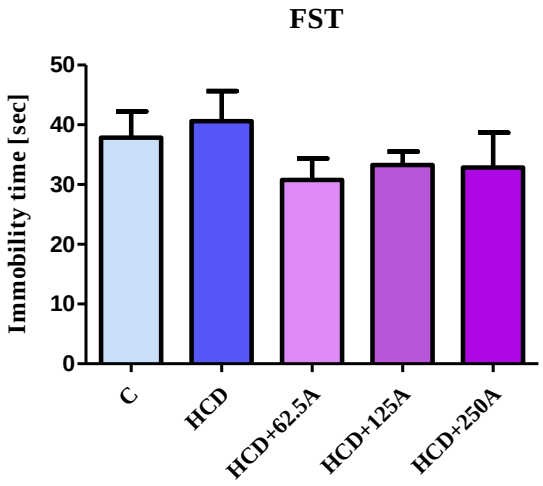

Figure 7: Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on immobility time in the forced swim test (FST)

2.4. Effect of anethole in an object place recognition test

Student's t-test showed a significant decrease ($p<0.001$) in the recognition index in the HCD group (0.39 ± 0.033) compared to the control group (0.57 ± 0.028), presented in **Table 6** and Figure 8. A significant effect of anethole treatment on spatial memory was shown by ANOVA ($F = 3.802$, $P = 0.0182$) analysis. Dunnet's post-test showed a significantly higher location recognition index in the HCD+125A (0.59 ± 0.041) and HCD+250A (0.61 ± 0.058) groups compared to the HCD group (0.39 ± 0.033), $p < 0.05$. Post-test for linear trend found a significant concentration-dependent effect of anethole ($P = 0.0028$). The location recognition index of all anethole-treated groups was not significantly different from that of the control group (ANOVA: $F = 0.5806$, $P = 0.6318$).

Table 6. Location recognition index values from the object location recognition test in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001 vs. control group (C); #p<0.05 vs. HCD

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
B/(A+B) (mean ± SEM)	0.57 ± 0.03	0.39 ± 0.03***	0.52 ± 0.06	0.59 ± 0.04 #	0.61 ± 0.06 #

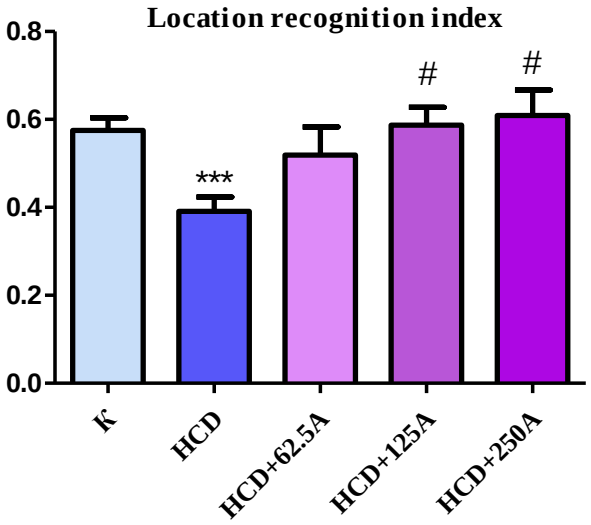

Figure 8. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the location recognition index in the object location recognition test. ***p<0.001 vs. control group (C); #p<0.05 vs. HCD

2.5. Discussion

Changes in motor activity or sedation are important behavioural conditions to consider when interpreting the results of other behavioural tests.

The available literature reveals single experimental studies of the effects of anethole in a model of obesity, despite its known beneficial effects on obesity. According to the available documentation, the present study is the first searching for the effects of anethole, administered in

increasing doses, on motor activity and spatial memory in rats receiving a high-calorie diet. In confirmation of the result obtained in the present study, Ritter et al also reported the absence of change in locomotion in an open field test when rats were treated with different doses of anethole (Ritter et al., 2014). Hassanzadeh et al reflected the same result in NMRI mice treated with trans-anethole at doses of 25 mg/kg and 50 mg/kg intraperitoneally (Hassanzadeh et al., 2022). Administration of anethole for 21 days in Swiss albino mice also did not alter locomotion (Dhingra et al., 2019). A similar result was obtained when fennel essential oil containing 82.08% anethole was administered intraperitoneally in albino mice at doses of 100-400 mg/kg (Abbasi-Maleki et al., 2021).

Anxiety disorders, which affect one-eighth of the world's population, are a major target of psychopharmacological research. Visceral obesity in humans leads to a high likelihood of developing anxiety-depressive disorders and cognitive impairment (Tang et al., 2021; Fulton et al., 2022). Many psychodynamic, psychoanalytic, behavioral, cognitive, genetic, and biological hypotheses have been proposed to explain the heterogeneous etiology of anxiety disorders (Shri et al., 2010). The present experiment showed that anethole at a dose of 62.5mg/kg prevented the development of anxiety in experimental animals on a high calorie diet, whereas at doses of 125 and 250 mg/kg showed no similar effect. Niksokhan et al. observed in rats anxiolytic effect of 250 and 300 mg/kg hydroalcoholic extract of Pimpinella annisum seeds whose major component is anethole (Niksokhan et al., 2015). In vivo study showed that methanolic extract of Illicium verum (Chinese star anise) fruits administered at a dose of 200mg intraperitoneally in rats had anxiolytic effect (Chouksey et al., 2013). Fennel oil administered at a dose of 0.5 ml/day for 3 weeks showed significant anxiolytic effect in rats (Perveen et al., 2017). In a study conducted with 60 postmenopausal women, fennel intake showed no significant decrease in anxiety and depression (Ghazanfarpour et al., 2017).

The study conducted revealed that the administration of HCD resulted in a non-significant increase in immobility time. The administration of anethole showed a trend to decrease the immobility time giving information of antidepressant effect. In another study, trans-anethole induced significant antidepressant activity in non-stressed and stressed mice, with inhibition of brain monoamine oxidase-A and reduction of oxidative stress considered as a likely mechanism. In addition, the antidepressant effect of trans-anethole (25 mg/kg) was comparable to that of the

antidepressant fluoxetine (Dhingra et al., 2019). The antidepressant effect of fennel essential oil at doses of 100-400 mg/kg in mice is described by Abbasi-Maleki et al. 2021. Repeated administration of fennel oil in a volume of 0.5 ml/day containing 60-90% trans-anethole may have an antidepressant effect comparable to that of fluoxetine (Perveen et al., 2017). Hassanzadeh et al. also demonstrated the antidepressant effect of trans-anethole 25 mg/kg and 50 mg/kg (Hassanzadeh et al., 2022). The authors also found that administration of dopamine antagonists to anethole-treated animals significantly inhibited its antidepressant effect in the forced swim test. This finding provides information that the dopaminergic system may play a role in the antidepressant-like action of anethole (Hassanzadeh et al., 2022).

Experimental animals that consume a diet high in calories have impaired spatial memory and develop anxiety (Reyzov et al., 2019). In the present study, SCD induced spatial memory impairment and reduced antioxidant defense mechanisms in experimental animals. Multiple lines of evidence suggest that obesity is associated with memory impairment and oxidative stress. Heyward et. al. found that diet-induced obesity resulted in a decline in hippocampus-dependent spatial memory, but object recognition memory was unaffected (Heyward et al., 2016). Insulin resistance is thought to impair hippocampal physiological function and synaptic plasticity (Stranahan et al., 2008). This finding provides the link between spatial memory and obesity. Another major mechanism of cognitive decline is the decrease in hippocampal brain neurotrophic factor (Stranahan et al., 2008).

In the present study, treatment with anethole improved spatial memory impairment in a dose-dependent manner. The treatment duration (9 weeks) was long enough for the aromatic compound to exert its effect in improving spatial memory. The memory-enhancing and neuroprotective properties of anethole have been demonstrated in a number of studies (Marinov and Valcheva-Kuzmanova, 2015). Anethole is a key compound of *Shanzhuyu* (a Chinese herb) that has been shown to regulate amyloid deposition, oxidative stress, and apoptosis by regulating signaling pathways involved in Alzheimer's disease pathogenesis (Qu et al., 2020). Anethole as one of the main components of *Uncaria rhynchophylla* is considered a potential candidate for a future anti-amnesic drug as it has been shown to improve memory in tests such as Morris water

maze and passive avoidance (Shin al., 2013). In harmony with the previous claim, treatment with anethole at a dose of 250 mg/kg improved rotenone-induced cognitive dysfunction in both a novel object recognition test and a passive avoidance test (Vasteganiet al., 2023). Administration of trans-anethole at a dose of 50 mg/kg for 14 days improved memory retention potential in a rat model of Huntington's disease (Rajan et al., 2016). Trimethyltin chloride-induced impairment of long-term potentiation (LTP) can be reversed by trans-anethole through action on the N-methyl-D-aspartate (NMDA) receptor (Chang et al., 2022).

3. Effect of anethole on biochemical and clinical-laboratory parameters

3.1. Effect of anethole on metabolic parameters

3.1.1 Blood glucose in the course of glucose tolerance test (GTT)

Figure 9 presents the fasting plasma glucose levels, presented as mmol/l, in control and experimental animals. No statistical significance was found between the different groups.

GTT results are shown in **Figure 10** and **Table 7** presented as mmol/l at the respective time intervals. The administration of HCD resulted in an increase in blood glucose levels at the 30th minute. Two-way ANOVA followed by Bonferroni post test analysis revealed that at 30th minute glucose levels were significantly elevated compared to the control group in all experimental groups with the exception of HCD+250A. For the HCD+65A and HCD+125 groups, the significance was $p<0.001$ compared to the control, with the HCD+125 group also showing a significant increase compared to the HCD group ($p<0.01$). At the 60th and 90th minute, there were no significant differences in blood glucose levels between groups. The area under the curve (AUC) calculated for the quantitative total increase in blood glucose during GTT (**Figure 11** and **Table 8**) also revealed an impairment of glucose tolerance by the consumption of HCD ($p<0.05$). Treatment with anethole at doses of 62.5 and 125 mg/kg did not prevent this change ($p<0.01$ and $p<0.001$ versus control, respectively). In the group receiving the highest dose of anethole 250 mg/kg, the value for the area under the glucose level curve was not significantly different from that of the control group.

Table 7. Blood glucose values during the course of glucose tolerance test presented as absolute values (mmol/l) and percentage (%) of baseline value at the 30th, 60th and 90th min in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001 vs. control group (C) and ##p<0.01 vs. HCD group

Group	30-th min		60-th min		90-th min	
	(mmol/l)	(%)	(mmol/l)	(%)	(mmol/l)	(%)
C	12.28 ± 0.73	260.6 ± 16.1	8.39 ± 0,30	177.62 ± 5.92	7.96 ± 0.36	168.25 ± 6.50
HCD	15.25 ± 0.60***	333.45 ± 14.53***	9.42 ± 0,31	206.30 ± 8.46	8.49 ± 0.31	185.77 ± 7.62
HCD+62.5A	15.50 ± 1***	349.98 ± 20.28***	9.74 ± 0,51	219.60 ± 8.68	8.42 ± 0,34	191.29 ± 9.79
HCD+125A	17.31 ± 0,40***##	392.83 ± 14.30***##	9.17 ± 0,34	212.16 ± 12,74	8.63 ± 0,27	191.77± 8.74
HCD+250A	13.84 ± 0,99	300.12 ± 22.13	8.82 ± 0,39	206.98 ± 18.81	8.19 ± 0,29	185.36± 10.87

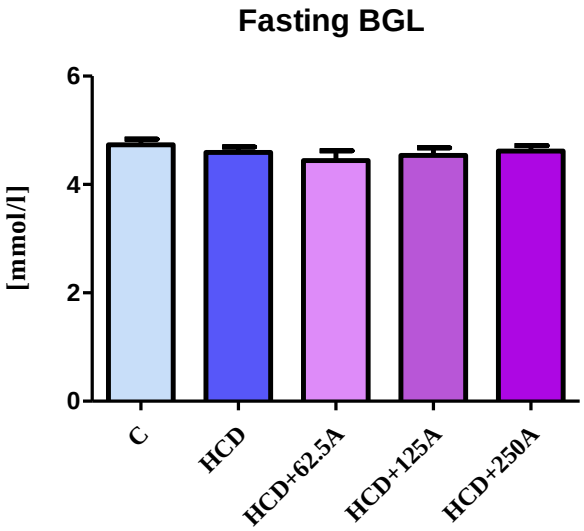

Figure 9. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on fasting blood glucose

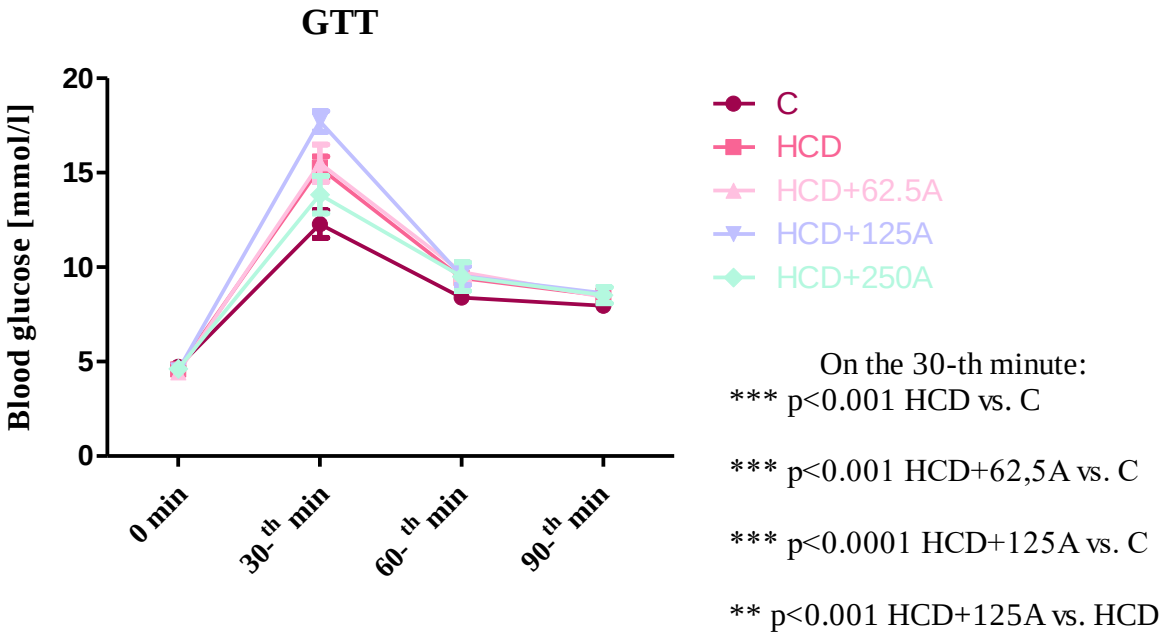

Figure 10. Effect of anethole (A) administered to rats on a high-calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A), and 250 mg/kg (HCD+250A) on blood glucose in the course of a glucose tolerance test

Table 8. Area under the curve (AUC) of blood glucose values over the course of glucose tolerance test in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05, **p<0.01, ***p<0.001 vs. control group (C)

Area under the curve (AUC)	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
Blood glucose	788,3 ± 21,23	936,4 ± 27,54 *	950,4 ± 49,56 **	1016 ± 30,31 ***	854,7 ± 38,54

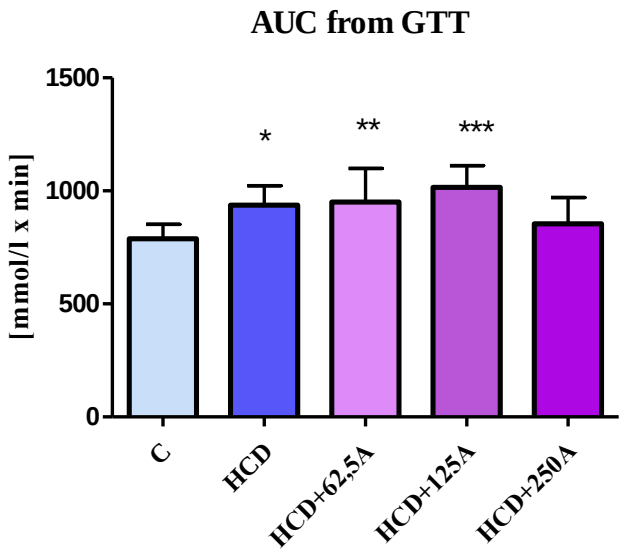

Figure 11. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on area under the curve (AUC) during glucose tolerance test; *p<0.05, **p<0.01,***p<0.001 vs. control group (C)

3.1.2. Effect of anethole on lipid profile

Serum triglyceride levels are presented in **Figure 12** and **Table 9**. There was no significant change in this parameter in the HCD group compared to the control group. Treatment of experimental animals with anethole did not result in a statistically significant change in serum triglycerides compared to the HCD group.

Serum cholesterol levels are presented in **Figure 13** and **Table 9.** The results of the statistical analysis were similar to triglycerides. There was no significant effect of either HCD or anethole treatment.

Table 9. Lipid profile in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M

Group	**Lipid profile**	
	Serum triglycerides	Serum cholesterol
C	0.67 ± 0.70	1.72 ± 0.08
HCD	0.80 ± 0.06	1.92 ± 0.10
HCD+62.5A	0.89 ± 0.08	2.18 ± 0.17
HCD+125A	1.07 ± 0.08	1.79 ± 0.03
HCD+250A	0.67 ± 0.07	2.01 ± 0.11

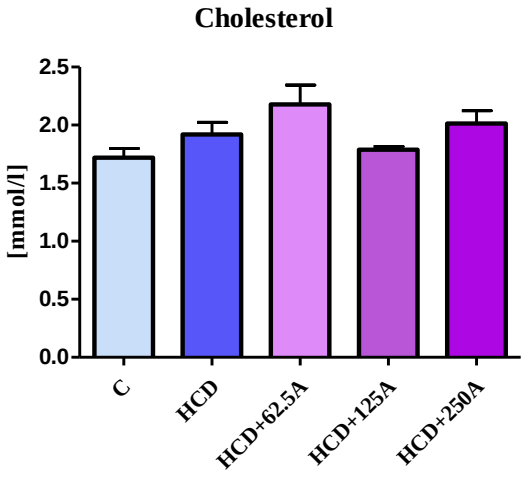

Figure 12. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on serum cholesterol

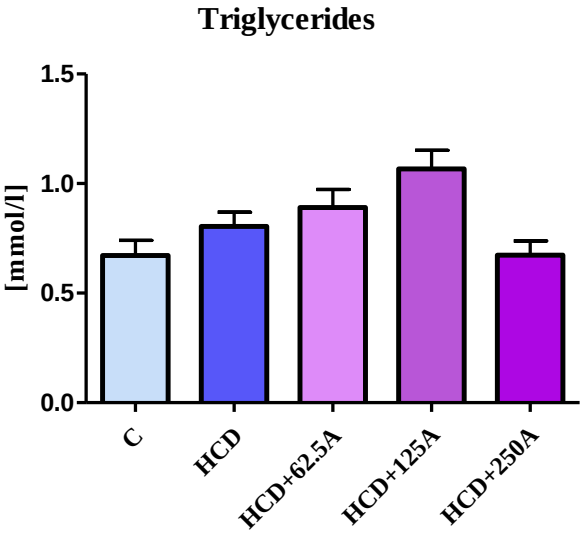

Figure 13. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on serum triglycerides

3.1.3. Effect of anethole on clotting time

The measured values of clotting time are presented in **Table 10** and **Figure 14.**

The clotting time was significantly decreased by the consumption of HCD with values of 86.86 ± 9.72 s compared to the control level of 103.7 ± 8.32 s. Administration of anethole resulted in no change in the levels of the parameter, as they remained similar to those of the HCD group. (ANOVA: F = 2.161, P = 0.0952).

Table 10. Time values of blood coagulation in rats with diet-induced obesity by HCD treated with anethole at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
Clotting time (sec)	103,7 ± 8,32	86,86 ± 9,72	76,50 ± 9,18	103,6 ± 8,40	81,88 ± 7,30

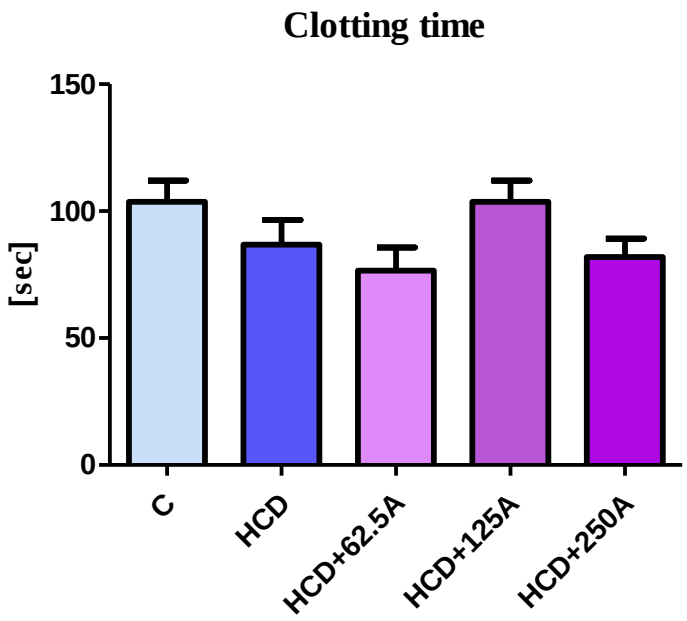

Figure 14. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on clotting time

3.1.4. Discussion

The condition known as lipotoxicity occurs when metabolites of ectopically accumulated lipids interfere with the action of insulin in peripheral tissues and insulin synthesis in the pancreas

(Unger. 2002). Adiposity is an approximate indicator of diabetes risk (Unger and Scherer Gluttony, 2010). In fact, research has shown that excess weight can increase levels of proinflammatory molecules such as inducer of kappa B kinase (IkK), nuclear factor kappa B (NF-kB), and c-Jun N-terminal kinase (JNK), which interfere with intracellular insulin signaling and cause insulin resistance (Shoelson et al., 2003).

However, it is believed that one mechanism alone cannot explain insulin resistance. Thus, oxidative stress has been suggested to play a role in the mechanism of insulin resistance (Pieri et al., 2023). Results from a study by Genever et al. suggest that obesity associated with oxidative stress may be a mechanism causing insulin resistance. However, other studies contradict this. Loh et al. showed that mice lacking glutathione peroxidase 1 (Gpx1), a key enzyme involved in the scavenging of physiological reactive oxygen species (ROS), were protected from the development of insulin resistance induced by a high-fat diet (Loh et al., 2009).

In the present study, HCD altered glucose metabolism - GTT demonstrated impaired glucose tolerance in the HCD group compared to the control group. The blood glucose level differed significantly at the 30th minute of the test. Fasting blood glucose levels were not altered but glucose tolerance was affected. The HCD used in the model was associated with insulin resistance, hyperglycaemia and other biochemical changes (Gancheva et al., 2015). GTT is a more sensitive indicator of early problems in glucose regulation than fasting plasma glucose or HbA1c (Ceriello. 2005).

The liver is considered a central metabolic organ in the body with an important role in glucose homeostasis (Petersen et al., 2017). The effects of anethole on carbohydrate metabolism were studied by Sheikh et al. in rats subjected to a streptozotocin-induced type 2 diabetes model (Sheikh et al., 2015). In this study, administration of trans-anethole at a dose of 80 mg/kg was associated with a significant decrease in plasma glucose and glycated haemoglobin levels, an increase in insulin level and an improvement in glucose tolerance. Analysis of serum glucose data in broilers showed a statistically significant reduction in the group receiving 0.5 g/kg anise with a decrease of about 5.7% compared to the control group (Soltan et al., 2008).

Administration of trans-anethole to rats with streptozocin-induced diabetes resulted in a significant antihyperglycemic effect, reducing glucose levels while those of insulin were normalized. These changes are comparable to the results obtained with treatment with the

antidiabetic drug glibenclamide, even in terms of HbA1c. A major advantage of trans-anethole is that it does not affect blood glucose in healthy non-diabetic rats (Bashir et al., 2015). Administration of trans-anethole prevents impaired glucose tolerance in diabetic rats (Bashir et al., 2015). The exact mechanism of action has not been established, but restoration of levels of key enzymes in carbohydrate metabolism, normalization of glycogen content in liver and muscle are thought to be underlying mechanisms. Anethole has been shown to possess amylase inhibitory activity. This effect is even more pronounced than that of the amylase inhibitor acarbose (Jurado et al., 2003). Futhermore, anethole affects key enzymes involved in carbohydrate metabolism and can be used as a beneficial bioactive compound in the development of therapeutic agents against type 2 diabetes mellitus (Samyal et al., 2011).

In another study, Vellapandian et al. reported that an anethole-rich fraction (100 mg/kg) derived from aromatic herbs lowered blood sugar levels and improved lipid profiles (Vellapandian et al., 2022). Ethanolic extract of the aerial and root part of tarragon (250 mg/kg body weight) significantly lowered blood sugar, cholesterol, triglyceride and plasma LDL levels compared to control group in mice with induced diabetes and hyperglycemia, while significantly increasing HDL levels. This antihyperglycemic effect was comparable to the conventional drug glibenclamide. The increase in insulin secretion by pancreatic beta cells is one of the potential mechanisms by which the ethanolic extract of tarragon rich in estragole (an isomer of anethole) (Ayoughi et al., 2011) reduces blood glucose levels (Samyal et al., 2021). Normally, elevated glucose causes abnormalities in lipoprotein metabolism, leading to increased triglycerides and cholesterol, indicating that glucose from peripheral tissues transfers fat from adipose tissue (Diabetes Control and Complications Trial Research Group, 1993). In the present experiment, there was no effect of anethole on serum lipid levels. The groups receiving anethole did not differ from the HCD group, but in this experiment HCD did not induce hyperlipidaemia. In experiments conducted by other researchers, anethole demonstrated antidyslipidemic effects. Such has been reported by Samadi-Noshahr et al. in rats with streptozotocin-induced diabetes (Samadi-Noshahr et al., 2021). In healthy rats treated with anise oil containing 80-90% trans-anethole, beneficial changes in lipid profile with reduction in triglyceride and total cholesterol levels were also found (Helal et al., 2019). Supplementation with trans-anethole also improved lipid profile in a study conducted on healthy male broilers (Yu et al., 2021). The addition of anethole to the diet of high-

fat rats significantly reduced the levels of triglyceride, LDL and VLDL concentrations in their blood ($p<0.05$) (Noreen et al., 2023). Administration of fennel, which is rich in anethole, to rats with diet-induced obesity also resulted in a reduction in serum cholesterol and triglyceride levels, also reducing the body weight of the experimental animals (Nawal et al., 2019). In humans, the effect of anethole on energy metabolism has not been studied. However, there are clinical studies examining the impact of fennel consumption. A double-blind randomized placebo-controlled trial conducted on postmenopausal women showed that fennel intake did not lead to significant differences in lipid profile between the study groups, a result similar to that observed in our experiment. However, women supplemented with fennel showed some improvement in HDL-cholesterol levels at the end of the study compared with baseline values (Afiat et al, 2018). Soltan et al. found that different concentrations of aniseed solution (0.25, 0.5, 0.75, 1, 1.25, and 1.5 percent) had no effect on plasma cholesterol, LDL triglycerides, VLDL, and HDL ($p>0.05$) (Soltan et al., 2021). Both fennel and anethole reduced serum triglyceride levels, total cholesterol, LDL fraction and increased HDL levels (Samadi-Noshahr Z, et al, 2021). Sedláková et al. reported that the presence of anethole, thymol and carvone in fennel and caraway plants resulted in a decrease in cholesterol and triglyceride levels, which was most likely related to reduced fat resorption in the intestine (Sedláková et al., 2003).

In the present experiment, HCD did not alter the time of blood coagulation. Anethole also showed no significant effect. Similar results were observed when the aqueous extract of cinnamon essential oil was tested, with the presence of trans-anethole 64.12%, which showed no effect on blood clotting time of healthy volunteers. However, the pure cinnamon extract significantly reduced clotting time compared to the control group (Hossein et al., 2013).

Oral administration of hydroalcoholic fennel extract at doses of 250, 500, 750 and 1000 mg/kg body weight for a period of 30 days did not result in significant changes in bleeding time compared to the control group (Mansouri et al., 2015). With regard to platelets, an absence of antiplatelet effect was reported by Imbabi et al. who found no statistically significant difference in platelet counts when rabbits were treated with fennel oil for 8 weeks (Imbabi et al., 2021). On the other hand, in the available literature there is evidence of an anti-aggregant effect of anethole. A study identifying essential oils rich in phenylpropanoids (A. dracunculus, F. vulgare, O. quixos, O. basilicum) found them to be most effective in inhibiting platelet aggregation. This

suggests a correlation between these chemical components of essential oils and their ability to inhibit this biological activity. Therefore, it can be suggested that the antiplatelet activity of fennel oil may be due to its phenylpropanoid moiety, which consists mainly of anethole (about 75%) (Tognolini et al., 2006).

Fennel oil and its main constituent anethole show a similar level of effectiveness in preventing aggregation caused by arachidonic acid, collagen, ADP in guinea pigs. Moreover, anethole inhibits the process of thrombin-induced clot constriction by having a destabilizing effect on it (Tognolini et al., 2007). Vasorelaxant activity is also important for antiplatelet action as it is associated with the suppression of increased intracellular calcium activity in the vascular endothelium (Xiong et al., 1995). Excessive influx of calcium into cells disrupts vascular tone by controlling vasoconstriction and promoting the production of vasoactive substances (Xiong et al., 1995). The effect of anethole on rat aorta with or without endothelium is vasorelaxant, being independent of nitric oxide. These effects have been observed at doses that have been shown to have antiplatelet properties and are not cytotoxic in vitro. Anethole administered orally at a dose of 30 mg/kg/day for 5 days showed antithrombotic activity. At the antithrombotic dose, no prohemorrhagic side effects were observed, unlike acetylsalicylic acid, which was used as the reference drug (Tognolini et al., 2007).

Trans-anethole suppressed the concentration of plasminogen activator inhibitor-1 (PAI-1) in isolated preantral ovarian follicles of goats (Sá et al., 2017).

3.2. Effects of anethole on indices of antioxidant protection and oxidative stress

3.2.1. Effects of anethole on serum superoxide dismutase (SOD) levels

As shown in **Figure 15** and **Table 11**, serum superoxide dismutase (SOD) activity in the SCD group (0.23±0.02 U/ml) was significantly lower ($p<0.05$) compared to the control level (0.31±0.01 U/ml). Antioxidant enzyme levels remained low in the HCD+62.5A (0.23±0.02 U/ml) and HCD+125A (0.22±0.01 U/ml) groups. SOD activity in the HCD+250A group (0.29±0.02 U/ml) showed no statistically significant difference compared to the control group.

Table 11. Serum superoxide dismutase (SOD) levels in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05, **p<0.01 relative to control group (C)

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
SOD levels measured in U/ml	0.31 ± 0.01	0.23 ± 0.02*	0.23 ± 0.02*	0.22 ± 0.01**	0.29 ± 0.02

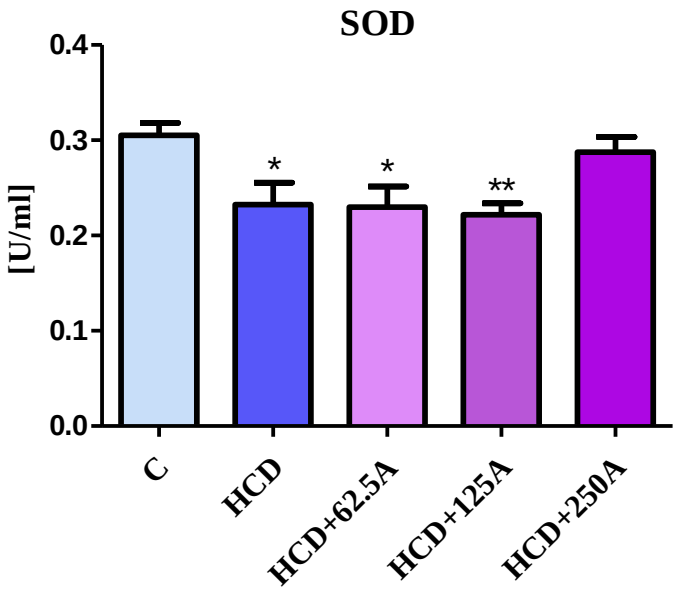

Figure 15. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on serum superoxide dimutase SOD; *p<0.05, **p<0.01 vs. control group (C)

3.2.2 Effect of anethole on thiobarbituric acid reactive substances (TBARS) levels in serum and brain tissue homogenate

TBARS values in serum are shown in **Figure 16** and **Table 12.** Serum TBARS levels were elevated (p<0.05) in the HCD+62.5 group (15.64 ± 1.47 nmol/g) compared to the control

level (11.26 ± 0.92 nmol/g). TBARS levels in the HCD+250A group (11.73 ± 1.09 nmol/g) showed no statistically significant difference compared to the control group.

Figure 17 and **Table 13** show the brain TBARS levels. Consumption of HCD resulted in a significant increase in brain TBARS levels with values of 13.78±1.05 nmol/g, compared to the control level of 9.91±0.89 nmol/g ($p<0.05$, Student's t-test). Administration of anethole resulted in a reduction of TBARS levels in brain homogenate. Measured levels of TBARS in the brain were 11.52±1.53 nmol/g for the HCD+62.5A group, 11.61±1.05 nmol/g for the HCD+125A group and 11.32±1.26 nmol/g for the HCD+250A group, and these values showed no significant difference compared with the control group (ANOVA: F=0.4218, P=0.7386).

Table 12. Serum TBARS levels in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *$p<0.05$ vs. control group (C)

Index	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
Serum TBARS level measured in nmol/g	11.26 ± 0.92	12.98 ± 1.08	15.64 ± 1.47 *	15.38 ± 0.97	11.73 ± 1.09

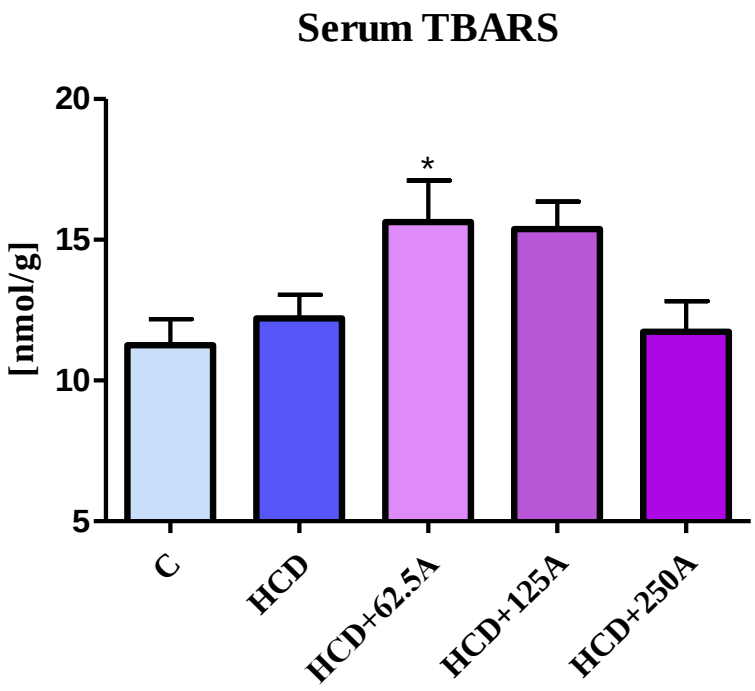

Figure16. Effect of anethole administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on serum TBARS levels; *p<0.05 vs. control group (C)

Table 13. TBARS levels in brain tissue homogenate of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05 vs. control group (C)

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
TBARS levels in brain tissue homogenate measured in nmol/g	9.9 ± 0.89	13.78 ± 1.05*	11.52 ± 1.53	11.61 ± 1.05	11.32 ± 1.26

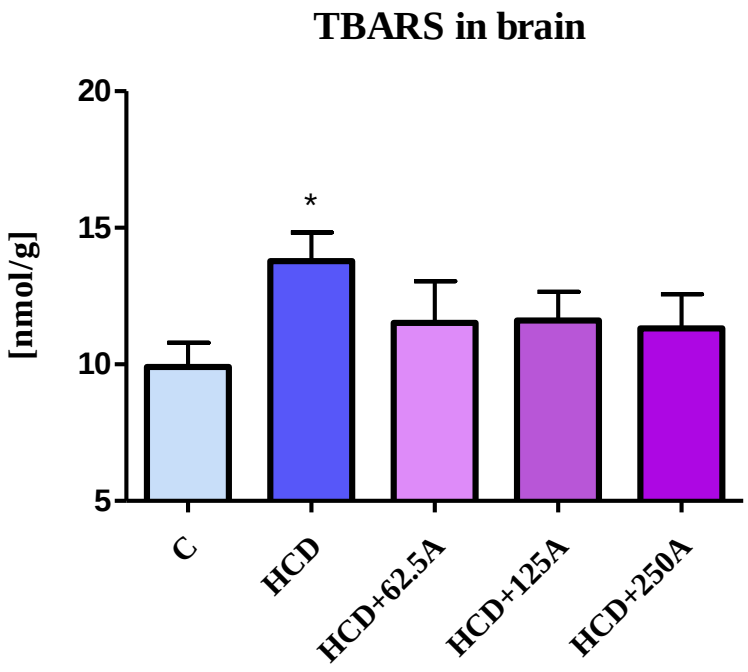

Figure 17. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on TBARS levels in brain tissue homogenate; *p<0.05 versus control group (C)

3.2.3. Discussion

Obesity is often associated with a diet that lacks essential nutrients and does not provide sufficient antioxidants. Inadequate levels of plasma micronutrients such as folic acid, vitamin B, vitamin C, iron and zinc may mimic DNA damage caused by radiation and oxidative damage (Ames et al., 2001). Increased lipid peroxidation products in the brain and decreased superoxide dismutase (SOD) activity, in the serum in the present experiment indicate that HCD causes oxidative stress and decreased antioxidant protection. Treatment of rats with anethole may counteract these effects of feeding rodents with HCD. Endogenous antioxidant enzymes, including catalase (CAT) and SOD, are essential for the elimination of reactive oxygen species and the protection of cells from the deleterious effects of oxidative stress. Several studies have

shown that a high-fat diet is associated with lower blood levels of antioxidant enzymes such as glutathione S-transferase, SOD, and KAT (Emami et al., 2016). Evidence has been obtained by Maciejczyk et al. that the hypothalamus and cerebral cortex of rats have higher concentrations of malondialdehyde (MDA) after receiving a high-fat diet for an extended period of time (Maciejczyk et al., 2018). Administration of anethole to rats on a high-fat diet for a period of 6 weeks resulted in a significant increase in the activity of the enzymes SOD and CAT in the blood, and a decrease in serum MDA levels was also observed in the animals (Maciejczyk et al., 2018). Anethole treatment resulted in a significant increase in SOD activity in the brain and a decrease in brain MDA levels in rats with rotenone-induced Parkinson's disease (Aazza et al., 2011). When rats with diabetic nephropathy that have consumed an unhealthy diet ("junk food") are given an anethole-rich fraction, their levels of SOD and reduced glutathione increase significantly (Vellapandian et al., 2022). Administration to rats of 200 mg/kg/day of Foenicum vulgare fruit, which contains trans-anethole as a major constituent, for three weeks significantly increased SOD and CAT activity (Choi E et al., 2004). In addition, lipid peroxidation has been shown to be significantly reduced as expressed by a decrease in MDA (Choi E et al., 2004). MDA is an aldehyde that is formed during oxidation of polyunsaturated fatty acids. It is widely recognized as a reliable indicator of oxidative stress (Olusi, 2002). The reaction of MDA with organic compounds, such as DNA and proteins, can lead to the formation of adducts. These adducts have toxic effects on biological molecules (Mas-bargues, 2021). After three weeks of treatment with trans-anethole, stressed rats showed a significant decrease in MDA levels in the brain (Dhingra et al., 2019). Ryu et al. demonstrated that due to its antioxidant and mitochondrial protection defenses, anethole pproduced neuroprotective effects (Ryu et al., 2014). In an animal model of epilepsy, anethole has been shown to reduce the amount of nitrite and MDA present in the brain (Raman et al., 2020). Furthermore, Raman et al. showed that memory was improved in socially isolated rats administered anethole treatment, indicating that this effect may be due to antioxidant action, inhibition of the enzyme cholinesterase, and hippocampal enhancement of long-term potentiation (LTP) (Salimian et al., 2022). Furthermore, rats given anethole at a dose of 80 mg/kg of body weight had significantly increased SOD activity. This study also showed that the reduction of oxidative stress in polycystic ovary rats was dose-dependent (Moradi Negahdari et al., 2022). There is evidence that fennel oil acts as an antioxidant due to its ability to inhibit lipid

peroxidation and reduce oxidative stress in gamma-irradiated mice. Treatment of animals before radiation resulted in cytoprotective effect and normalization of serum MDA and SOD (Mohamadet al., 2011).

4. Effects of anethole on adipose tissue

4.1. Fat tissue indices

The indices of different types of adipose tissue are presented in **Table 14** and **Figure 18.** One-way ANOVA analysis revealed that the retroperitoneal adipose tissue index in the HCD group (2.16 ± 0.08) was significantly higher ($p<0.001$) compared to the control (1.32 ± 0.11). The index levels in the HCD+250A group (1.61 ± 0.08) were significantly lower ($p<0.05$) compared to the HCD group. There were no significant differences between all groups in perigonadal adipose tissue index ($F = 1.523$).

The results of the analysis of mesenteric adipose tissue index showed that the consumption of HCD resulted in a significant increase in the index with values of 1.10 ± 0.05, compared to the control level of 0.65 ± 0.05 ($p<0.001$). Administration of anethole did not lead to a decrease in the index, which remained higher than the control: 1.15 ± 0.09 ($p<0.001$) for the HCD+62.5A group ($p<0.001$), 1.15 ± 0.07 ($p<0.05$) for the HCD+125A group and 0.90 ± 0.05 for the HCD+250A group (ANOVA: $F = 10.86$).

There was no significance in paranephric adipose tissue index between HCD and control. Low doses of anethole HCD+62.5A ($p<0.05$) increased the index, whereas no significance was found in the other anethole-treated groups.

Total adipose tissue index was used to assess obesity. The analysis of variance One-way ANOVA showed a significant treatment effect: $F(4,43)=3.868$, $P=0.0090$. Dunnet's post test revealed an increase in the index in the HCD group compared to the control group ($P<0.05$). The index also remained significantly elevated ($p<0.05$) compared to control in the HCD+62.5A and HCD+125A groups. In the HCD+250A group receiving the highest dose of anethole, the total adipose tissue index was not significantly different from that of control animals.

Table 14. Retroperitoneal, perigonadal, mesenteric, paranephric and total adipose tissue indices in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001, *p<0.05 vs. control group (C); #p<0.05 vs. HCD

Group	Retroperitoneal adipose tissue index	Perigonadal adipose tissue index	Mesenteric adipose tissue index	Paranephric adipose tissue index	Total adipose tissue index
C	1.32 ± 0.11	1.10 ± 0.10	0.65 ± 0.05	0.23 ± 0.02	3.29 ± 0.19
HCD	2.16 ± 0.08 ***	1.32 ± 0.06	1.10 ± 0.05***	0.29 ± 0.02 *	4.79 ± 0.18 *
HCD+62.5A	2.16 ± 0.19 ***	1.40 ± 0.18	1.15 ± 0.09 ***	0.36 ± 0.05	4.80 ± 0.58 *
HCD+125A	1.87 ± 0.13 *	1.28 ± 0.09	1.15 ± 0.07 ***	0.29 ± 0.03	4.76 ± 0.34 *
HCD +250A	1.61 ± 0.08 #	1.10 ± 0.06	0.91 ± 0.05 *	0.29 ± 0.01	3.89 ± 0.17

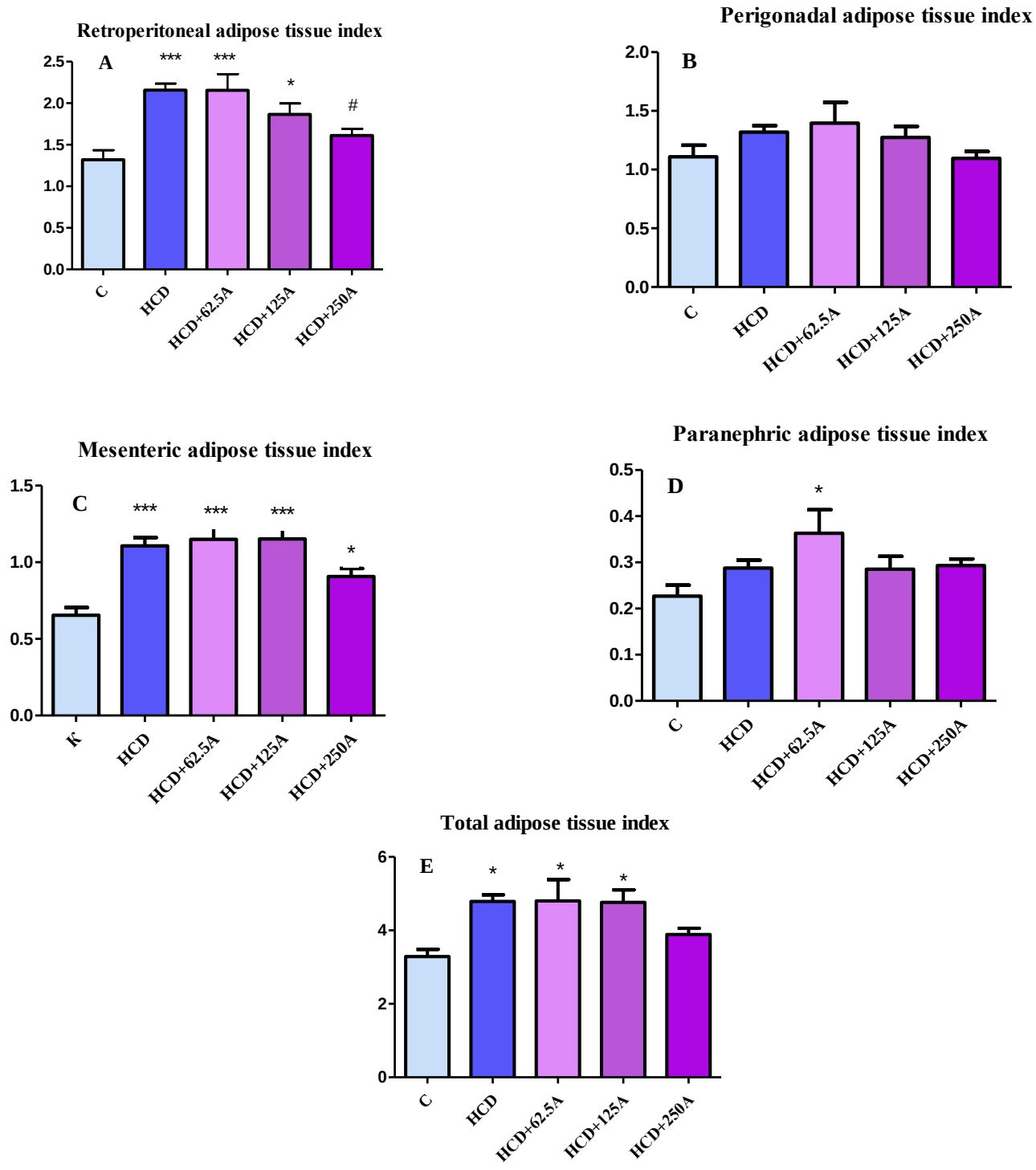

Figure 18. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62. 5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the indices of retroperitoneal (A), perigonadal (B), mesenteric (C), paranephric (D) and total adipose tissue (E); ***p<0.001, *p<0.05 vs. control group (C); #p<0.05 vs. HCD

4.2 Histology of retroperitoneal adipose tissue

The histological results for retroperitoneal adipose tissue are presented in **Figure 19**. In the control group of animals, adipocytes of medium size and oval or polygonal phrma with a peripherally located nucleus predominated (**Figure 19**, panel A). Large-sized adipocytes predominated in the retroperitoneal adipose tissue of rats in the obese group (**Figure 19**, panel B). In the retroperitoneal adipose tissue of the animals of the HCD+62.5A group, medium to large-sized adipocytes were present with a predominance of large-sized adipocytes (**Figure 19**, panel C), which did not distinguish them from the HCD group. In the HCD+125A group, the size of adipocytes decreased, fat lobules with medium to large sized adipocytes were observed, and tissue with inflammatory changes was observed in places (**Figure 19**, panel D). In the group treated with the highest dose of anethole (HCD+250A), the size of adipocytes was similar to that of the control group, medium and large sized adipocytes were observed (**Figure 19**, panel E).

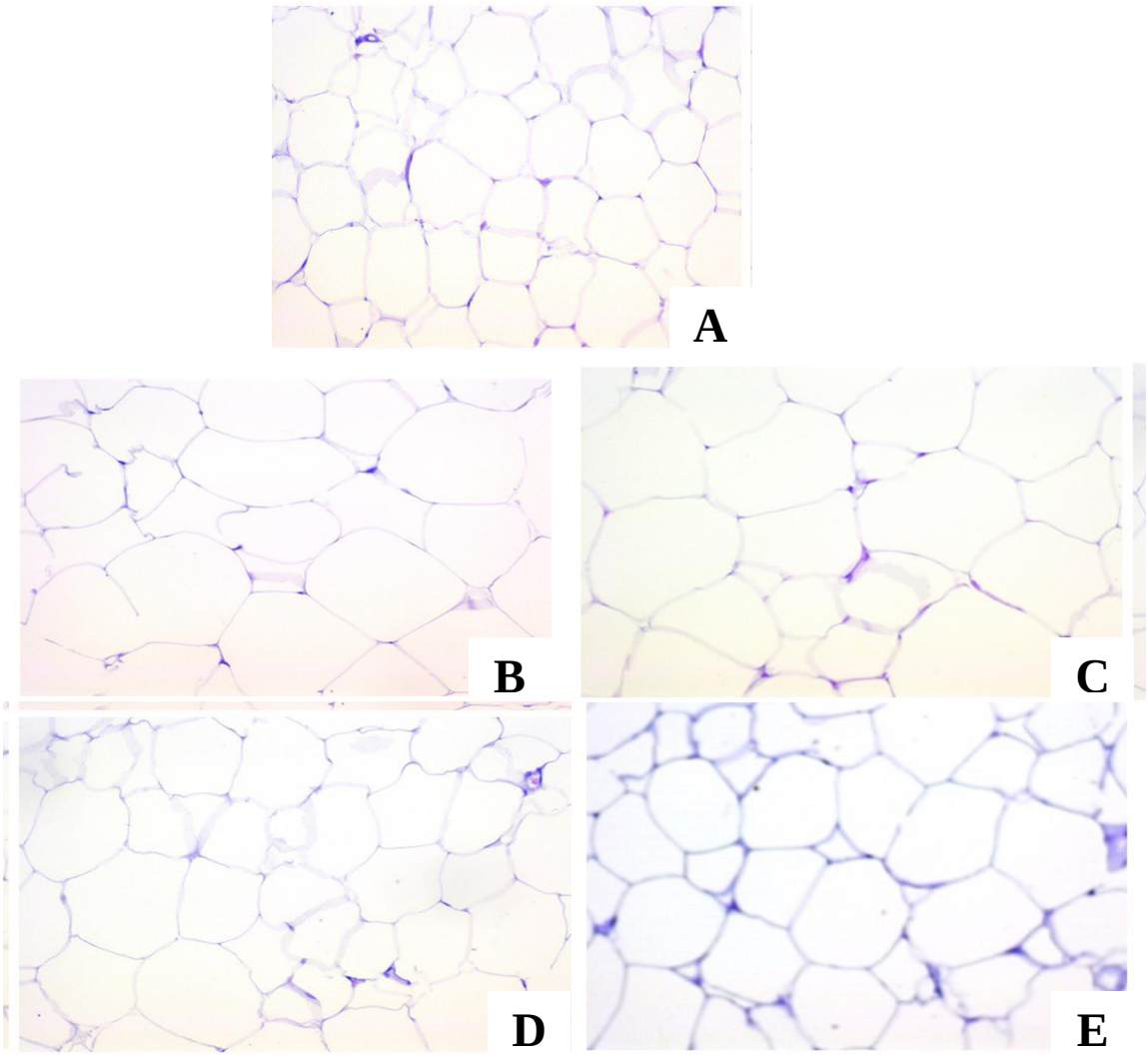

Figure 19. Histology of retroperitoneal adipose tissue in rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D), and 250 mg/kg (panel E); contralateral group (panel A); hematoxylin-eosin staining; magnification x 200

4.3. Immunohistochemistry of retroperitoneal adipose tissue

4.3.1. BAX expression

Figures 20 and **21** and **Table 15** show BAX expression in retroperitoneal adipose tissue. BAX expression was significantly increased in the HCD group with values of 0.47 ± 0.02 compared to the control level of 0.19 ± 0.03 ($p<0.001$). The administration of anethole at a dose of 125 mg/kg antagonized the diet-induced effect, which was reflected by a significant decrease in the levels of

the indicator compared to the HCD group: 0.39 ± 0.03 ($p<0.05$), however, this indicator remained high compared to the control group ($p<0.001$). Administration of anethole at a dose of 250 mg/kg, on the other hand, showed the opposite effect - BAX expression was significantly increased relative to the HCD group, and also relative to the control ($p<0.001$) (ANOVA: $F=126.4$, $P<0.0001$).

Table 15. Expression of the apoptotic marker BAX in retroperitoneal adipose tissue of rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001 vs. control group (C); #p<0.05 vs. HCD, ###p<0.001 vs. HCD

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BAX expression score in retroperitoneal adipose tissue	0.19 ± 0.03	0.47 ± 0.02 ***	0.39 ± 0.03 ***	0.36 ± 0.02 ***#	0.99 ± 0.01 ***###

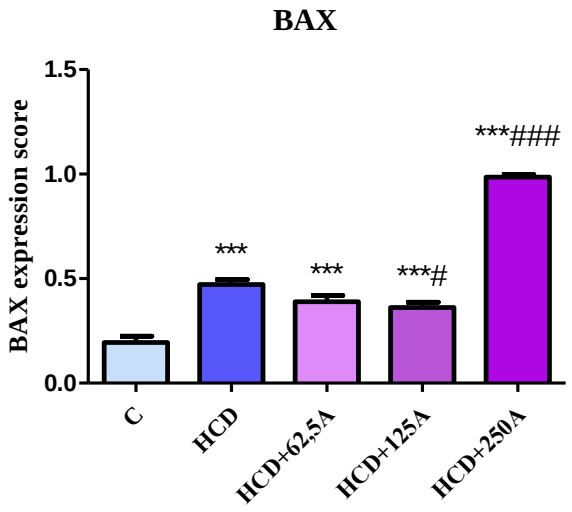

Figure 20. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the

expression of the apoptotic marker BAX in retroperitoneal adipose tissue; ***p<0.001 vs. control group (C); #p<0.05, ###p<0.001 vs. HCD

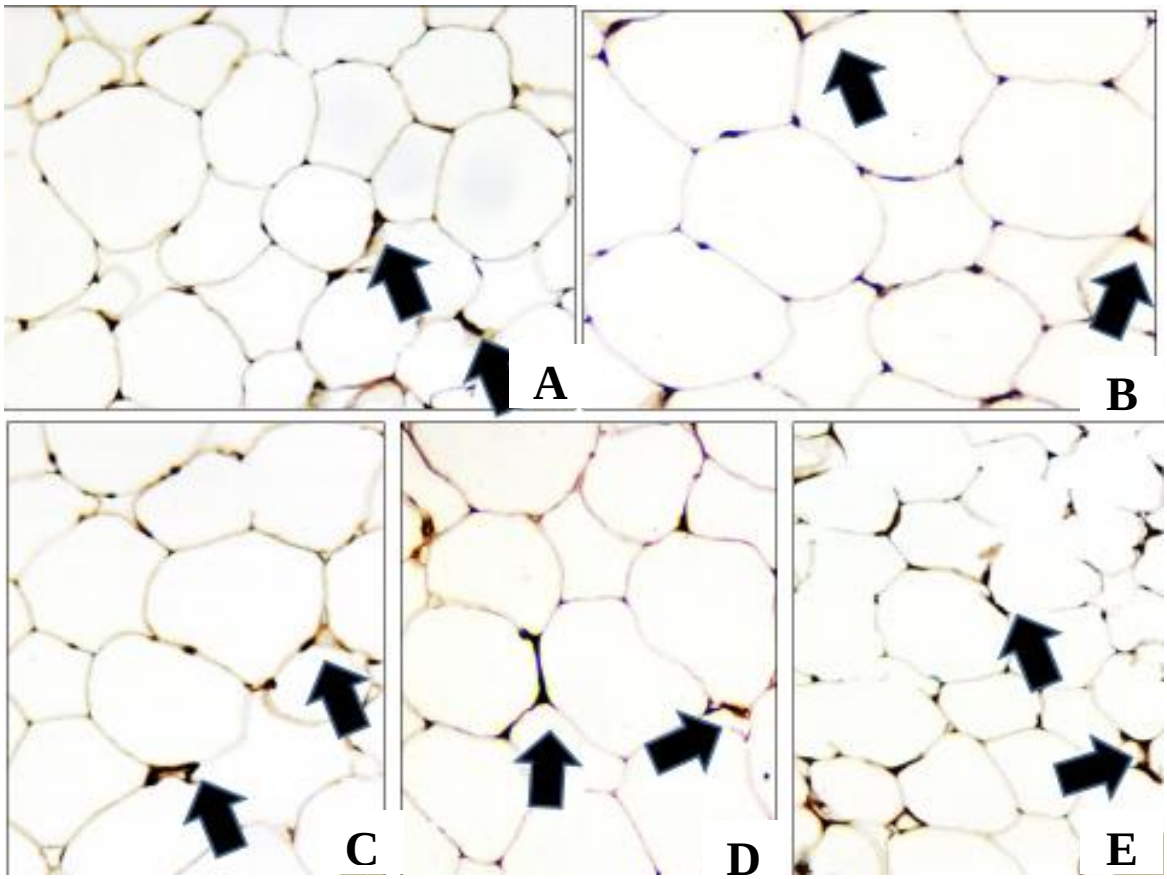

Figure 21. Expression of the apoptotic marker BAX in retroperitoneal adipose tissue of rats with diet-induced obesity by a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D) and 250 mg/kg (panel E); control group (panel A); black arrow - cytoplasm of adipocytes; magnification x 320

4.3.1. BCL-2 expression

Figures 22 and **23** and **Table 16** show the expression of BCL-2 in retroperitoneal adipose tissue. There is no significance between BCL-2 levels in the HCD group and the control group. There was a tendency for the values of the parameter to decrease from the anethole treatment without statistical significance (ANOVA: F = 1.883, P = 0.1261).

Table 16. Expression of the antiapoptotic marker BCL-2 in retroperitoneal adipose tissue of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125, and 250 mg/kg. Results are presented as mean ± S.E.M

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BCL-2 expression score in retroperitoneal adipose tissue	0.1632 ± 0.02	0.1680 ± 0.02	0.1142 ± 0.02	0.1354 ± 0.01	0.1650 ± 0.01

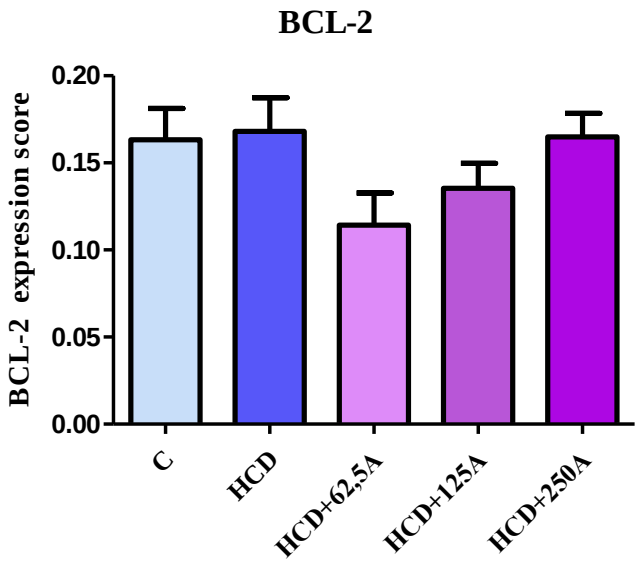

Figure 22. Effect of anethole (A) administered to rats on a high-calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the expression of the apoptotic marker BCL-2 in retroperitoneal adipose tissue

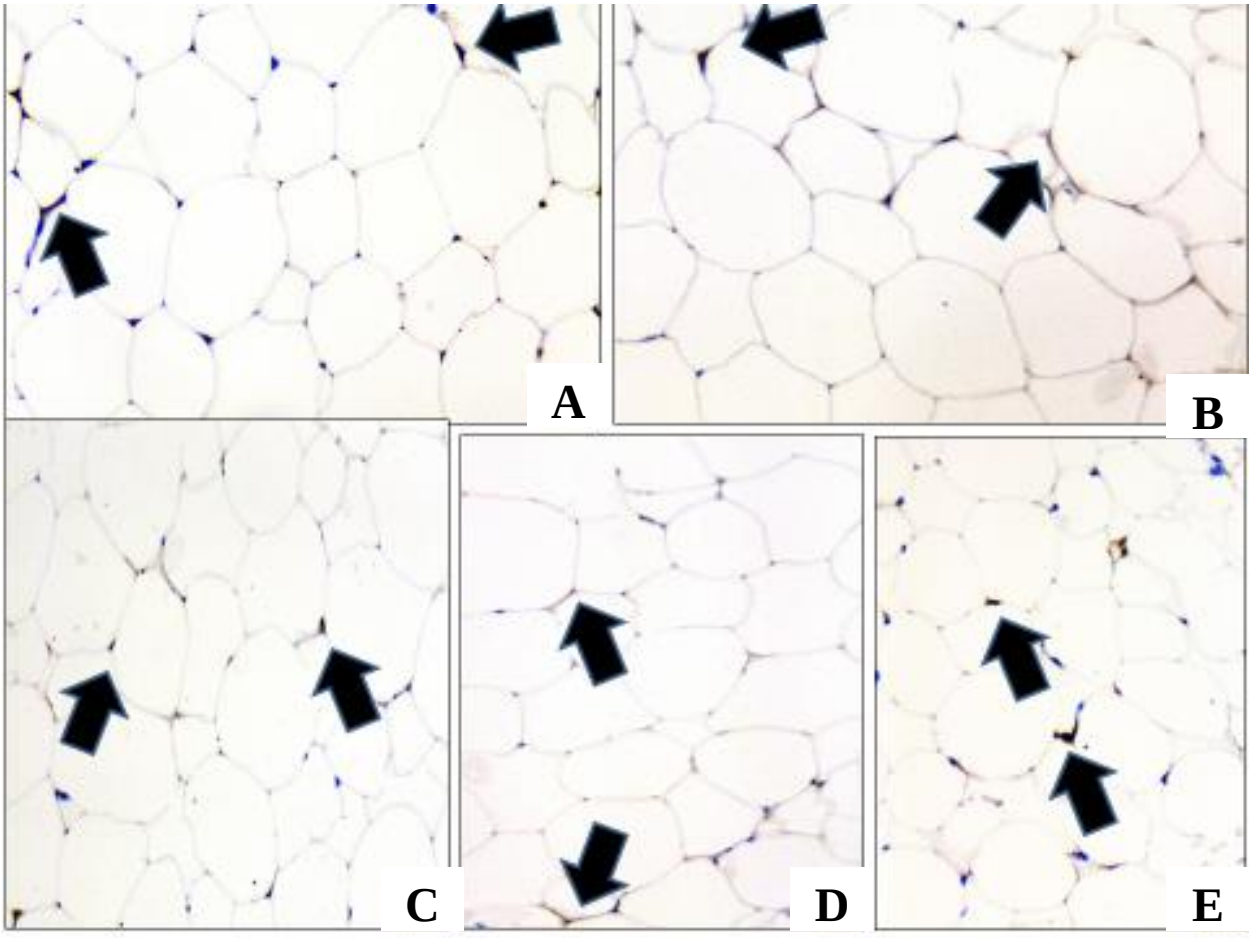

Figure 23. Expression of the antiapoptotic marker BCL-2 in retroperitoneal adipose tissue of rats with diet-induced obesity by high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D), and 250 mg/kg (panel E); control group (panel A); black arrow - cytoplasm of adipocytes; magnification ×320

4.3.3.BAX/BCL-2 ratio

One-way ANOVA analysis revealed that the BAX/BCL-2 ratio was significantly higher in the HCD group compared to the control group ($p<0.05$), $F=12.98$, $P<0.0001$. Treatment with anethole at 62.5 and 250 mg/kg doses increased the index compared to control ($P<0.001$), the ratio at 250 mg/kg dose remained high also compared to HCD group ($P<0.01$). No statistical difference was found between the values of HCD+125A and control. The results are presented in **Table 17** and **Figure 24.**

Table 17. BAX/BCL-2 ratio in retroperitoneal adipose tissue of rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05 , ***p<0.001 vs. control group (C); ##p<0.01 vs. HCD

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BAX/ BCL-2 ratio	1.232 ± 0.27	3.375 ± 0.43 *	4.857 ± 0.73 ***	2.958 ± 0.37	6.258 ± 0.52 ***###

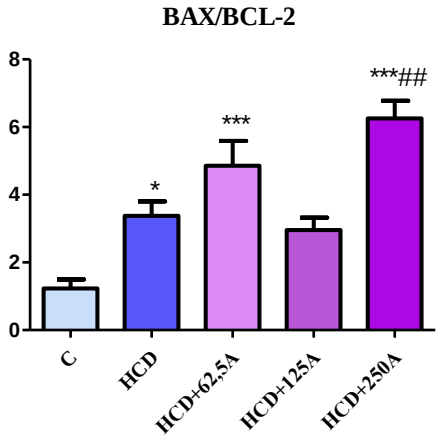

Figure 24. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on BAX/BCL-2 ratio in retroperitoneal adipose tissue; *p<0.05 , ***p<0.001 vs. control group (C); ##p<0.01 vs. HCD

4.3.4. MAC387 expression in retroperitoneal adipose tissue

One-way ANOVA analysis reported a statistical difference between the groups, with Dunnet's post test showing an increase in MAC387 marker expression by consumption of a high calorie diet (p<0.05). As a consequence of the anethole treatment, MAC387 expression did not

differ with that of the HCD group, while remaining elevated relative to control: $p<0.05$ for HCD+250A and $p<0.01$ for HCD+62.5A and HCD+125A (**Table 18** and **Figures 25** and **26**).

Table 18. MAC387 expression in retroperitoneal adipose tissue in rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05 , **p<0.01 vs. control group (C)

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
MAC387 expression score in retroperitoneal adipose tissue	0,05 ± 0,01	0,14 ± 0.02 **	0,13 ± 0.02 **	0,14 ± 0.01 **	0,13 ± 0.02 *

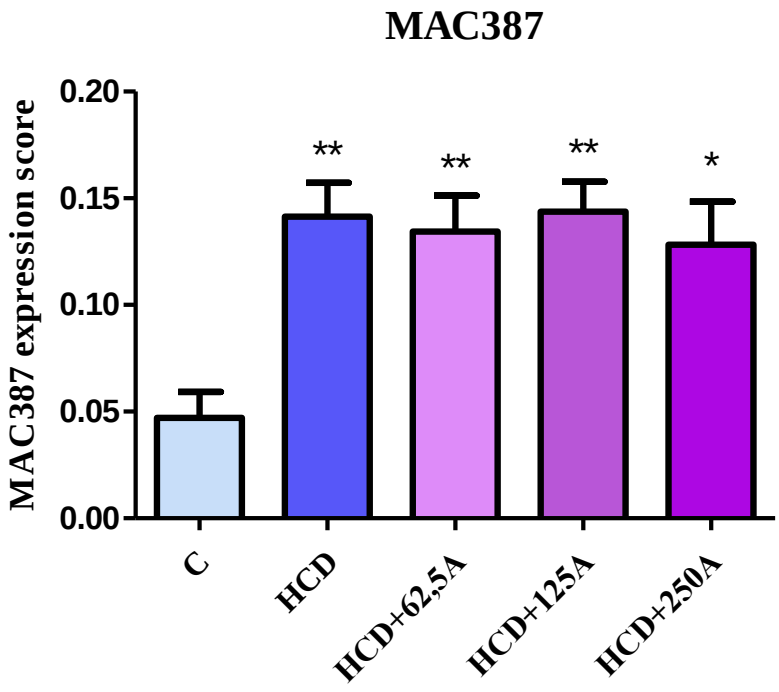

Figure 25. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on MAC387 expression in retroperitoneal adipose tissue; *p<0.05 , **p<0.01 vs. control group (C)

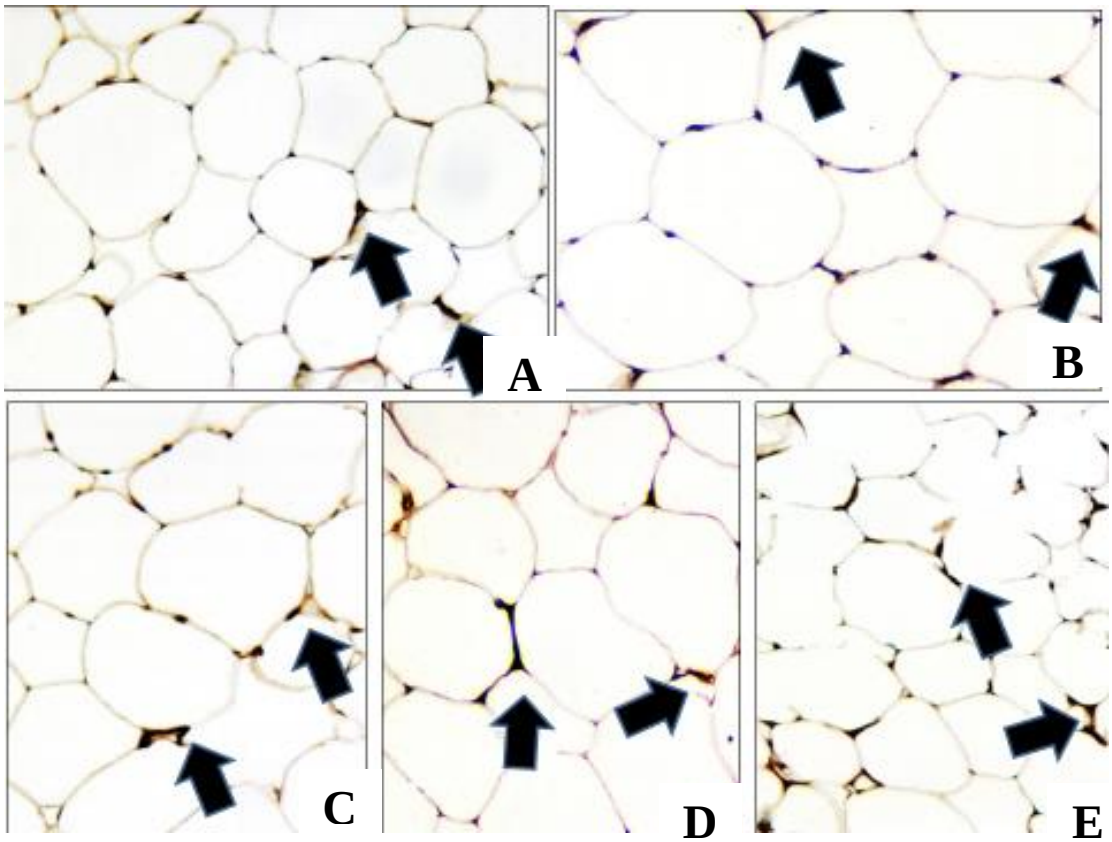

Figure 26. Expression of the proinflammatory marker MAC387 in retroperitoneal adipose tissue of rats with diet-induced obesity by high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D) and 250 mg/kg (panel E); control group (panel A); black arrow - cytoplasm of adipocytes; magnification ×320

4.4. Discussion

Adipose tissue is a highly dynamic organ that plays several roles, such as endocrine, paracrine and autocrine functions, in human physiology and disease (Trayhurn and Beattie, 2001). By performing metabolic, cellular and endocrine processes, adipose tissue is critical in controlling energy balance in the body. Historically, adipose tissue has been divided into catabolic brown adipose tissue and anabolic white adipose tissue (Lee et al., 2014). The traditional function of white adipose tissue has been described as energy storage, with fatty acids being released when 'fuel' is required. However, white adipose tissue plays a complex role in

metabolism. This tissue is necessary for healthy glucose homeostasis and is thought to be involved in inflammatory processes. The discovery of leptin, an important hormone for energy balance that is predominantly produced by white adipose tissue, gives the tissue an endocrine function. Among the proteins that white adipocytes secrete along with leptin are angiotensinogen, adipsin, acylation-stimulating protein, adiponectin, retinol-binding protein, TNF-alpha, IL-6, and tissue plasminogen activator inhibitor-1. Some of these proteins function as inflammatory cytokines, while others are involved in lipid metabolism, vascular hemostasis, or the complement system. The effects of certain proteins may be autocrine, paracrine, or distant from adipose tissue depending on where they act. Fibrinogen-angiopoietin-related protein, metallothionein, and resistin are the most recently identified adipocyte secretory proteins. An adipose tissue-specific factor called resistin is thought to cause insulin resistance, which is the pathogenetic link between diabetes and obesity. Metallothionein, a metalloprotein that binds and responds to stress, may play an antioxidant role. Discovering the full range of secreted proteins, determining their function and pathophysiological effects of adipose tissue changes (obesity, starvation, cachexia) are the major challenges in determining the secretory functions of white adipose tissue. A growing body of evidence confirms that the metabolic and cardiovascular effects of obesity are associated with increased synthesis of several adipocyte components. White adipose tissue is an important secretory and endocrine organ that performs a variety of tasks other than fat storage (Trayhurn and Beattie, 2001).

These findings suggest that focusing on the cellular and metabolic adaptability of adipose tissue may open new therapeutic approaches for obesity-related disorders (Lee et al., 2014). Regulating adipogenesis through its key mechanisms may help combat obesity. Recent studies have shown that activation of AMPK can help reduce obesity. AMPK is a key pathway for lipid metabolism, regulating lipogenesis, insulin secretion, and glucose uptake in many tissues. Exercise or drug intake can induce AMPK activation, potentially being used to treat obesity or correct metabolic abnormalities associated with type 2 diabetes (Hardie. 2010).

In the present study, the administration of HCD increased the weight of retroperitoneal, mesenteric tissue as well as total adipose tissue index. The diet consumed by the rodents was

more caloric than the conventional diet and induced visceral obesity, although no increase in body weight was observed.

Reducing body fat to a certain extent can improve the balance of harmful molecules and the overall well-being of DNA in obese individuals (Usman et al., 2018). Single experimental studies examining the potentially beneficial effects of anethole on obesity are found in the available literature. The results of the present experiment suggested that anethole, administered at a sufficiently high dose, was able to prevent the development of visceral obesity in experimental animals. Similar results were reported by Kang et al. who administered trans-anethole to mice treated with a fat-rich diet (Kang et al., 2018). According to the authors of this study, trans-anethole stimulates the conversion of white adipose tissue to brown, activates brown adipocytes, and promotes lipid catabolism by affecting gene expression in fat cells, and by this complex mechanism is able to prevent the development of obesity (Kang et al., 2018). Similar effects on gene expression were found in a liver cell line by Song et al. who found that trans-anethole increased the expression of lipolytic genes and decreased that of lipogenic genes (Song et al., 2020).

Obese mice fed a high-calorie diet reduced body weight gain by about 27% compared with controls when treated with anethole at doses of 100 mg/kg. Anethole regulates lipid metabolism by decreasing white adipose tissue (WAT) weight and increasing brown adipose tissue (BAT) weight while upregulating brown adipocyte-specific genes and proteins (Kang et al., 2018). Histologically, the reduction in adipocyte size is also confirmed (Kang et al., 2018).

According to Masoudzadeh et. al. al, the addition of fennel at a concentration of 1% to the lamb diet quantitatively increased DLK1 gene expression in adipose tissue and decreased backfat thickness (mobilization or body reserve recovery index) compared to the control group, indicating that DLK1 functions as an inhibitor of adipogenesis. Contrary to expectations, at concentration 2%, where quantitatively higher gene expression of DLK1 was observed, fennel did not induce a difference in backfat thickness (Masoudzadeh et al., 2020). The amount of abdominal fat in broiler chickens decreased insignificantly compared to the control group when tarragon powder was used at concentrations of 0.125, 0.25 and 0.5 percent (Hosseinzadeh et al., 2014). Administration of trans-anethole at doses of 105 to 550 mg/kg body weight per day reduces fat accumulation in rats (Truhaut et al., 1989). Anethole is also an inhibitor of lipid

peroxidation (Stohs et al., 1986). ROS formation is essential for both the onset of adipocyte differentiation and the activation of glycogen synthase kinase in glucose metabolism (Rhee et al., 2016). According to Rhee et al, the anti-adipogenic effects of anethole were due to its antioxidant activity and modulation of energy metabolism through suppression of mTOR (Rhee et al., 2018). Higuchi et. al. al found that antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) are increased in hMSCs during adipogenesis. In cells undergoing adipogenesis, there is a balance between ROS production and endogenous antioxidants. Approaches targeting ROS in adipocytes may lead to new ways to prevent and treat obesity and metabolic syndrome (Higuchi et al., 2013). Under conditions of adipogenic induction, anethole reduced lipid accumulation in hMSCs without altering cytotoxicity. It also inhibits phosphorylation of mTOR, PPAR and Akt. It is extremely crucial that activation of AMPK e has been found during adipogenic differentiation of hMSCs in the presence of anethole (Rhee et al., 2018). The mTOR has been identified as a significant downstream effector in PI3K/Akt signaling (Soumya et al., 2013), while AMPK has anti-mTOR activity (Zhao et al., 2014). Anethole decreasesd phosphorylation of mTOR while increased phosphorylation of AMPK (Rhee et al., 2018), indicating that ROS had an effect on the metabolic pathway between mTOR and AMPK which can be controlled by anethole (Rhee et al., 2018). Administration of anethole reduced overexpression of adipogenic markers and restored AMPK activation (Rhee et al., 2018). All these interactions lead to the conclusion that anethole is not only a ROS scavenger but also an inhibitor of adipogenic pathways, such as Akt-mTOR-PPAR. Apoptosis is controlled by several molecular signaling pathways, including mitochondrial mechanisms. The BCL-2 family of proteins strictly regulates the permeability of the mitochondrial outer membrane by various mechanisms. BCL-2 has been identified as a crucial regulator of apoptosis because it has the ability to protect cells from death caused by various forms of damage, such as radiation, chemotherapy, or lack of growth factors (Kalkavan et al., 2017). The integration of many intracellular signals from the BCL-2 family to determine whether a cell will undergo cell death or survival is a crucial issue. Currently, a total of thirty members belonging to the BCL-2 family have been found. Proteins in the BCL-2 family can be categorized into three categories based on their structural similarities and functional characteristics. The first group consists of BAX, BAK and BOK, which are the active effectors of apoptosis. The second group includes proteins with

only BH3, while the third group includes antiapoptotic proteins such as BCL-2, BCL-xL, BCL-W, MCL-1, and A1 (Kalkavan et al., 2017). In healthy cells, BAX is thought to be located in the cytosol or continuously retrotranslocated by proteins to avoid apoptosis (Kalkavan et al., 2017).

BCL-2 is found in self-renewing populations of long-lived stem cells that are found along the basement membrane of various epithelial tissues, such as skin, colon, and prostate. However, it is not present in the fully developed cells that are found on the surface of these tissues. These surface cells are thought to undergo programmed cell death known as apoptosis (Hockenbery,1991).

Recent findings suggest that adipose tissue functions as a dynamic organ, constantly generating new cells and removing adipocytes through apoptosis. This occurs both in normal physiological states and during disease (Prins et al. 1994). In the present experiment, a high-calorie diet increased the apoptotic potential of adipocytes in retroperitoneal adipose tissue. Administration of anethole in high doses significantly increased this potential. This result may explain the reduction in retroperitoneal adipose tissue observed in the current study. According to Zhu et al. (2015), induction of mitochondrial apoptotic events in adipose tissue was associated with the upregulation of BCL-2 family proteins and etc. may be a promising therapeutic approach for obesity treatment by reducing the number of adipocytes through induction of apoptosis.

Hyperglycemia is associated with a fourfold increase in reactive oxygen species (ROS) formation in adipocytes, which in turn promotes inflammation (Lin et al., 2005).

5. Effects of anethole on myocardium and coronary vessels

5.1. Myocardial histology

The histological results are shown in **Figure 27**.

The myocardium of the control group had cardiomyocytes of relatively uniform thickness. HCD caused myocardial damage, cardiomyocytes were of different thickness, spaced apart and vacuolated cells appeared between them, and cardiomyocytes with degenerative changes were also present. Administration of anethole, regardless of dose, failed to prevent the changes induced by obesity in the myocardium and they persisted.

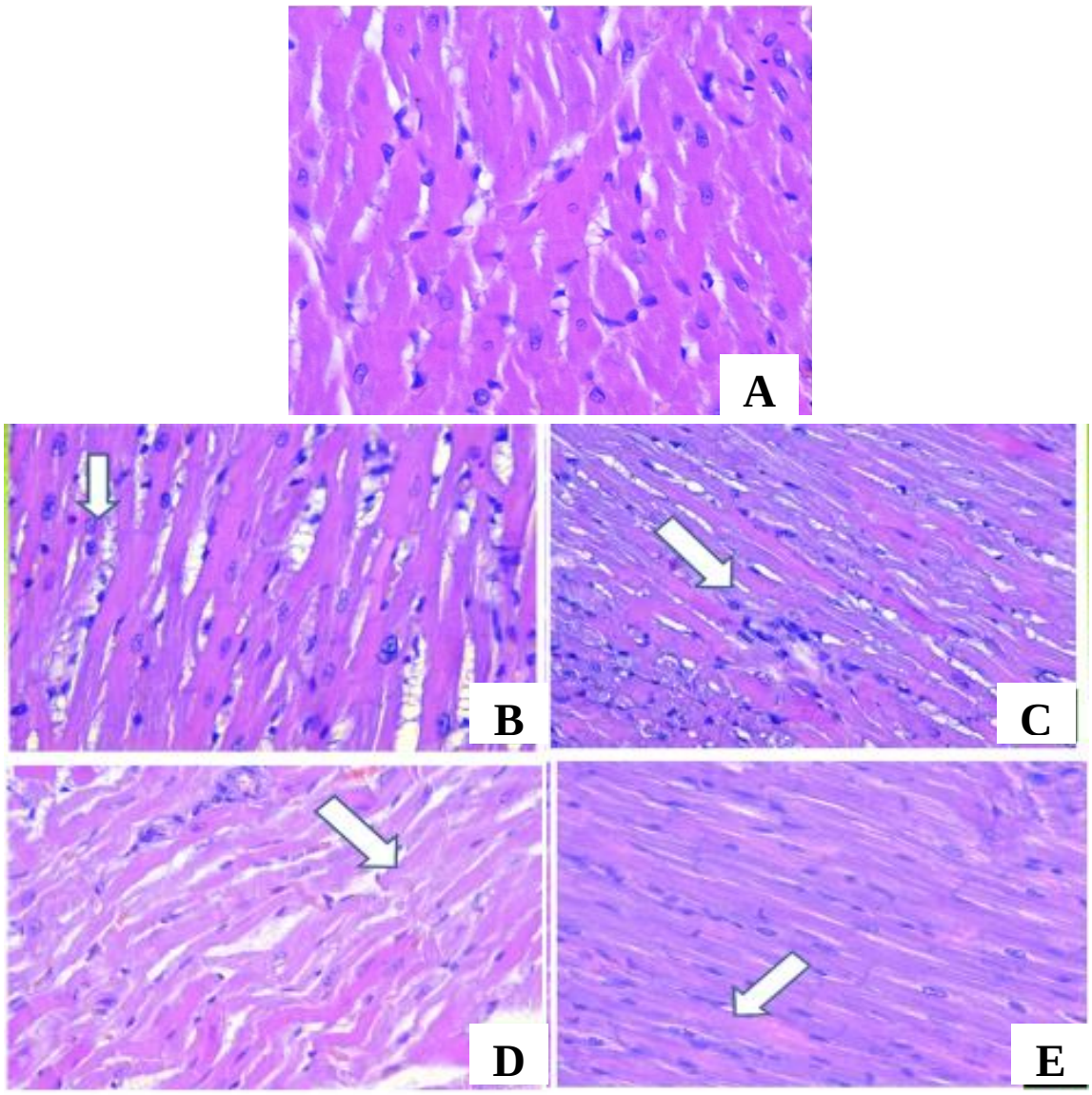

Figure 27. Histology of myocardium in rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D), and 250 mg/kg (panel E); contrpl group (panel A); hematoxylin-eosin staining; magnification × 200

5.2 Coronary histology

The histology results are presented in **Figure 28.**

The endothelial layer is well expressed with preserved architectonics in the control group rats. Obesity induced endothelial damage and focal cell necrosis occurred. Administration of anethole regardless of dose failed to prevent the effects of the high calorie diet, endothelial cells remained activated, vacuolization was observed and there was loss of endothelial cells.

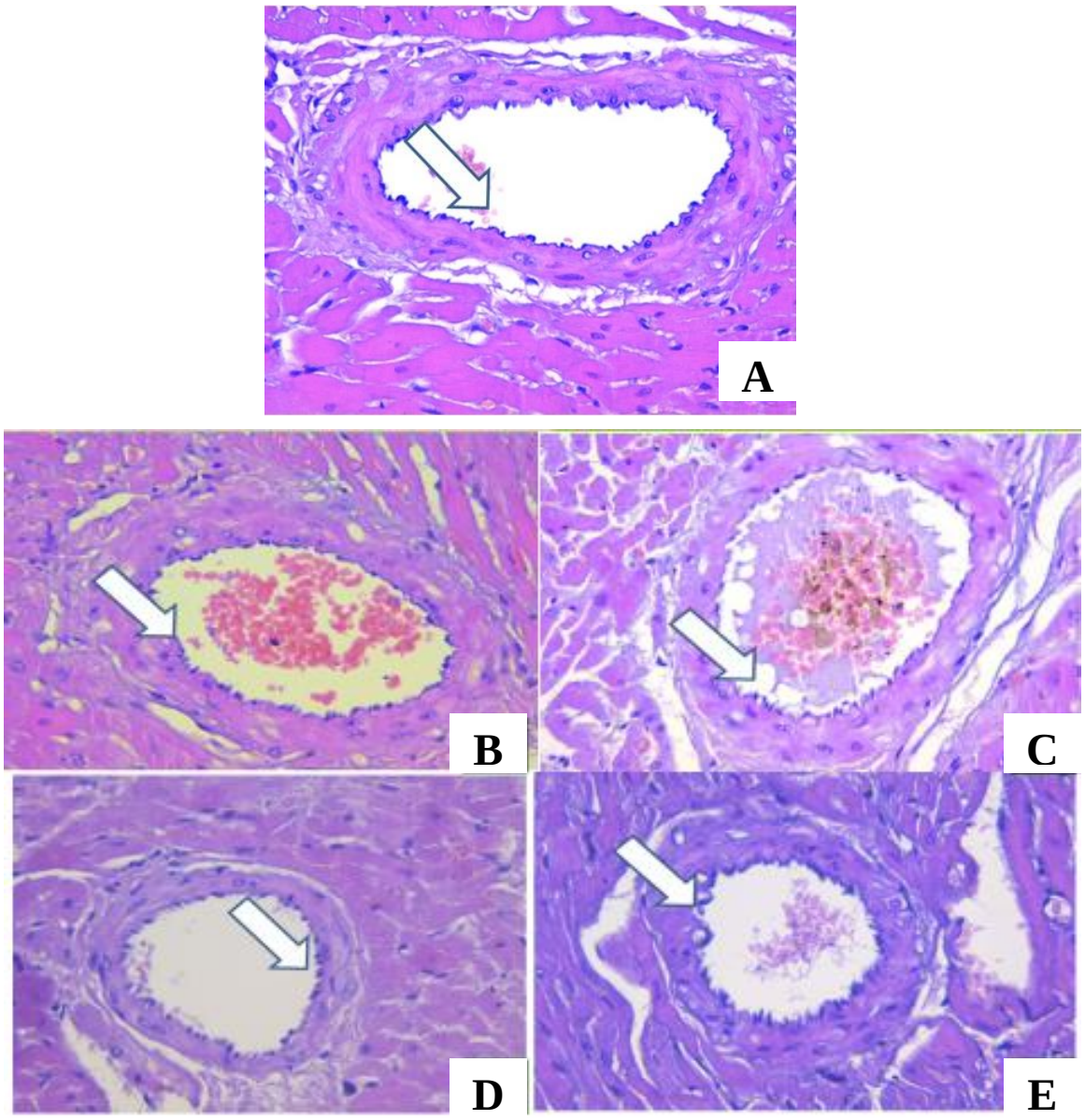

Figure 28. Coronary vessel histology of rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D), and 250 mg/kg (panel E); contol group (panel A); hematoxylin-eosin staining; magnification × 200

5.3 Effects of anethole on thiobarbituric acid reactive substances (TBARS) levels in heart tissue homogenate

Figure 29 and Table 19 show the levels of TBARS in tissue homogenate from heart. Consumption of HCD resulted in a slight increase in TBARS levels with values of 25.17 ± 2.14 nmol/g, compared to the control level of 22.90 ± 0.72 nmol/g. Administration of anethole did not alter TBARS levels in cardiac homogenate, although the levels of the parameter were increased in the 62.5 mg/kg anethole dose group as this change was statistically insignificant. TBARS values in the HCD+125A and HCD+250A groups did not differ from the control.

Table 19. TBARS levels in heart homogenate of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M

Index	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
TBARS levels в heart homogenate measured in nmol/g	22.90 ± 0.72	25.17 ± 2.14	29.79 ± 1.22	22.67 ± 1.79	24.06 ± 1.97

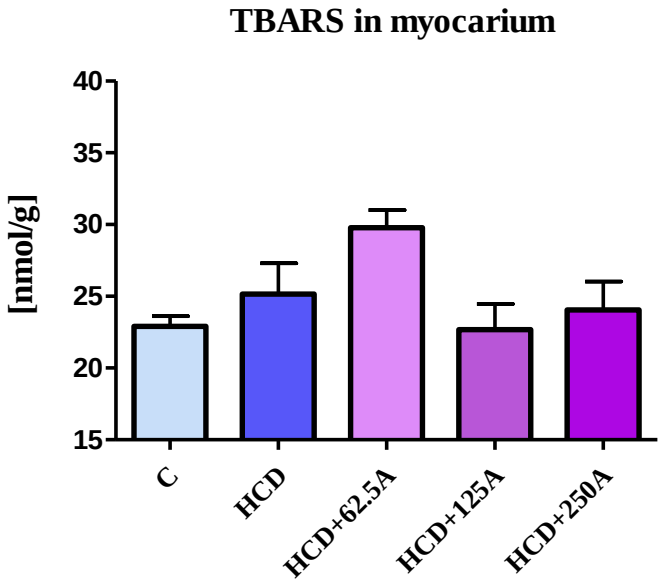

Figure 29. Effect of anethole (A) administered to rats on a high-calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A), and 250 mg/kg (HCD+250A) on TBARS levels in heart tissue homogenate

5.4. Discussion

Researches suggested the possibility that dysregulation of immunoinflammatory pathways is the underlying cause of many cardiovascular diseases, including myocardial infarction. This opens up new, non-traditional therapeutic options for these conditions (Wagsty et al., 2016). Cardiomyocytes take part in signaling pathways that regulate the damaging consequences caused by myocardial infarction (Young et al., 2021). These pathways include nuclear factor kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), and T cell receptor (TCR) signaling pathways (Matboli, 2020).

HCD in the current study injured myocardium and vascular endothelium, with no biochemical increase in myocardial lipid peroxidation. Histologically, anethole did not exert a protective effect against diet-induced changes. Biochemically, TBARS levels were not significantly affected by anethole. Studies demonstrate that anethole exhibits a dose-dependent bimodal effect on contractility of isolated rat aorta (S. Ryu, 2014). Anethole reduced levels of the

cardiac enzymes CK-MB and troponin, also demonstrating improvements in the histological characteristics of cardiac muscle accompanied by a significant reduction in cardiac fibrosis. These effects were observed in an ischemia/reperfusion model, and also anethole-treated rats showed a significant increase in VAV3 iRNA expression accompanied by a significant decrease in TNF-κ protein level in cardiac tissues. Furthermore, higher doses of anethole had a greater degree of efficacy with respect to the described cardioprotective changes. The experimental substance ameliorated cardiac tissue damage by suppressing the activity of the cardiac inflammatory response, reducing TNF- α levels in cardiac tissue (Matboli. 2022).

6. Effects of anethole on the liver

6.1. Liver weight and liver index

As shown in **Table 20** intake of HCD does not cause an increase in liver weight. Anethole treatment also had no effect on the liver weight values.

Liver index values were not increased by the consumption of HCD. Dunnet's post-test showed that the index was increased in the HCD+125A group ($p<0.05$) compared to the control group, while the values in the other anethole-treated groups did not differ from those of the HCD and control groups (**Table 20** and **Figure 30**).

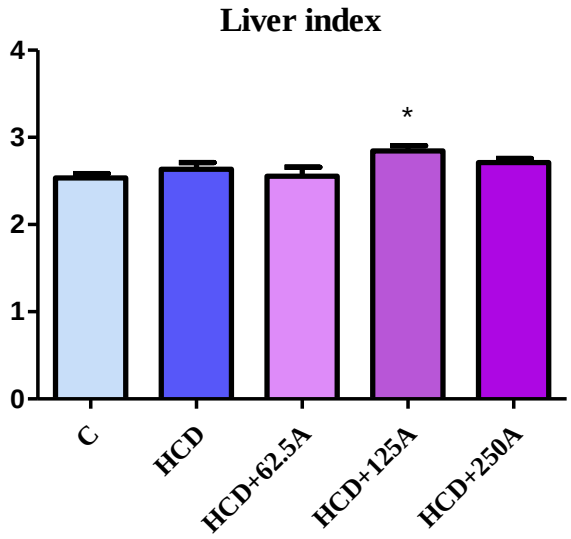

Figure 30. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on liver index; *p<0.05 vs.control group (C)

Table 20. Liver weight (g), liver index values and TBARS levels in heart homogenate of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250). Results are presented as mean ± S.E.M; *p<0.05 vs. control group (C)

Group / Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
Liver weight (g)	8.53 ± 0.4	8.98 ± 0.3	9.18 ± 0.4	9.66 ± 0.3	8.34 ± 0.3
Liver index values	2.54 ± 0.05	2.63 ± 0.08	2.56 ± 0.10	2.84 ± 0.06 *	2.71 ± 0.05
TBARS levels в liver homogenate (nnmol/g)	54.29 ± 3.7	56.48 ± 3.8	47.35 ± 2.0	49.32 ± 2.7	51.01 ± 2.2

6.2 Effects of anethole on levels of thiobarbituric acid reactive substances (TBARS) in liver tissue homogenate

Administration of a high calorie diet for 10 weeks showed no significant changes on liver TBARS levels (**Table 20**) Treatment with anethole showed no reliable difference for the parameter (ANOVA: F = 1.491, P = 0.2212).

6.3. Liver enzymes - aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP)

The values of serum AST, ALT, AP concentration are shown in **Figure 31** and **Table 21.** ALT levels were not induced by the intake of HCD. ANOVA analysis showed statistically significant differences between experimental groups in serum ALT levels: F= 4.811, P= 0.0033. Dunnet's post hoc test showed a statistically significant increase in the index in the HCD+125A group (66.4 ± 1.09 U/L) (p<0.05) compared with the HCD (61.4 ± 1.06 U/L) and C (60.92 ± 0.89

U/L) groups. Serum AST levels were also not increased by the consumption of HCD. One-way ANOVA showed a significant effect: $F(4, 40) = 3.093$, $P = 0.0261$. Dunnet's multiple comparison test showed no significant differences in the anethole-treated groups. Serum AP values were significantly increased in the HCD group compared with the control group ($P<0.05$), and these changes persisted in the high-dose anethole-treated group ($P<0.05$ vs. C). HCD+62.5A and HCD+125A showed no statistically significant difference in enzyme levels compared to control.

Table 21. Levels of serum AST, ALT and AP of rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; *p<0.05 vs. control group (C); #p<0.05 vs. HCD group

Index	**C**	**HCD**	**HCD+62.5A**	**HCD+125A**	**HCD+250A**
AST	88.26 ± 0.88	87.60 ± 0.54	90.80 ± 1.33	89.74 ± 0.96	85.87 ± 1.41
ALT	60.92 ±0.90	61.40 ± 1.07	63.40 ± 1.38	66.40 ±1.10 *#	65.79 ±0.93 *
AP	111.3 ± 6.17	160.1 ± 13.63*	143.9 ± 13.6	142.7 ± 16.44	164.8 ± 10.88 *

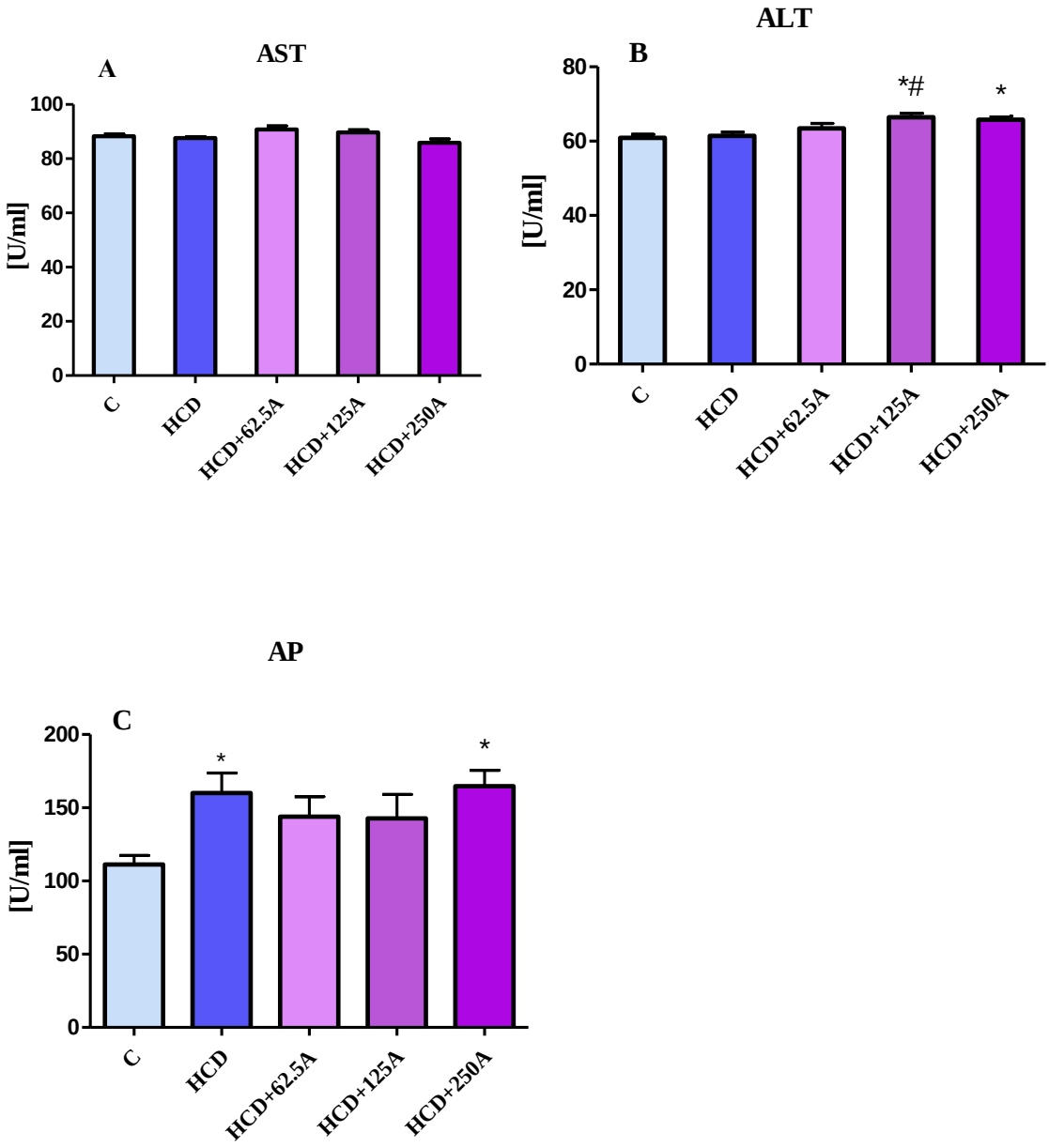

Figure 31. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on AST (Panel A), ALT (Panel B) AP (Panel C) levels; *p<0.05 vs. control group (C); #p<0.05 vs. HCD group

6.4. Liver histology

The hepatocytes of the rodents of the control group had preserved architectonics with well visible hepatic ridges. In obesity, small-droplet steatosis develops, involving all areas of the hepatic tissue as the changes were mainly pronounced around the v. centralis. The severity of changes in the low-dose anethole-treated group did not differ from those in the HCD group. Higher doses of anethole also did not significantly alter the structural changes of the liver. Hepatocytes with degenerative and inflammatory changes were observed (**Figure 32**).

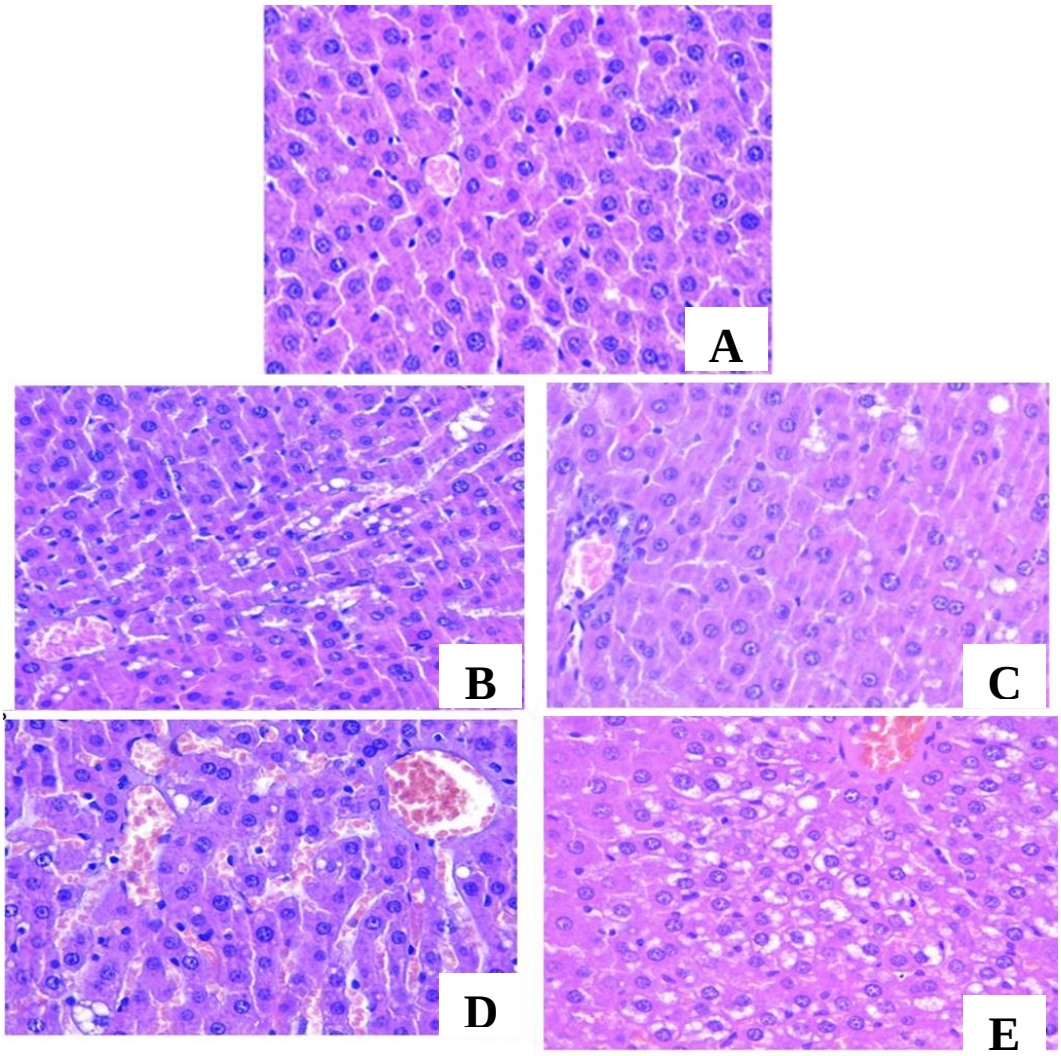

Figure 32. Liver histology of rats on high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg (panel C), 125 mg/kg (panel D) and 250 mg/kg (panel E); contol group (panel A); hematoxylin-eosin staining; magnification × 200

6.5 Liver immunohistochemistry

6.5.1. BAX expression in the liver

The marker BAX in the control group was expressed in sinusoids and in individual hepatocytes (**Figures 33** and **34, Table 22**). Consumption of HCD resulted in a significant increase of BAX apoptotic protein in hepatocytes ($p<0.001$ vs. control). The expression of BAX in the HCD+62.5A and HCD+125A groups was in hepatocytes and sinusoids, remaining similar to the HCD group, while in the HCD+250A group it was significantly reduced ($p<0.001$ compared to HCD) but remained higher than the control level ($p<0.001$).

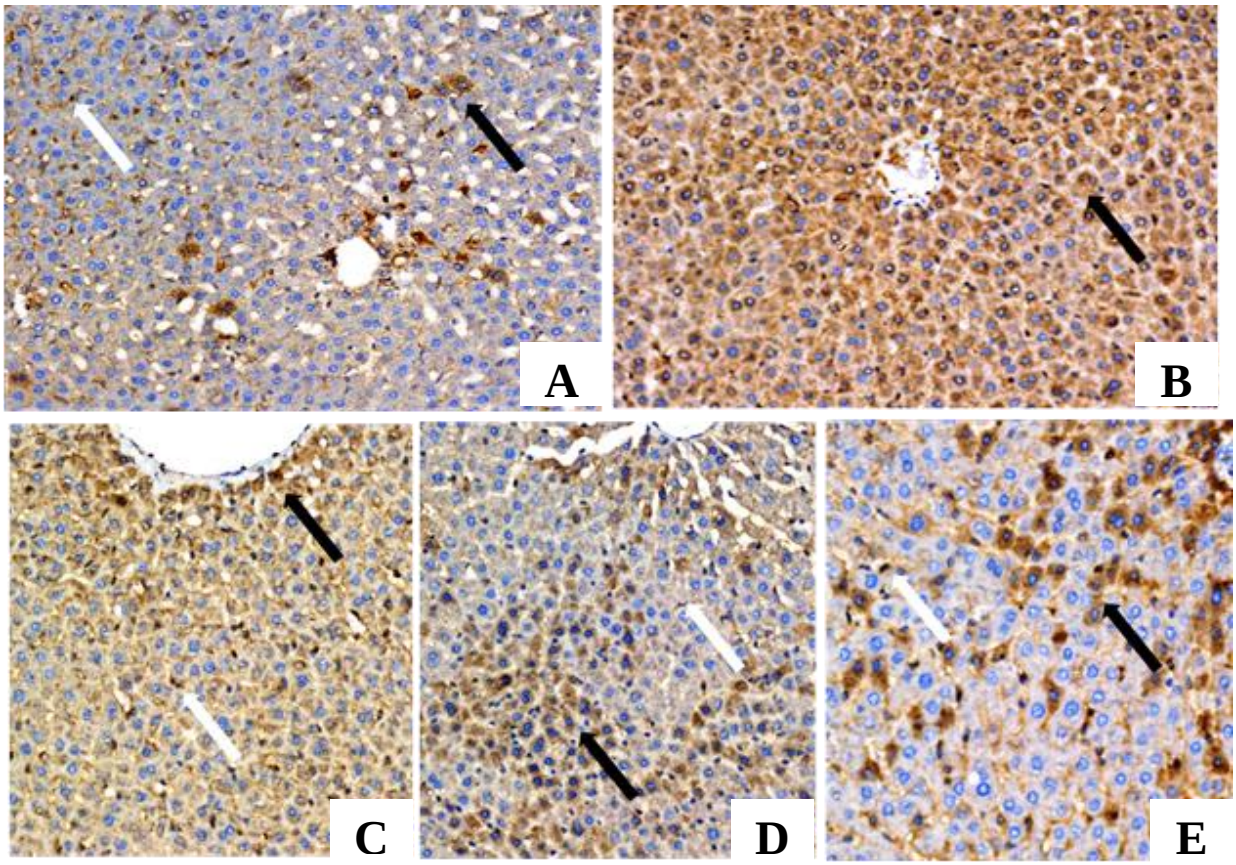

Figure 33. Expression of the apoptotic marker BAX in livers of rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg in panel C (HCD+62. 5A), 125 mg/kg in panel D (HCD+125A) and 250 mg/kg in panel E (HCD+250A) expression of in liver; contol group (panel A); white arrow - sinusoidal cells, black arrow - hepatocytes; magnification × 20

Table 22. Expression of the apoptotic marker BAX in livers of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.0001 vs control group (C), ###p<0.0001 vs HCD group

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BAX expression score in liver	0.18 ± 0.05	2.48 ± 0.10 ***	2.30 ± 0.07 ***	2.30 ± 0.06 ***	1.50 ± 0.16 ***####

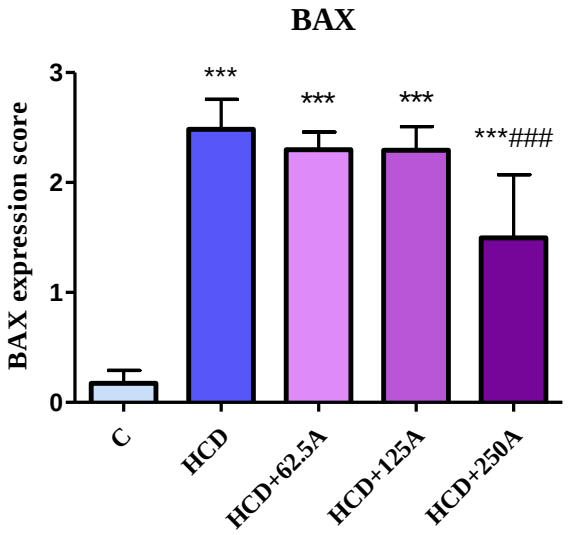

Figure 34. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the apoptotic marker BAX in liver; ***p<0.001 vs. control group (C), ###p<0.001 vs. HCD group

6.5.2 BCL-2 expression in liver

BCL-2 expression in the liver parenchyma of the control group was found predominantly in sinusoids and in individual hepatocytes (**Figures 35** and **36**, **Table 23**). According to One-way ANOVA, the anti-apoptotic protein BCL-2 was significantly increased (p<0.001 vs. control) in

the HCD group, being expressed predominantly in hepatocytes. Administration of anethole decreased BCL-2 expression compared to HCD: for HCD+62.5A and HCD+125A groups with significance of $p<0.05$ and for HCD+250A - $p<0.001$.

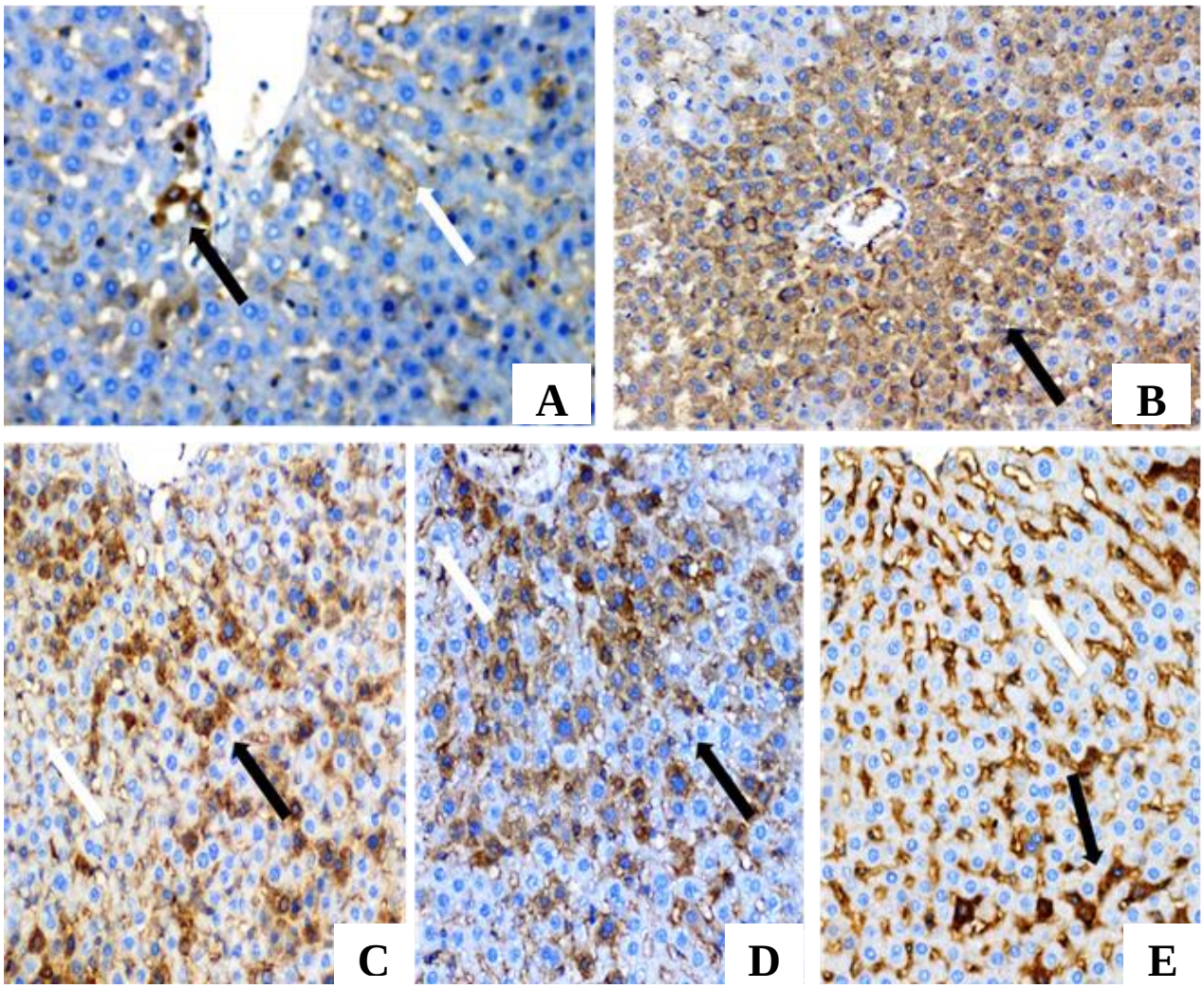

Figure 35. Expression of the antiapoptotic marker BCL-2 in livers of rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg in panel C (SCD+62. 5A), 125 mg/kg in panel D (BCD+125A) and 250 mg/kg in panel E (BCD+250A) expression of in liver; contol group (panel A); white arrow, sinusoidal cells - black arrow - hepatocytes; magnification × 200

Table 23. Expression of the antiapoptotic marker BCL-2 in livers of rats with diet-induced obesity by a high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125, and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001 vs. control group (C); #p<0.05, ###p<0.001 vs. HCD group

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BCL-2 expression score in liver	0.31 ± 0.07	1.33 ± 0.17 ***	0.70 ± 0.11 #	0.92 ± 0.12 #	0.08 ± 0.02 ###

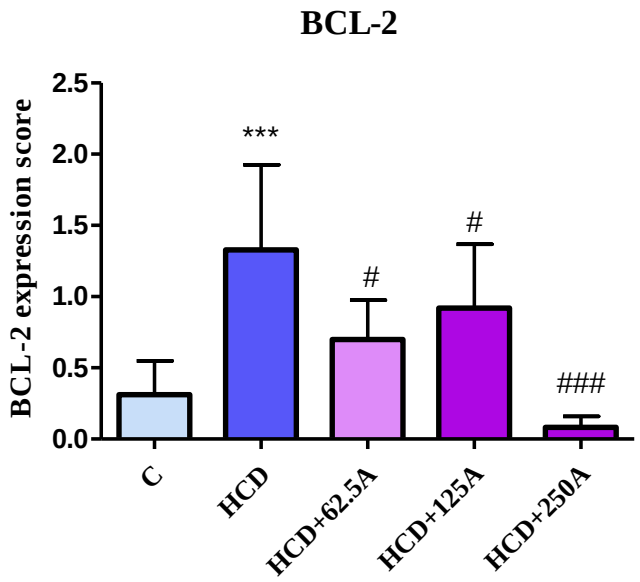

Figure 36. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on the anti-apoptotic marker BCL-2 in liver; ***p<0.001 vs. control group (C); #p<0.05, ###p<0.001 vs. HCD group

6.5.3. BAX/BCL-2 ratio

Dunnet's multiple comparison post-hoc test showed no significant increase in BAX/BCL-2 ratio in the HCD group. There were no significant differences in the BAX/BCL-2 ratio between the anethole-treated groups, with the exception of the HCD+250A group, for which the ratio was significantly increased ($p<0.001$) compared to both the control and HCD groups (**Table 24** and **Figure 37**).

Table 24. BAX/BCL-2 ratio in livers of rats with diet-induced obesity by high calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; ***p<0.001 vs. control group (C); ###p<0.001 vs. HCD group

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
BAX /BCL-2 ratio	0.86 ± 0.20	2.10 ± 0.45	3.16 ± 0.69	3.00 ± 0.56	29.62 ±6.72 ***###

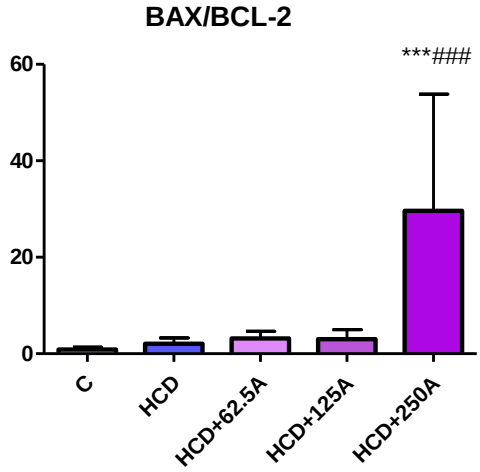

Figure 37. Effect of anethole (A) administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on BAX/BCL-2 ratio in liver; *p<0.05 vs. control group; ###p<0.001 vs. HCD group

6.5.4. MAC387 expression

As shown in **Table 25** and **Figures 38** and **39**, MAC387 in the control group was expressed in sinusoidal cells (white arrow) and in individual hepatocytes (black arrow). High-calorie diet

induced an increase in MAC387 expression relative to control ($p<0.001$). Treatment with anethole at 62.5 mg/kg prevented the induced changes ($p<0.01$) - the marker was expressed in sinusoidal cells and single hepatocytes. Values of the high anethole dose of 250 mg/kg remained similar to those of the contol with marker expression in sinusoidal cells and hepatocytes.

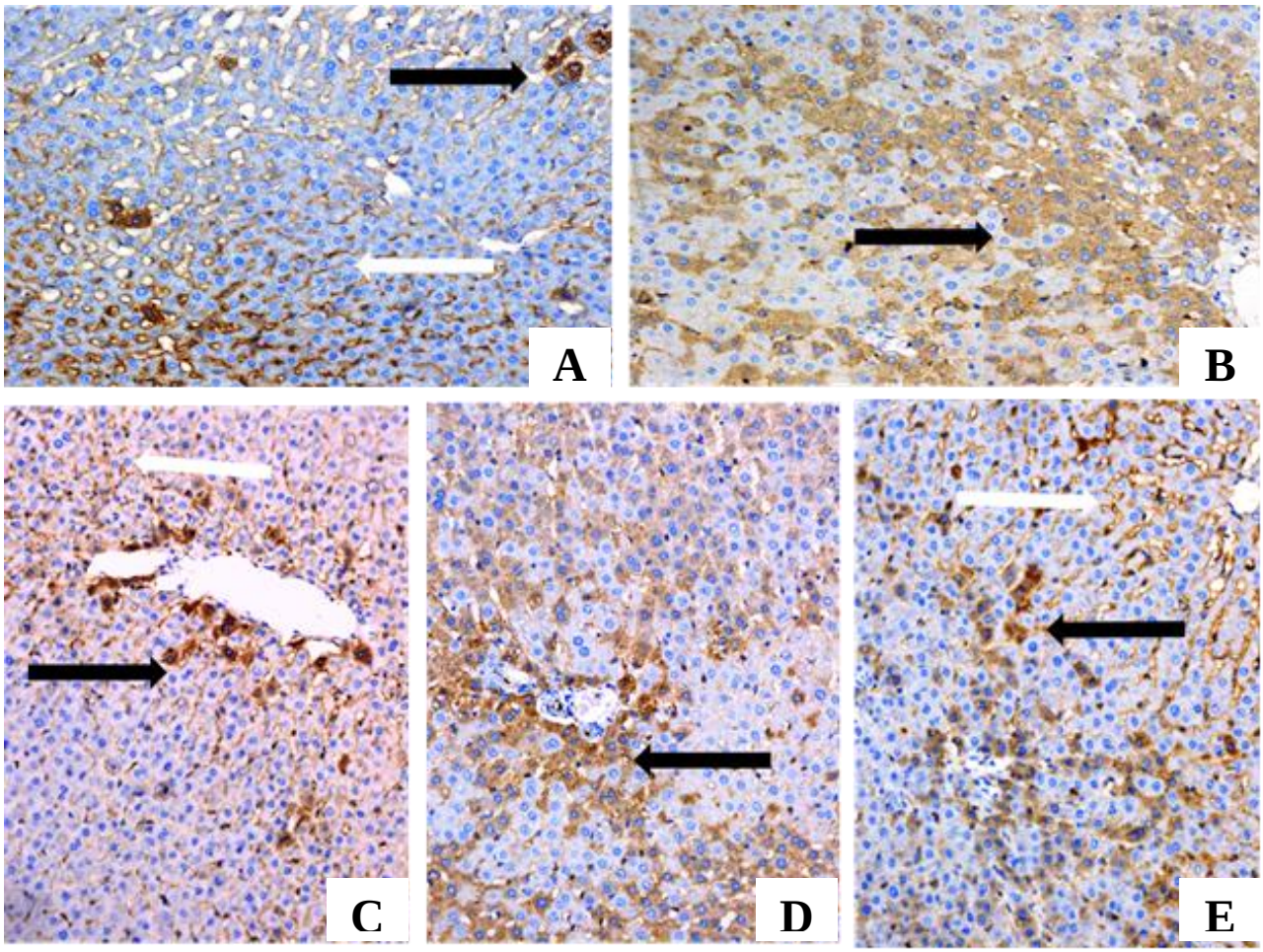

Figure 38. Expression of the proinflammatory marker MAC387 in the liver of rats on a high-calorie diet (panel B) treated with anethole at doses of 62.5 mg/kg in panel C (HCD+62.5A), 125 mg/kg in panel D (HCD+125A), and 250 mg/kg in panel E (HCD+250A); control group (panel A); white arrow - sinusoidal cells, black arrow - hepatocytes

Table 25. MAC387 expression in liver of rats with diet-induced obesity by high-calorie diet (HCD) treated with anethole (A) at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M; **p<0.01, ***p<0.001 vs. control group (C); ##p<0.01 vs. HCD

Index	C	HCD	HCD+62.5A	HCD+125A	HCD+250A
MAC387 expression score in liver	0.69 ± 0.09	1.88 ± 0.22 ***	0.70 ± 0.21 ##	1.78 ± 0.23 **	1.23 ± 0.07

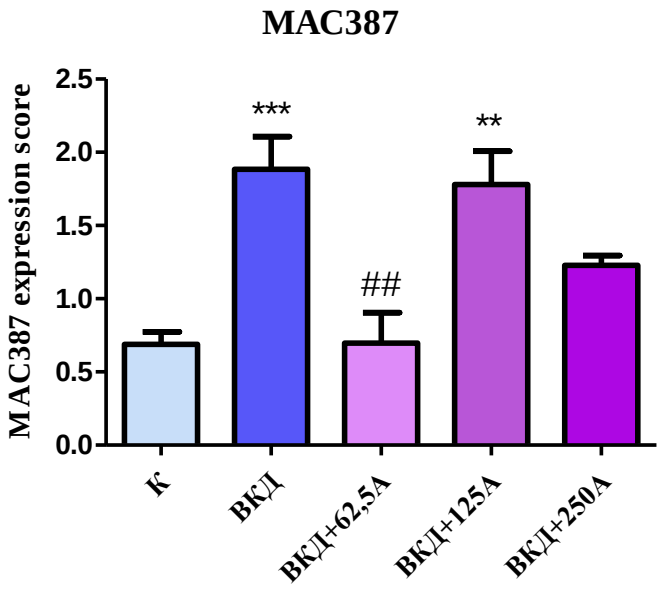

Figure 39. Effect of anethole (A) administered to rats on a high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on MAC387 expression in liver; **p<0.01 , ***p<0.001 vs. control group (C); ##p<0.01 vs. HCD

6.6. Discussion

Liver injury and dysfunction are widely recognized as significant health problems that have reached pandemic levels worldwide. Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease with an incidence of up to 25% worldwide. Its incidence is closely associated with obesity (Kim et al., 2018). NAFLD and obesity share common pathophysiological mechanisms, with insulin resistance being a key factor. In hepatocytes, hyperinsulinemia increases fatty acid synthesis "de novo". Hepatic triglyceride synthesis is induced by increased free fatty acid content in hepatocytes and insulin-mediated "up"-regulation of lipogenic enzymes (Kim et al., 2018). ALT and AST are used as a measure of normal liver

status. AST is found in various tissues, including muscles (Bovera, 2007), but is primarily recognized as a marker of liver function. Alkaline phosphatase (AP) and gamma-glutamyltransferase (GGT) are widely recognized as indicators of bile flow obstruction, particularly cholestatic pressure (Al-Amoudi. 2017). When liver cell membranes are damaged, the leakage of intracellular enzymes into the circulation leads to an increase in serum ALT and AST levels. ALT is highly specific for liver tissue and its high serum concentration is an indicator of liver injury (Engelmann et al., 2017; Sookoian et al., 2015). AST distribution is predominantly mitochondrial (Engelmann et al., 2017).

Consumption of HCD did not induce hepatomegaly, alteration in hepatocyte lipid peroxidation or increase in AST and ALT levels in the experimental animals. Instead, steatosis, elevated levels of AP, and increased MAC387, a marker of inflammation, were emphasized. Processes such as lipotoxicity, oxidative stress and hepatic de novo lipogenesis are responsible metabolic effects of fructose such as insulin resistance, obesity, type 2 diabetes mellitus, elevated blood pressure. lipid accumulation in the liver leading to hepatomegaly (Tappy et al., 2010). The administration of anethole does not antagonize the histological consequences of the diet, and in terms of the biochemical indicator ALT, it even worsened the changes. Regarding the inflammatory marker MAC387, low doses of anethole prevented obesity-induced inflammation.

According to the literature, anethole contributes to a decrease in serum levels of IL-1β, TNF-α, ALT and AST, the production of which is increased in liver inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS) and increases the level of IL-10. In addition, the amounts of IL-6 and TNF-α were decreased and those of IL-10 were increased in the livers of the trans-anethole groups. Furthermore, in inflamed broiler livers, levels of nuclear factor kappa B p65 (NF-κB p65) and TNF-α mRNA expression were decreased by anethole, while levels of IL-10 and inhibitor of NF-κB alpha (IκBα) were increased (Tong et al., 2022).

Trans-anethole at doses of 600 mg/kg also prevented LPS-induced acute jejunal inflammation in broilers. The mechanisms by which this occured were reduced NF-κB activation, increased IL-10 level and decreased IL-1β level in the tissue (Tong et al., 2022). Antioxidant indices such as SOD, CAT had higher concentrations compared to the control group in liver tissue of rabbits treated with fennel oil (Imbabi et al., 2021).

AST, ALT and AP blood levels were significantly reduced ($p \leq .05$) in rats on high fat diet supplemented with 20 mg/kg anethole (Noreen et al., 2023). Trans-anethole affected CCl4-induced liver injury by reducing AST, ALT and AP activity at doses of 160 mg/kg body weight. This effect was observed during a 4-week treatment (Pandit et al., 2022). Pretreatment with hydroalcoholic extract of Foeniculum vulgare (fennel) seeds decreased ALT, AL and AST levels in rabbit serum in paracetamol-induced hepatotoxicity (Nazir et al., 2020).

In other studies, fennel oil treatment did not alter serum ALT levels of healthy rabbits, while AST values were significantly decreased (Imbabi et al., 2021).

Hosseinzadeh and Farhoomand (2014) concluded that the addition of 0.5% tarragon powder (high in estragole and anethole) to broiler feed resulted in a non-significant reduction in liver weight (Hosseinzadeh et al., 2014). The addiministration of fennel oil containing 75% anethole showed no statistically significant effects on liver weight (Imbabi et al., 2021).

Other studies obtained similar results to ours. According to a 1989 study, prolonged consumption of large doses of trans-anethole in female rats caused liver damage and a small number of liver tumors (Truhaut et al., 1989). Several hepatic abnormalities were found histologically when anethole was administered at doses of 105 to 550 mg/kg bw per day for about 117 weeks, affecting mainly adult rats. Lesions included modified cell clusters, nodular hyperplasia, benign and malignant tumours, with the former more common at the highest dose of 1% and the female sex predominating (Truhaut et al., 1989). Studies have shown that persistent consumption of large doses of trans-anethole leads to a number of deleterious effects on cells, including cytotoxicity, cell necrosis, and cell growth. Hepatotoxicity has been reported in rats during long-term dietary experiments when the estimated daily production of anethole in the liver exceeded 30 mg of anethole per kilogram of body weight. In female rats, chronic hepatotoxicity and a low incidence of liver tumours were observed when consuming chow containing 550 mg trans-anethole per kilogram body weight each day.

Oxidative stress has the effect of disrupting lipid peroxidation in hepatocytes, which can lead to their massive accumulation. Hepatocytes are damaged by chronic oxidative stress-induced upregulation of cytochrome P450 2E1 (CYP2E1), although the exact mechanism leading to CYP2E1-related cell death is not yet known (Seki et al., 2005). Hepatic steatosis affects the production of proteins that are both pro- and anti-apoptotic (Wang et al., 2017). Hepatocyte

apoptosis contributes to liver injury and fibrosis, while apoptosis of activated stellate cells (HSCs) helps limit liver fibrosis (Canbay et al., 2004). Similarly, increased expression of BCL-2 and decreased expression of BAX play a role in reducing the negative effects of reperfusion-induced apoptosis. These conclusions were reached by examining the liver hemi-artery (Jin et al., 2012). A large percentage of cholangiocarcinomas stained positive for BCL-2, whereas hepatocellular carcinomas were negative for BCL-2. Furthermore, half of metastatic adenocarcinomas express BCL-2. Also, BCL-2 protein serves as a hallmark of cholangiocarcinoma but not hepatocellular carcinoma. This protein could potentially help to differentiate these two types of primary liver malignancies (Charlotte et al., 1994). Expression of BCL-2 is observed in bile ducts and epithelium of small bile ducts in the normal liver and therefore may have an extended lifespan, whereas hepatocytes and epithelium of large bile ducts do not show any BCL-2 expression. Proliferated ductal epithelial cells in cirrhosis and focal nodular hyperplasia are BCL-2-positive.

In healthy cells, BAX is presumed to be inactive and does not need to be controlled by antiapoptotic BCL-2 proteins (Kalkavan et al., 2017). In the present experiment, anethole at high doses increased the BAX/BCL-2 ratio, providing information on a proapoptotic phenotype. The available literature lacks data on the effect of anethole on markers of liver apoptosis/antiapoptosis in a model of obesity. Anethole reduced the proapoptotic protein p53 and the oncogene Cyclin D (associated with an aggressive form of hepatocellular carcinoma) immunohistochemically in carbon tetrachloride-induced liver injury in rats (Pandit el al., 2022).

7. Effects of anethole on carrageenan-induced hind paw edema

7.1. Results

The results regarding the volume of hind paw oedema of the experimental animals are presented in **Tables 26** and **27** and **Figure 40**.

Injection of carrageenan into the hind paw of rats induced acute inflammation in all groups. Carrageenan-induced edema in the HCD group increased gradually from 0.16 ± 0.05 at the 30th minute to 0.78 ± 0.04 at the 5th hour. There was no statistical significance of edema values in all groups at all hours.

Table 26. Values of hind paw oedema volume of rats with diet-induced obesity by HCD treated with anethole at doses of 62.5, 125 and 250 mg/kg. Results are presented as mean ± S.E.M

Group	Paw oedema on the 30-th min	Paw oedema on the 1-st h	Paw oedema on the 2-nd h	Paw oedema on the 3-rd h	Paw oedema on the 4-th h	Paw oedema on the 5-th h
HCD	0.16 ± 0.05	0,21 ± 0.05	0.25 ± 0.03	0.72 ± 0.04	0.84 ± 0.04	0.78 ± 0.04
HCD+62.5A	0.11 ± 0.03	0,20 ± 0.05	0.34 ± 0.06	0.70 ± 0.04	0.84 ± 0.05	0.90 ± 0.06
HCD+125A	0.20 ± 0.03	0,22 ± 0.03	0.38 ± 0.14	0.69 ± 0.06	0.83 ± 0.07	0.97 ± 0.06
HCD+250A	0.15 ± 0.05	0,23 ± 0.05	0.35 ± 0.04	0.59 ± 0.05	0.76 ± 0.06	0.79 ± 0.05

Table 27. Inhibition of paw oedema (%) in rats with diet-induced obesity by HCD treated with anethole at doses of 62.5, 125 and 250 mg/kg.

Group	Inhibition of paw oedema on the 30-th min	Inhibition of paw oedema on the 1-st h	Inhibition of paw oedema on the 2-nd h	Inhibition of paw oedema on the 3-rd h	Inhibition of paw oedema on the 4-th h	Inhibition of paw oedema on the 5-th h
HCD+62.5A	31 %	22%	-34%	3%	липсва	-15%
HCD+125A	-27%	12%	-51%	3%	1%	-24%
HCD+250A	8%	10%	-38%	18%	9%	-1%

7.1. Discussion

The inflammation model is commonly used to assess the efficacy of anti-inflammatory drugs (Winter et al., 1962; Grau, 1991). Cuzzocrea et al. (1998) found that histamine, platelet-

activating factor and arachidonic acid metabolites are generated when carrageenan-induced oedema occurs. Neutrophils undergo migration and aggregation at the site of inflammation as part of the delayed inflammatory response. During this process, neutrophils release reactive oxygen species, proteolytic enzymes, and other mediators.

Low-grade inflammation is the link between obesity and insulin resistance, leading to subsequent damage in the majority of organs (Khanna et al., 2022). Adipose tissue secretes multiple factors such as adiponectin, TNF-alpha and others, the cause of low-grade inflammation. In the present experiment, obesity did not induce inflammation (Trayhurn and Beattie, 2001). Anethole treatment did not suppress the inflammatory response.

A number of studies have demonstrated the anti-inflammatory effect of anethole. Anethole leads to a decrease in the amount of pro-inflammatory macrophages and neutrophils as well as pro-inflammatory mediators (Marinov and Valcheva-Kuzmanova, 2015). Furthermore, anethole study in a pain model shows a decrease in the release of inflammatory mediators (Alessandra et al., 2014). Anethole has been found to have inhibitory properties with respect to NO and PGE2 generation, which are involved in the regulation of non-immune acute inflammatory diseases. Pleural exudate volume and leukocyte count were reduced (Domiciano et al., 2015). It modulated inflammatory cytokines (TNF-alpha, IL-6, IL-1 beta), nuclear factor kappa B (NF-kappa B) and apoptotic state in brain homogenate and serum. Anethole decreased aquaporin 1 (AQP1) expression and stimulates protein kinase A (PKA) activity. A signaling pathway associated with synoviocytes that effectively suppresses cellular inflammation and death in a model of H_2O_2-induced inflammation (Huang et al., 2024). Despite the fact that these effects were undoubtedly induced by anethole, it is possible that they were triggered by pharmacologically active hepatic metabolites of the substance itself (Domiciano et al., 2013). Anethole exerted its anti-inflammatory effects in peridontitis by suppressing pro-inflammatory molecules including IL-1β and TNF-α, and this effect was comparable to the effects observed with ketoprofen (Moradi et al., 2014).

Anethole mediated cellular responses to TNF. It efficiently inhibits TNF-induced NF-κB activation. It further inhibits NF-κB activation induced by various inflammatory agents (Chainy et al., 2000).

Anethole suppressed carrageenan-induced oedema at doses of 3, 10 and 30 mg/kg in the time range of 60 to 240 minutes after induction. The time range indicated that anethole prevented the edema caused by substance P, bradykinin, histamine and TNF-α (Ponte et al., 2012). Administration of anethole at doses of 62.5 mg/kg, 125 mg/kg and 250 mg/kg effectively reduced the number of leukocytes including mononuclear and polymorphonuclear leukocytes in rats with fungal induced arthritis (Wisniewski et al., 2024).

Anethole was associated with a T cell receptor signaling pathway by inhibiting T cell proliferation and IL-2 generation. This suppression was mediated in part by downregulation of nuclear factor of activated T cells (NF-AT) and activator protein-1 (AP-1) (Yea et al., 2006).

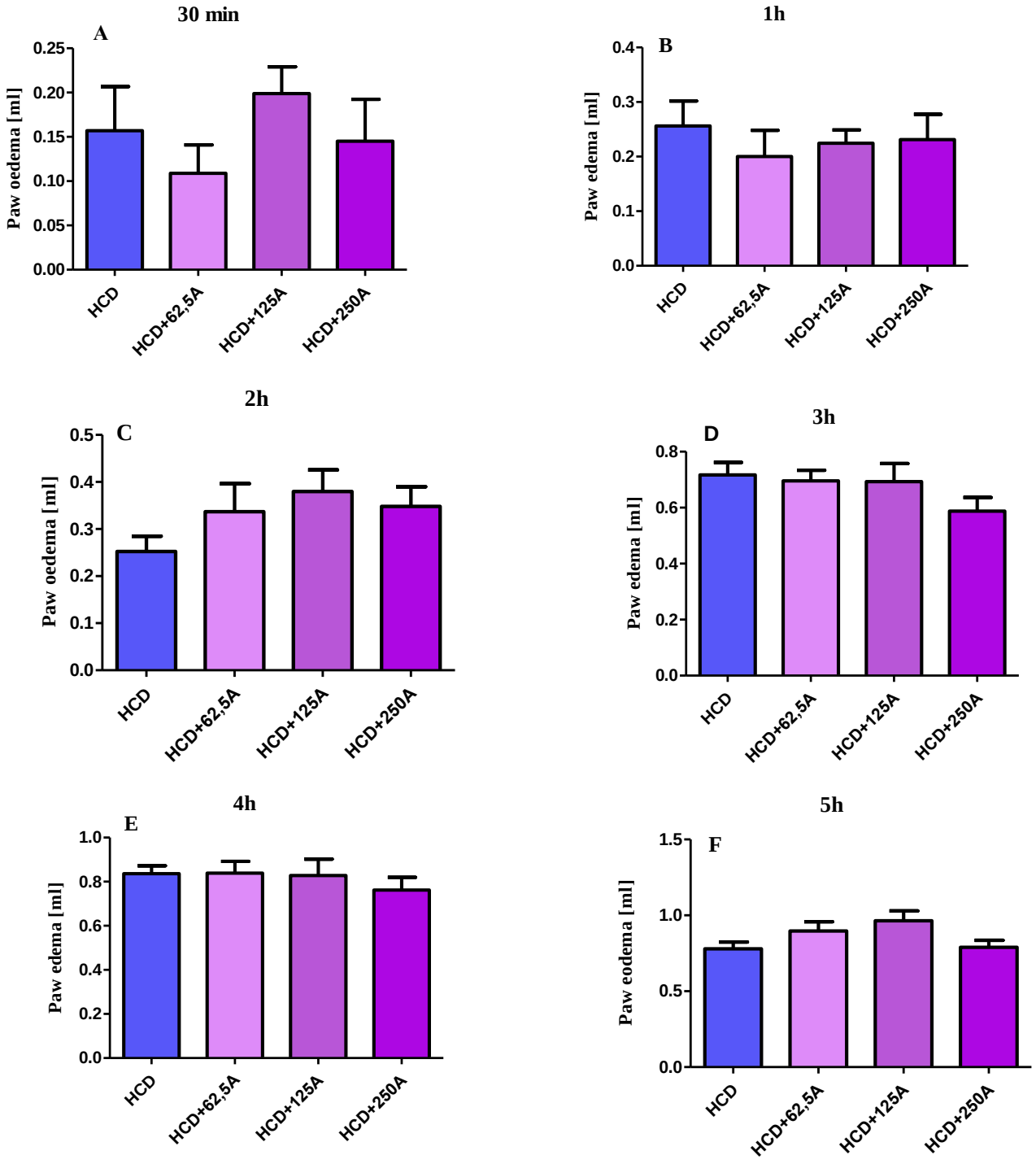

Figure 40. Effect of anethole administered to rats on high calorie diet (HCD) at doses of 62.5 mg/kg (HCD+62.5A), 125 mg/kg (HCD+125A) and 250 mg/kg (HCD+250A) on carrageenan-induced edema at 30 min (A), 1st hour (B), 2nd hour (C), 3rd hour (D), 4th hour (E) and 5th hour (F)

V.CONCLUSIONS

1. Administration of a high calorie diet for 10 weeks in male Wistar rats induced obesity. Caloric intake was increased in the experimental animals resulting in:

1.1. Behavioral disturbances - anxiety and impaired spatial memory;

1.2. Biochemical disorders - impaired glucose tolerance, increased alkaline phosphatase values, decreased serum superoxide dismutase levels, increased lipid peroxidation in the brain;

1.3. Visceral obesity;

1.4. Organ changes proven histopathologically:

1.4.1. In the myocardium - dilatation of cardiomyocytes and vacuolated cells, presence of degenerative changes;

1.4.2. In coronary vessels - focal necrosis of endothelial cells;

1.4.3. In the retroperitoneal adipose tissue - adipocyte hypertrophy;

1.4.4. In the liver - small cell steatosis;

1.5. Increased apoptosis and inflammation, proven immunohistochemically:

1.5.1. In retroperitoneal adipose tissue:

1.5.1.1. Increased expression of the pro-apoptotic BAX marker, without change in the anti-apoptotic BCL-2 marker; Increased BAX/BCL-2 ratio;

1.5.1.2. Diffuse and significant expression of the pro-inflammatory marker MAC387;

1.5.2. In the liver:

1.5.2.1. Diffuse and significant expression of both BAX and BCL-2;

1.5.2.2. Increased expression of the pro-inflammatory MAC387.

2. Anethole, when subchronically administered during the course of obesity induction, affects some of the energy metabolism indices, behaviour and organ changes induced by the high calorie diet:

2.1. Anethole significantly increases caloric intake:

2.1.1. Significantly dose-dependently increases food consumption in all intakes compared to untreated groups;

2.1.2. Significantly increased intake of fructose solution at a dose of 125 mg/kg.

2.2. Anethole reduced weight gain at a dose of 250 mg/kg, independent of increased caloric intake.

2.3. Anethole produces some behavioural changes:

2.3.1. It does not affect motor activity;

2.3.2. Prevented high-calorie diet-induced anxiety at a dose of 62.5 mg/kg, found by increasing the time of social interaction between experimental animals;

2.3.3. Exhibited a tendency to reduce depressive symptomatology at all doses administered, as evidenced by the shortening of immobility time in the forced swim test;

2.3.4. Dose-dependently improved spatial memory, significantly at doses of 125 and 250 mg/kg, by increasing the object location recognition index.

2.4. Anethole induces changes in some biochemical and clinical-laboratory parameters:

2.4.1. It does not affect fasting blood glucose, but in the course of the glucose tolerance test at a dose of 250 mg/kg it normalizes impaired glucose tolerance from a high-calorie diet;

2.4.2. Does not affect serum triglyceride and total cholesterol levels;

2.4.3. Administered at a dose of 125 mg/kg, significantly increases alanine aminotransferase levels and does not alter aspartate ammonotransferase and alkaline phosphatase levels;

2.4.4. It improves the antioxidant protection of the organism by normalizing the levels of serum superoxide dismutase decreased by a high-calorie diet at a dose of 250 mg/kg;

2.4.5. Normalizes high-calorie diet-induced lipid peroxidation in the brain in all doses administered;

2.4.6. Does not alter lipid peroxidation levels in myocardial homogenate, liver and serum;

2.4.7. Does not affect the time of blood coagulation.

2.5. Anethole induces changes in tissue indices:

2.5.1. Lowers the retroperoneal adipose tissue index at a dose of 250 mg/kg and achieves total adipose tissue index values close to control values at the same dose;

2.5.2. Did not significantly change the mesenteric, perigonadal and paranephric adipose tissue indices relative to the high calorie diet group, but at a dose of 62.5 mg/kg increased the paranephric adipose tissue index relative to control;

2.5.3. Did not significantly change the hepatic index relative to the high calorie diet group, but at a dose of 125 mg/kg significantly increased the hepatic index relative to control;

2.6. Anethole affected some of the organ changes demonstrated histologically compared to the high calorie diet group:

2.6.1. It does not prevent myocardial damage;

2.6.2. Does not antagonize focal necrosis in the endothelium of coronary vessels;

2.6.3. At a dose of 250 mg/kg significantly reduces the size of adipocytes in retroperitoneal adipose tissue, achieving the morphology of control adipose cells;

2.6.4. Did not show a protective effect against small-cap steatosis in the liver of obese rats.

2.7. Anethole alters the expression of markers of apoptosis/antiapoptosis and inflammation as demonstrated immunohistochemically:

2.7.1 In retroperitoneal adipose tissue compared to the high calorie diet group:

2.7.1.1 Expression of the apoptotic marker BAX was decreased from a dose of 125 mg/kg and increased from a dose of 250 mg/kg;

2.7.1.2. There was a tendency to decrease the antiapoptotic marker BCL-2 from doses 62.5 and 125 mg/kg;

2.7.1.3. The BAX/BCL-2 ratio was significantly increased at the 250 mg/kg dose;

2.7.1.4. MAC387 expression was not altered by anethole.

2.7.2. In the liver compared to the high calorie diet group:

2.7.2.1. BAX expression was significantly decreased at a dose of 250 mg/kg;

2.7.2.2. BCL-2 expression was significantly decreased at all doses used, with the most pronounced effect at the 250 mg/kg dose;

2.7.2.3. The BAX/BCL-2 ratio was significantly increased at a dose of 250 mg/kg;

2.7.2.4. MAC387 expression was significantly decreased at a dose of 62.5 mg/kg.

2.7. Anethole did not affect carrageenan-induced hind paw oedema in rats on a high calorie diet.

VI. CONTRIBUTIONS

1. For the first time, data were obtained on the effects of anethole on food, fluid, and caloric intake in rats in an experimental model of diet-induced obesity, establishing:

1.1. Increase in food consumption;

1.2. Increase in fluid intake;

1.3. Increasing calorie intake.

2. For the first time, the effects of anethole on behavior were investigated in rats with an experimental model of diet-induced obesity, finding:

1.1. Antianxiogenic effect;

1.2. Tendency to reduce depressive symptomatology;

1.3. Improvement of spatial memory.

3. For the first time, data were obtained on the effect of anethole on glucose tolerance and lipid peroxidation in the brain of rats with an experimental model of diet-induced obesity, establishing:

2.1. Glucose tolerance-enhancing effect;

2.2. Suppression of lipid peroxidation in the brain.

4. For the first time, data on anethole on histological findings in retroperitoneal adipose tissue were obtained in rats with an experimental model of diet-induced obesity, and an antagonizing effect was found against high-calorie diet-induced adipocyte enlargement.

5. For the first time, data were obtained on the effects of anethole on programmed cell death in retroperitoneal adipose tissue and liver in rats with an experimental model of diet-induced obesity, and an enhancement of the proapoptotic phenotype was found in retroperitoneal adipose tissue and liver.

6. For the first time, data were obtained for a decrease in the expression of the inflammatory marker MAC387 in the liver by anethole in an experimental model of diet-induced obesity.

VIII. LIST OF PUBLICATIONS AND FORUMS RELATED TO THE THESIS

List of publications:

1. E. Rafailova, S. Gancheva, K. Moneva, M. Todorova, M. Eftimov, M. Reyzov, M. Effect of anethole on liver aminotransferases in rats with diet-induced metabolic syndrome. Proceedings of the XVIII National Conference with International Participation "Natural Sciences'2020"; 50-54.

2. E. Rafailova, S. Gancheva, K. M. Todorova, M. Eftimov, M. Reizov, M. Zhelyazkova-Savova, St. Valcheva-Kuzmanova. Effect of anethole on visceral obesity and serum triglyceride and cholesterol levels in rats on a high-calorie diet. Varna Medical Forum, 10, 2021, Suppl. 3, 27-32.

3. Rafailova E, Todorova M, Moneva K, Reizov M, Gancheva S, Eftimov M, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Effects of anethole on behavior in a rat experimental model of obesity. Science and youth 2022. application of the III th National scientific conference "Courage and youth in pharmacology 2022"; 133-138.

4. E. Rafailova, S. Kuzmanova-Vulcheva. Antiobesity effect of selected monoterpenes - a review. Proceedings of the Twenty-first National Scientific Session for Students and Teachers "With a Vision to the Future", Dedicated to the 75th Anniversary of the Medical College of Medical University - Pleven, 27-28 October 2023; 215-223.

5. E. Rafailova, S. Valcheva-Kuzmanova. Health consequences of obesity - a review. Varna Medical Forum, vol. 13, 2024, Online First; https://journals.mu-varna.bg/index.php/vmf/issue/view/582.

List of forums:

1. K. Moneva, D. Pavlov, K. Kuzmanov, M. Todorova, S. Gancheva, **E. Rafailova**, M. Eftimov, M. Reyzov, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova. Evaluation of the effect of anethole on carrageenan-induced paw edema in metabolic rat. Book of abstracts from Jubilee Scientific Conference “45 years Medical University - Pleven”, 2019; 12:123.

2. S. Gancheva, K. Kuzmanov, D. Pavlov, M. Todorova, K. Moneva, M. Eftimov, M. Reyzov, **E. Rafailova**, V. Kuzmanova, A. Kuzmanov, S. Todorov, M. Zhelyazkova-Savova. Effect of metabolic syndrome on carrageenan-induced paw edema in rats. Book of abstracts from Jubilee Scientific Conference “45 years Medical University - Pleven”, 2019; 12:116-117.
3. **E. Rafailova,** S. Gancheva, K. Moneva, M. Todorova, M. Eftimov, M. Reyzov, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova. Effect of anethole on liver aminotransferases in rats with dietary induced metabolic syndrome. XVIII National Conference with International Participation "Natural Sciences'2020", 2-4 October 2020;
4. **E. Rafailova,** S. Gancheva, K. Moneva, M. Todorova, M. Eftimov, M. Reizov, M. Zhelyazkova-Savova, St. Valcheva-Kuzmanova. Effect of anethole on visceral obesity and serum triglyceride and cholesterol levels in rats on a high-calorie diet. Jubilee scientific conference "60 years of Pathophysiology- Medical University Varna" , 24-25.09.2021.
5. M. Tsaneva, **E. Rafailova,** M. Eftimov, M. Reizov, K. Moneva, M. Todorova, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova; Effect of anethole on histopathological changes in heart, liver and adipose tissue in rat model of metabolic syndrome. XIII National Congress of Pathology, September 10-12, 2021. Burgas. Proceedings, pp. 57-58.
6. **Rafailova E,** Todorova M, Moneva K, Reizov M , Gancheva S, Eftimov M, Zhelyazkova-Savova M, Valcheva-Kuzmanova S. Effects of anethole on behavior in rats with experimental model of obesity. III th National Scientific Conference "Courage and Youth in Pharmacology 2022", 30 September - 02 October 2022, Tsigov Chark.
7. **Rafailova, E.,** Gancheva, S.,Eftimov, M.,Reyzov, M.,Moneva, K.,Todorova, M.,Zhelyazkova-Savova, M.,Valcheva-Kuzmanova, S.; Effect of anethole on anxiety in rats on a high-calorie diet . 35th ECNP Congress, 15-18 October 2022, Vienna, Austria. Neuroscience Applied, Volume 1, Supplement 2, 2024, p.315.
8. **Rafailova, E**., Gancheva, S.,Eftimov, M.,Reyzov, M.,Moneva, K.,Todorova, M.,Zhelyazkova-Savova, M.,Valcheva-Kuzmanova, S. Effect of anethole on spatial memory in rats on a high-calorie diet ; 35th ECNP Congress, 15-18 October 2022, Vienna, Austria. Neuroscience Applied, Volume 1, Supplement 2, 2024, p.183.

9. **Rafailova E**, Valcheva-Kuzmanova S. Obesity and organ damage – a review. Eighth Pharmaceutical Business Forum and Scientific and Practical Conference "Pharmacists and Physicians - United in Support of the Patient", 28-29 October, Varna, Bulgaria. Scripta Scientifica Pharmaceutica, 9, Suppl. 1, 2022, p. 26.
10. **E. Rafailova**, S. Gancheva, M. Todorova, K. Moneva, M. Reizov, M. Eftimov, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova. Effects of anethole on blood glucose level in glucose tolerance test in rats with experimental model of obesity. Eighth Congress of Pharmacy with International Participation, 27-30 April 2023, k.k. Borovets; p. 198; file:///E:/START.html.
11. **Rafailova E**., Gancheva S., Todorova M., Moneva K., Reyzov M., Eftimov M., Zhelyazkova-Savova M., Valcheva-Kuzmanova S. Anethole dose-dependenltly affects liver apoptosis/anti-apoptosis by regulating BAX/BCL-2 signaling pathways in rats on a high-calorie diet; 5th ICNPU: from Plants to Pharmacy Shelf, 30 May – 2 June, Sts. Constantine & Helena resort, Bulgaria. p.232
12. **E. Rafailova** , S. Gancheva , K. Moneva-Marinova , M. Reyzov , M. Eftimov , M. Todorova , M. Zhelyazkova-Savova , S. Valcheva-Kuzmanova. Anethole prevents high-calorie diet-induced brain lipid peroxidation in rats and normalizes superoxide dismutase activity. 36th ECNP Congress, 7-10 October 2023, Barcelona, Spain. Neuroscience Applied, Volume 2, Supplement 2, 2023, 103315.
13. **E. Rafailova,** St. Valcheva-Kuzmanova. Potential anti-cancer activity of anethole – a review. Ninth Pharmaceutical Business Forum and Scientific and Practical Conference "Faculty of Pharmacy - 15 Years of Traditions, Innovations, Perspectives" 20 - 21 October 2023, Sofia, Bulgaria. Varna, resort. "Sunny Day". Scripta Scientifica Pharmaceutica, 2023;10, suppl. 2, p.31.
14. **Elis Rafailova**, Stefka Kuzmanova-Vulcheva. Antiobesity effect of selected monoterpenes - a review. Twenty-first National Scientific Session for Students and Teachers "With a Vision to the Future", Dedicated to the 75th Anniversary of the Medical College of Medical University - Pleven, 27-28 October 2023.

Conference Awards:

1. Excellence award at 35 ECNP, 2022, Vienna, Austria for the abstract "Effect of anethole on spatial memory in rats on a high-calorie diet"- E. Rafailova, S. Gancheva, M. Eftimov, M. Reyzov, K. Moneva, M. Todorova, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova

2. Excellence award at 36 ECNP, 2023, Barcelona, Spain for the abstract "Anethole prevents high-calorie diet-induced brain lipid peroxidation in rats and normalizes superoxide dismutase activity"- E. Rafailova, S. Gancheva, K. Moneva-Marinova, M. Eftimov, M. Reyzov M. Todorova, M. Zhelyazkova-Savova, S. Valcheva-Kuzmanova

3. 3rd Prize for PhD Students' Papers at the Ninth Pharmaceutical Business Forum and Scientific and Practical Conference "Faculty of Pharmacy - 15 Years of Traditions, Innovations, Perspectives" 20 - 21 October 2023, Sofia, Bulgaria. Varna, resort. "Sunny Day" for the report "Potential anti-cancer activity of apethol - a review" - E. Rafailova, St. Valcheva-Kuzmanova