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THE EFFECT OF HYDROXYTYROSOL (HXT) AND A LOCAL OLIVE OIL EXTRACT ON THE LEVEL OF HEPCIDIN HORMONE AND PATHOLOGICAL HISTOLOGICAL CHANGES WITH IRON DEPOSITION IN THE AORTA RESULTING FROM INDUCED HYPERLIPIDEMIA IN MALE RATS

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Abstract

Hydroxytyrosol (HXT) is a polyphenol present in olive oil, known for its antioxidant effects. For this, the present study was designed to examine the role of both Local Olive Oil (LOO) and HXT in improving the level of lipid profile and the hepcidin hormone and iron, as well as the histological study of the aorta in male white rats type Sprague Dawley Experimental hyperlipidemia and comparison of results with Atorvastatin (ATOR). This study used 30 male white rats that were distributed to six groups and weights close. The first group (control) was given the standard diet and the second group (cholesterol) a diet containing 2% cholesterol throughout the eight-week trial period, while the third, fourth, fifth and sixth groups were given a high-cholesterol diet for two weeks and then gavage with LOO only, HXT only, LOO + HXT and ATOR, respectively, for six weeks while continuing on the diet rich in cholesterol. The results of the study showed a significant increase ($P < 0.05$) in the level of total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C) and Atherogenic Index (AI) and hepcidin hormone and a significant decrease ($P < 0.05$) in the level of high-density lipoprotein cholesterol (HDL-C) and iron, As well as many of the imbalances histopathological in the aorta, included the deposition of Cholesterol Crystals (CC), formation of plaques (PL), Lymphocytes Infiltration (LI), and Iron Deposition (ID) in the wall layers with an increase in wall thickness In the infected animal group compared to the healthy control group. While the groups of infected animals that were Gavage with LOO, HXT, LOO + HXT, and ATOR showed positive improvement in all the above variables, LOO + HXT surpassed all treatments. Current results suggest that the synergistic effect of HXT with LOO may enhance the antioxidant system and thus reduce the negative effects of hyperlipidemia.

***Keywords*:** Local Olive Oil, Hydroxytyrosol, Hepcidin Hormone, Iron Deposition.

Introduction

The term Hyperlipidemia (HLD) refers to an abnormally high level of lipids or lipoproteins due to imbalance in the metabolism and function of lipids, due to dietary disorders, obesity, and genetic diseases such as Familial hypercholesterolemia and Diabetes diseases (Sudhakaran *et al*., 2018; Yao *et al*., 2020). Hyperlipidemia patients are more likely to increase to cardiovascular disease. Therefore, hyperlipidemia is a very important risk factor for predicting Atherosclerosis (AS) and coronary artery diseases (Elhissi *et al*., 2014; Zhang *et al*., 2018). This is a disease, as indicated by the World Health Organization (WHO) (WHO, 2017). The number one cause of death in the world. Changes in lipid parameters associated with Atherosclerosis include elevated (TC), (TG), (LDL-C), low (HDL-C). As high cholesterol in the blood contributes to the development of Atherosclerosis (Schwingshackl and Hoffmann, 2014).

Monocyte macrophages in an environment of oxidized Low-Density Lipoprotein by removing LDL-C and generating foam cells (FoC), which is a major type of cell found within the fatty streaks and fibrous plaque (Napoli *et al*., 1997). And that iron overloads an important role in the development of (AS) (Kim *et al*., 2017). High expression of hepcidin increases Iron Deposition (ID) in Macrophage Atherosclerosis plaques And enhances the instability of plaque, This indicates that hepcidin is closely related to the deposition of iron in plaque and the stability of plaque in Atherosclerosis (Habib *et al*., 2015; Pechlaner *et al*., 2016). Functional nutrients and functional nutrients beneficial to vascular health may be beneficial compounds that can reduce the overall cardiovascular risk caused by fat disorders by acting in parallel with statins or as auxiliary substances (Scicchitano *et al*., 2014). It is confirmed that Studies indicate that the introduction of virgin olive oil in the diet leads to a significant decrease in the levels of inflammatory vital signs associated with Atherosclerosis (Casas *et al*.,2016; Medina-Remon *et al*., 2017). The anti-oxidant and Anti-atherogenic effects of polyphenols in olive oil, such as Oleuropein and Hydroxytyrosol, have been significantly confirmed in studies (Carluccio *et al*., 2003). The current study aims to test the efficacy of LOO extract and HXT in improving the lipid profile and the concentration of the hepcidin hormone and iron, and histological dysfunctions of the aorta in rats with experimental hyperlipidemia.

Materials and Methods

Materials

LOO was obtained from the Kamaran laboratory in Kirkuk governorate, and the rats were treated with a concentration of 1/2 ml/kg of body weight. HXT was

obtained from Shaanxi Bolin Biotechnology - Shaanxi of China, and the rats were treated with a concentration of 50 µl/kg of body weight according to the concentration determined by the study experiments. The Atorvastatin drug used in this experiment was made by the International Pharmaceutical Industries / Amman / Jordan, and the rats were treated with a concentration of 2.06 mg/kg (Nair and Jacob, 2016).

The Animals

Was used (30) Sprague Dawley male white rats, Age (16-18) weeks and weigh (200-260) grams. Animals were placed in cages designed for this purpose. These animals were subjected to laboratory conditions that included 12 hours of light and 12 hours of darkness. The degree of heat was established at (22 ± 2) ^{0}C. Cages were taken into account, clean, and sterilized. The animals were left for two weeks to adapt to the new breeding conditions and to ensure that they were free of diseases, and they fed on the diet (25% wheat, 45% yellow corn, 20% soybeans, 10% concentrated animal protein, 1% powdered milk added to it 50 g / 100 kg vitamins And preservatives and anti-fungal materials) (Balducci-Roslindo *et al*., 2001). And given food and water continuously throughout the experiment.

Experiment Design

This study used 30 male from mature white rats distributed to 6 groups, each group included 5 animals with close weights. Healthy animals were fed on the standard diet during the eight-week trial period, while the infected animals feed on the diet containing cholesterol by 2% (Yang *et al*., 2019). For two weeks, he was then administered LOO, HXT, and ATOR for six weeks while continuing on the cholesterol-rich diet as follows.

1. The first group (control group): This group was given a standard cholesterol-free diet and gavage with distilled water.
2. The second group (the cholesterol group): This group was given a standard diet plus cholesterol at a rate of 2% and gavage with distilled water.
3. The third group (the group of cholesterol and the extract of LOO): This group was given a standard diet plus cholesterol at a rate of 2% and gavage with the extract of LOO at a concentration of (1/2 ml/kg) of body weight.
4. The fourth group (cholesterol group and HXT substance): This group was given a standard diet plus cholesterol at 2% and gavage with HXT at a concentration of (50µl / kg) of body weight.
5. The fifth group (cholesterol group and LOO extract + HXT substance): This group was given a standard diet plus cholesterol at 2% and gavage with LOO extract at a concentration of (2/1 ml/kg) + HXT substance at a concentration of (50µl / kg) Bodyweight.
6. The Sixth group (the cholesterol group and ATOR drug): This group was given a standard diet plus cholesterol at 2% of the weight of the diet and was gavage with ATOR at a concentration of (2.06 mg/kg) of body weight.

Collection of Blood Samples:

Blood samples were collected 8 weeks after the start of the experiment. The animals were starved for 12 hours and then drugged with Ketamine and Xylazine in doses of 5-35 mg/kg of body weight by intramuscular injection (Jaber *et al*., 2014). Then blood samples were drawn from the heart, the blood was placed in plastic tubes free of anticoagulant and left for 15 minutes at room temperature until blood coagulation, then the tubes were placed in a centrifuge at a speed of 3000 r / min for 15 minutes to obtain a serum blood. The serum was frozen by freezing at a temperature of 20- °C until the required chemical analysis, then the aorta was removed for histological study.

Biochemical Tests in Serum:

The TC concentration was estimated based on (Deacon and Dawson,1979), TG, and HDL-C using the analysis kit manufactured by the French company (BIOLABS SA, France) and calculating the LDL-C according to (Tietz *et al*., 1999). VLDL – C concentration was calculated based on (Burtis and Ashwood, 1999). And the AI account is according to (Dobiasova, 2004). The iron concentration was estimated by using the analysis kit manufactured by the French company (BIOLABS SA, France), and the estimation of the concentration of the hepcidin hormone based on the technique of Elisa sandwich using the analysis kit manufactured by (MyBioSource, USA).

Histological preparations:

After the animals were dissected, the aorta was removed and washed with a physiological solution. Samples were prepared using microscopic tissue sections (Suvarna *et al*., 2013). Using hematoxylin, eosin, and Prussian Blue Iron. After completing the preparation of the microscopic tissue sections, they were examined by optical microscopy.

Statistical Analysis:

Statistical analysis of the results was conducted by ANOVA Analysis of Variance. The significant differences were determined according to Duncan's multiple ranges and at a significant level ($P \leq 0.05$) (Elsahookie and Wuhaib, 1999).

Results and Discussion

Serum Lipid Profile

The results in Table 1 showed a significant increase ($P < 0.05$) in the level of TC, TG, LDL-c, VLDL-c, and AI and lower HDL-c level in the affected animal group compared to the healthy control group. It is noted that the groups of infected animals that were gavage with olive oil extract, HXT substance, olive oil extract + HXT and ATOR showed a significant increase ($P < 0.05$) in the level of HDL-c and a significant decrease in the level of TC, TG, LDL-c, VLDL- c and AI compared to the group of infected animals, as the olive oil extract + HXT exceeded all the treatments followed by ATOR and HXT in the second degree and then olive oil extract.

Table 1 : HXT, LOO extract, and ATOR drug in lipid profile and Atherogenic index in the serum of rats male treated with cholesterol.

Parameters / Groups	Total Cholesterol mg/dl	Triglyceride mg/dl	HDL-c mg/dl	LDL-c mg/dl	VLDL-c mg/dl	Atherogenic index
Control	119.53±9.12c	124.20±11.59c	43.60±5.31b	51.09±1.66d	24.84±2.32c	2.75±0.13c
HLD	210.80±11.58a	229.63±15.08a	29.90±6.9c	134.97±1.81a	45.93±3.02a	7.25±1.35a
Olive Oil+HLD	143.77±8.52b	162.47±12.01b	46.17±6.92b	65.11±1.03b	32.49±2.40b	3.14±0.29b
HXT +HLD	136.54±8.01b	153.70 ±8.74b	46.20±5.47b	59.59±0.9c	30.74±1.75b	2.97 ±0.17b
(Olive Oil+HXT) +HLD	123.33±7.72c	129.70±10.42c	53.73±8.56a	43.66±2.93f	25.94±2.08c	2.30±0.29c
ATOR +HLD	132.63±8.52b	150.57±9.82b	47.27±3.92a	55.25±5e	30.11±1.96b	2.81±0.18b

•Values are expressed in mean ± standard deviation.
•The number of rats (5) in each group.
• The numbers followed by vertically different letters indicate a significant difference at the probability level ($P \leq 0.05$).

These results are consistent with a study (Al-Obaidy and Al-Obaidy, 2018) in rabbits. The reason is due to an imbalance in the metabolism of lipid, or an imbalance in the absorption and excretion of steroids, or perhaps due to a decrease in the concentration of bile salts (Al-Ashlash, 2012). They indicated (Garg *et al*., 2018) that the reason for the high values of the Atherogenic index in serum is due to the deposition of macrophages and fats in major organs and blood vessels such as the liver, kidney, heart, aorta, and the coronary artery. Regarding the role of LOO extract in reducing lipid profile, the results of the current study were consistent with the study (AL-Azawiy, 2018) when induced diabetes and the use of olive oil that showed a significant decrease in the level of lipid profile. The role of olive oil in preventing high cholesterol in the blood is because it contains Monounsaturated fatty acids (MUFA) and its effect on the manufacturing process of cholesterol, which causes its prevention or contributes to the process of metabolizing factory cholesterol, thus reducing its level in the body (Pignatelli *et al*., 2012). And olive oil works to reduce or control the TG by containing olive oil high amounts of unsaturated fats that work to prevent AS, especially in the coronary arteries (Orsavova *et al*., 2015). Regarding the role of HXT, our results are consistent with a study (Jemai *et al*., 2008). when using HXT to treat hyperlipidemia in rats, which reduced the lipid profile and increased the HDL level and inhibited lipid peroxide by increasing the activity of CAT and SOD in serum compared to the affected control group. Moreover, HXT also exerts a beneficial effect on HDL-C (Berrougui *et al*., 2015). Our results also showed an improvement in the lipid profile in the group of gavage with LOO + HXT extract together significantly compared to other treatments through a decrease in lipid profile and AI values and a significant increase in HDL-C level. This positive change may be attributed to the LOO extract possessing several bioactive compounds in addition to HXT that can regulate the different mechanisms associated with cholesterol metabolism and Hypocholesterolemic by building the molecular mechanisms responsible for these changes (Meneses *et al*., 2016). The role of ATOR is to inhibit cholesterol production by regulating the build-up of LDL-C receptors on the surface of liver cells which leads to the removal of LDL-C from circulation (Gotto, 2002). Statins lower cholesterol levels through selective and competitive inhibition of the HMG-CoA reductase, in addition to this it works indirectly by increasing the median receptors and absorbing LDL-C and thereby reducing their level of blood plasma (Raghow, 2017).

Determination of Iron and Hepcidin Concentration in the Serum

The results in Table 2 showed a decrease in serum iron concentration and a significant increase in the concentration of hepcidin ($P < 0.05$) in the affected animal group compared with the healthy control group. The groups of infected animals that were gavage with LOO extract, HXT, LOO extract + HXT and ATOR showed a significant increase ($P < 0.05$) in the iron concentration and a decrease in the concentration of hepcidin in the blood serum compared to the group of infected animals, As the LOO extract + HXT exceeded all the treatments, followed by the HXT substance in the second class, then the LOO extract and ATOR drug.

Table 2 : the effect of HXT, LOO extract, and ATOR on iron concentration and hepcidin hormone In the blood serum of male rats treated with cholesterol.

Groups / Parameters	Control	HLD	Olive Oil +HLD	HXT +HLD	(Olive Oil +HXT) +HLD	ATOR +HLD
Iron (µg/dL)	102.3±9.34 a	81.6±4.7 c	93.4±5.98 b	95.3±9.44 b	102.27±8.04 a	92.3±9.07 b
Hepcidin (ng/dL)	444.45±78.25 b	651.11±94.44 a	579.25±63.88 a	493.71±65.05 a	455.82±25.49 b	546.78±71.9 a

•Values are expressed in mean ± standard deviation.
•The number of rats (5) in each group.
• The numbers followed horizontal different letters indicate a significant difference at the probability level ($P \leq 0.05$).

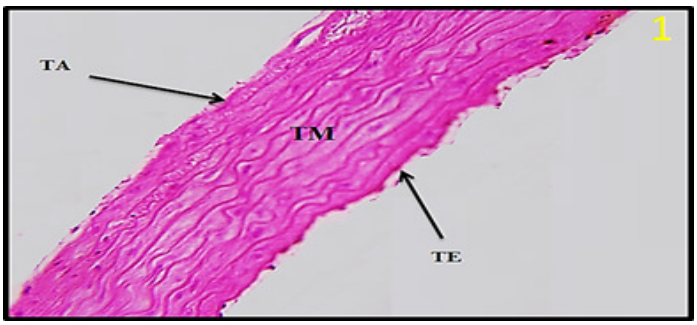
He has indicated (Prentice, 2017) that Hepcidin acts as the main regulator of iron balance by destroying Ferroportin, the cellular iron source that transports iron from cellular iron stores such as Enterocytes, Macrophages, and Hepatocytes to the blood, leading to a decrease in the circulating iron concentration. Iron balance is mainly regulated through the Hepcidin-Ferroportin axis, As it senses Hepcidin which is secreted by the liver systemic iron level and iron absorption is regulated by intestinal cells and release of iron from hepatocytes and macrophage cells by binding with Ferroportin (Ganz and Nemeth, 2011). Hepcidin stimulates the degradation of ferroportin by endocytosis, which reflects Hepcidin's ability to reduce iron absorption and recycling mechanisms (Ganz, 2012). It might help eat foods potentially to be heart-friendly, like olive oil to the diet to control these risk factors, and in reducing cytokines and inflammatory signs. Studies in vitro and in vivo indicate that phenolic compounds, such as HXT, Tyrosol, and Secoiridoid derivatives, may reduce the expression of adhesion molecules. And the consequences of that from immune cell migration, Thus, reducing levels of inflammatory signs in the blood, The daily consumption of olive oil modifies the cytokines and inflammatory markers related to cardiovascular diseases in individuals exposed to them (Souza *et al*., 2017). About the role of HXT in reducing the concentration of hepcidin due to its anti-inflammatory role, it was established that HXT reduced the expression of TNF-α and IL-6 in the liver, and increased expression of anti-inflammatory IL-10 (Cao *et al*., 2014; Carito *et al*., 2015). As for the role of the local olive oil extract + HXT in reducing the concentration of hepcidin due to its content of phenolic acids and their derivatives, phenolic alcohol, Secoiridoids, Lignans, and Flavones (Carluccio *et al*., 2015). As for the role of the ATOR drug, it is consistent with the results obtained (Masajtis-Zagajewska and Nowicki, 2018), as they confirmed a decrease in the concentration of hepcidin and IL-6 after patients were given the ATOR drug. As serum hepcidin is increased in inflammatory cases, this leads to decreased iron release from macrophages (Swinkels and Wetzels, 2008).

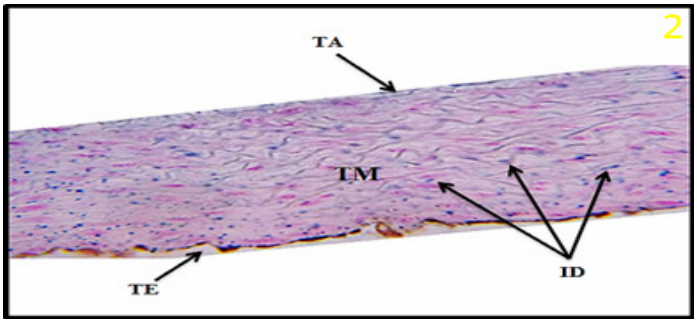
Histological Study of the Aorta:

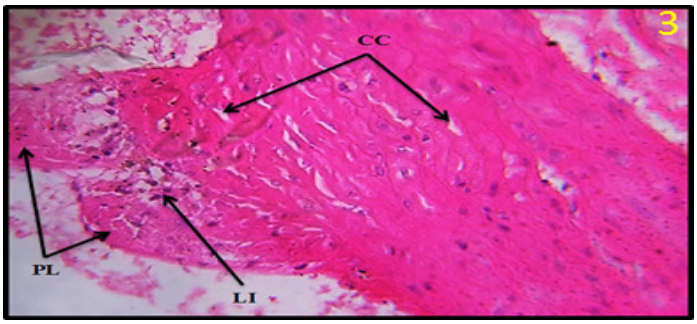
The results of the current study of the healthy control group showed the natural shape of the layers of the aortic artery, noting the Tunica Endothelium (TE), the Tunica Media (TM), the Tunica Adventitia (TA), and the Iron Deposition (ID) ratio. Trace rare and natural thickness in the wall layers as in Figure 1 and 2. Whereas in the group fed to the HLD many histological changes included Cholesterol Crystals (CC) deposition and plaques (PL) plaques formed with a high percentage within the endothelial and low Lymphocytes Infiltration (LI) and iron deposition (ID) A high percentage of the wall layers with an increase in the wall thickness of the aorta, as shown in Figure 3 and 4.

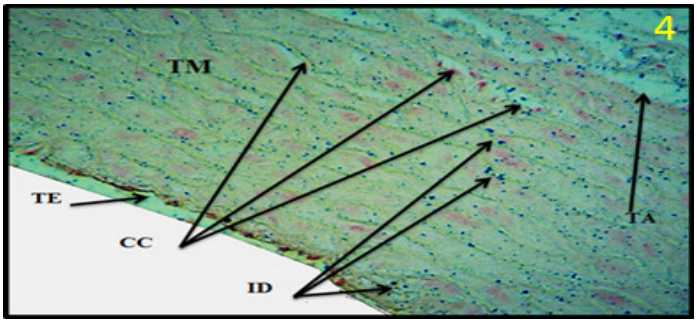
As for the group fed to the HLD and treated with olive oil extract, an improvement in aortic layers was observed. However, cases of cholesterol crystals (CC) continued at an average rate in the middle tunica and endothelium and iron deposition (ID) at a high rate with a decrease in the thickness of the aorta wall compared to the HLD group as in Figure (5, 6). The microscopic examination of the aortic sections of the group that were fed the HLD diet and their treatment with HXT showed a significant improvement in the artery layers, but cases continued deposition of the Cholesterol Crystals (CC) at an average rate, noting a decrease in the iron deposition (ID) to an average ratio and a decrease in the thickness of the wall of the aorta compared to the HLD group as well as the group of olive oil as shown in Figure 7, 8.

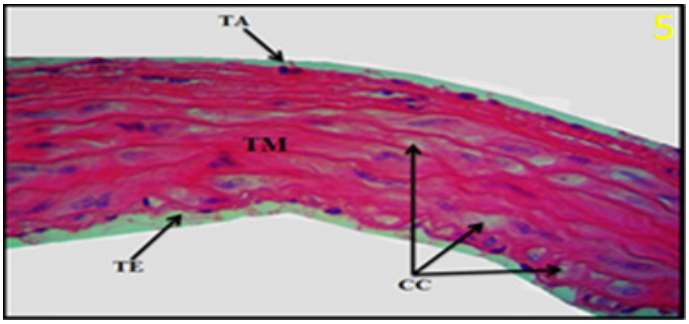
The histological examination of the aorta sections in the group fed on the HLD diet and its treatment with LOO extract + HXT showed a very significant improvement compared to all treated groups, as the deposition of Cholesterol Crystals (CC) and the Iron Deposition (ID) decreased to a low rate with no note plaques and Lymphocytes Infiltration (LI) with a decrease in wall thickness of the aorta compared to all groups as in the Figure (9, 10). In the group fed on the HLD diet and treatment with ATOR showed a significant improvement compared to the HLD group, but the deposition of Cholesterol Crystals (CC) continued by a high rate while the Iron Deposition (ID) decreased to a low percentage and not noticed the formation of plaques (PL) and Lymphocytes Infiltration (LI) with a significant decrease in aorta wall thickness compared to the HLD group as shown in Figure (11,12).

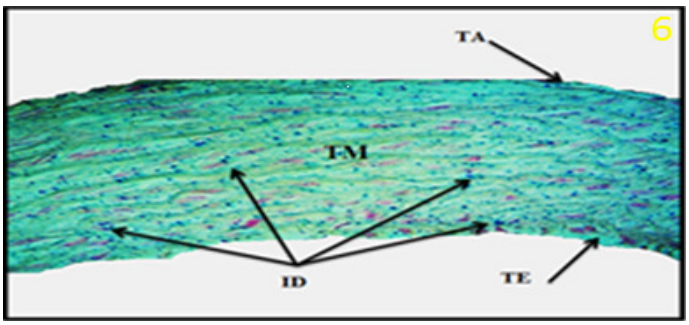


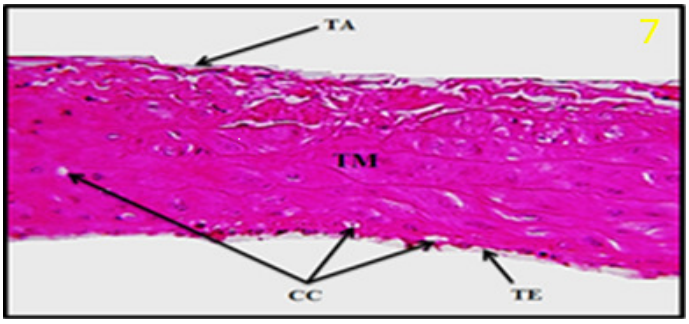


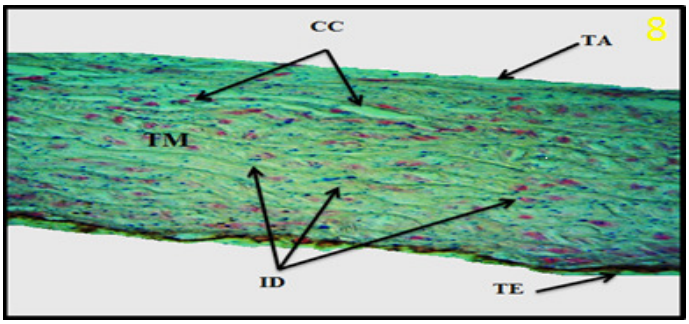


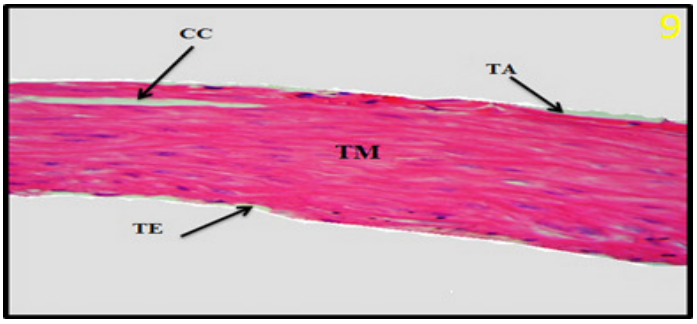


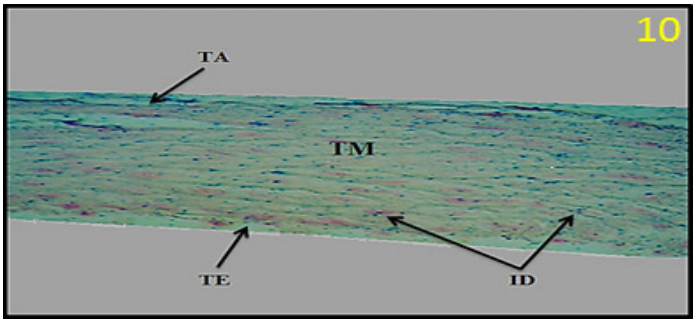


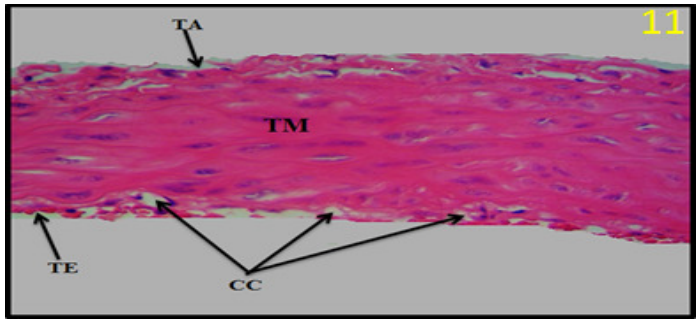












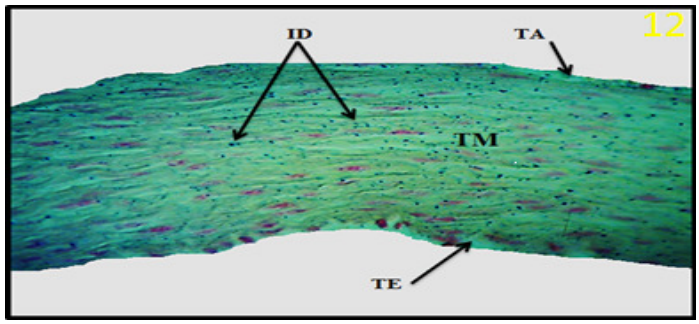


Figure (1) and (2) cross-section of the aorta of the control group showing the tunica endothelial (TE) and the tunica medial (TM) and the tunica adventitia (TA) with the iron deposition regions (ID). Figure (3) and (4) section of the aorta of the group treated with HLD shows deposition of cholesterol crystals (CC), Lymphocytes Infiltration (LI), and formation of plaques(PL) within tunica Endothelium (TE) with iron deposition (ID). Figure (5) and (6) cross-section of the aorta of the group treated with HLD and olive oil showing the deposition of the cholesterol Crystals (CC) in the middle and endothelial tunica with iron deposition (ID). Figure (7) and (8) cross-section of the aorta of the group treated with HLD and HXT showing the deposition of Cholesterol Crystals (CC) with iron deposition (ID). Figure (9) and (10) cross-section of the aorta of the group treated with HLD and olive oil extract + HXT showing the deposition of Cholesterol Crystals (CC) within the tunica (TA) with iron deposition (ID). Figure (11) and (12) cross-section of the aorta of the group treated with HLD and ATOR drug shows the deposition of Cholesterol Crystals (CC) in the Tunica Endothelium (TE) with the iron deposition (ID). The shapes are stained with H & E 400X and PBIS 400X, respectively.

The histological results of the current study showed that giving it an HLD diet led to the development of stiffness lesions within the aorta compared to the control group. The results of the current study were in agreement with a study (Al-Obaidy, 2018) that showed that rabbits treated cholesterol led to many histological changes in endothelial cells with deposition of cholesterol crystals and an increase in the thickness of the aorta's wall than in control. The reason for the emergence of histological changes with the increase in the thickness of the aorta wall mentioned in the current study is due to the role of the HLD diet with defective events in the concentration of lipid parameters compared to the control group. Concerning the high level of iron deposition, our results are in agreement with a study (Sun *et al.*, 2018) when they observed that the treatment of mice with cholesterol led to an increase in the concentration of hepcidin in the blood serum and the concentration of iron in the aorta extract compared with the control group, as they indicated that iron deposition is the main entry point to oxidative stress**.** Vascular endothelial dysfunction is one of the primary

events of cardiovascular disease and plays a crucial role in causing atherosclerosis as the HLD stimulates vascular endothelial dysfunction and oxidative stress which may lead to endothelial damage and atherosclerosis (Vanhoutte *et al*., 2017; Lv *et al*., 2017).

As for the role of local olive oil extract, our results agreed with (AL-Azawiy, 2018), as it confirmed that the rabbit gavage with olive oil reduced the effect of alloxan in causing damage to blood vessels, including the aorta, as olive oil is known to have high effectiveness as an antioxidant and a great benefit because it works to reduce heart disease due to the presence of Phenolic Anti-oxidants, amounts of Vitamin E and K, and an abundance of fatty acids in addition to monounsaturated and saturated fats (Brzosko *et al*., 2002). As for HXT, it returns to its normalizing role lipid profile disorders Caused by the HLD diet. HXT can inhibit the initiation and spread of lipid peroxidation and significantly delay the oxidation time of the LDL for a long time that may help suppress atherosclerosis. By scavenging free radicals, and that inhibiting the formation of foam cells resulting from LDL- oxidized and lowering the level of TC, TG, and LDL, by natural compounds, will delay the development of the atherosclerotic lesion (Fki *et al*., 2015). Concerning the role of local olive oil extract + HXT compared to all treatment groups in the current study, it is due to the synergistic role of these compounds and the increase in the concentration of polyphenols that give health benefit against cholesterol accumulation and thus delay the formation of pathogens of atherosclerosis and considered Polyphenols are antioxidants strong inhibitors to the oxidation of LDL which are a major mechanism in the development of atherosclerosis (Aviram *et al*., 2000). As for the role of ATOR in protecting the aorta against the HLD, a significant improvement is observed in the thickness of the inner layer of the aorta with a decrease in the accumulation of fat and macrophages compared to the control group infected when gavage with ATOR to treat hypercholesteremia caused by the use of cholesterol in rats.

We conclude from the current study that LOO and HXT have a very important effect in normalizing the values of lipid parameters and improving the parameters of histological changes, and this may come through the effective components of olive oil and HXT in enhancing the anti-oxidant system inside the body and reducing the formation and spread of free radicals responsible for many diseases, especially cardiovascular diseases.

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