Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Potential anti-dyslipidemia and hepatoprotection of functional food components represented by tetracosanol and mixture of policosanol in Triton X-100 induced dyslipidemic rats

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Abstract

The elevated plasma triglycerides (TGs), total cholesterol (TC) and low density lipoprotein-cholesterol (LDL-C) with concomitant reduction of high density lipoprotein-cholesterol (HDL-C) are designated as dyslipidemia. The aim of the present research was to study the protective effect of tetracosanol and policosanol mixture towards triton X-100 induced dyslipidemia along with their hepatoprotective effect in rats. The policosanol mixture was prepared from tetracosanol, hexacosanol, octacosanol and heptacosanol in the ratio of 3:1:1:1. Rats were divided into 4 groups; a normal control (NC), dyslipidemic control (DC) and two test groups with dyslipidemia and treated with either tetracosanol or policosanol mixture in the form of emulsions. The determined biochemical parameters were plasma TGs, TC, LDL-C, HDL-C, malondialdehyde (MDA) and the activities of alanine transaminase (ALT) and aspartate transaminase (AST). In addition to calculation of TC/HDL-C and LDL-C/HDL-C as the elevation of such ratios are considered risk factors for cardiovascular diseases (CVDs). Histopathological examination of liver was also carried out. Results showed significant dyslipidemia and elevated LDL-C/HDL-C, TC/HDL-C, MDA, ALT and AST with liver histopatological changes in the DC group compared to the NC group. Both tetracosanol and policosanol mixture reduced the risk factors for CVDs and improved the dyslipidemia, oxidative stress and liver function. Tetracosanol was superior in reducing TC, ALT and AST and improving liver histopathology while policosanol mixture was more efficient in reducing oxidative stress.

Keywords: Triton X-100; dyslipidemia; malondialdehyde; liver histopathology; tetracosanol; policosanol mixture; rats.

Introduction

Dyslipidemia is a term indicating elevated plasma TC, TGs, LDL-C and very low density lipoprotein cholesterol together with reduction of plasma HDL-C. The term dyslipidemia is a better indicator than hyperlipidemia of the aforementioned changes because the lipid disorder includes an increase in some lipid parameters together with a decrease in other. The elevated TC/HDL-C and LDL-C/HDL-C ratios together with high oxidative stress are the most prevalent indicators of the susceptibility to cardiovascular disease which is an increasing medical problem and the principal cause of mortality worldwide [1,2]. Although cholesterol is required for the production of steroid hormones, vitamin D, and bile acids together with the maintenance of cell membranes, however its increase, specially the LDL-C, has a bad prognosis to CVDs [3]. Therefore, protection from CVDs might involve mainly the control of both blood lipid profile and the reduction of oxidative stress. Although many drugs have been developed to treat hyperlipidemia with promising effect, however they have been shown to induce side effects. The world is now turning to bioactive components derived from natural sources which are expected to be free from side effects and easily available. Functional food components or nutraceuticals are example of such natural agents. Nutraceuticals are defined as being bioactive ingredients obtained from food, either from botanical or animal sources, and protect from or treat one or more diseases. Policosanol is among such nutraceuticals of possible health benefits.

Policosanol is a mixture of primary long chain aliphatic alcohols of high molecular weight containing from 22 to 38 carbon atoms. It is present in rice bran, sugar cane, fruits, nuts, wheat germ, bees wax, evening primrose oil and spinach [4-7]. The

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Receive Date: 13 February 2022; Revise Date: 25 February 2022; Accept Date: 28 February 2022.
DOI: 10.21608/EJCHEM.2022.121634.5452.

mixture of aliphatic alcohols in policosanol might differ according to its source. The aliphatic alcohols that could be present in policosanol are tetracosanol, hexacosanol, heptacosanol, octacosanol, nonacosanol, triacontanol, dotriacontanol and tetratriacontanol [8]. Policosanol was reported to have antiproliferative, antiaging, hypocholesterolemic and anti-inflammatory effect and was used in functional foods [9-11]. It can also be used to prevent atherosclerotic complications represented by platelet hyperaggregability, ischemia and thrombosis. Policosanol might also prevent gastric ulcers and improve male sexual activity [8]. However the aforementioned health benefits may differ according to the composition of policosanol.

Tetracosanol, a lignoceryl alcohol, demonstrated antiproliferative and antimutagenic effects [12,13]. It was reported that the carbon chain length and the hydroxyl group in tetracosanol served a critical role for the *in-vivo* glucostasis. Tetracosanol could also improve glycemic control via an effect on insulin receptor kinase activity that leads to glucose transporter translocation and improve glucose uptake. The insulin receptor kinase activity was also ascribed to the hydroxyl group [14]. In a previous work, when analyzing rice bran oil, that demonstrated multiple health benefits, the main aliphatic alcohol in policosanol was tetracosanol, followed by hexacosanol then octacosanol while heptacosanol showed the lowest level [6,15-17]. Therefore, it was hypothesized that health benefits might either be attributed to tetracosanol or to a mixture of tetracosanol, hexacosanol, octacosanol and heptacosanol in which tetracosanol was of the highest content. Consequently the objective of the present research was to study the anti-dyslipidemic effect and the oxidative stress reducing ability together with the hepatoprotective effect of 1-tetracosanol alone and when mixed with a mixture of 1-hexacosanol, 1-octacosanol and 1-heptacosanol in the ratio of 3:1:1:1 in rat model of triton X-100 induced dyslipidemia.

Experimental

Materials

Triton-X 100, used for induction of dyslipidemia, and Tween 80 (polyoxyethylene sorbitan mono-oleate), an emulsifying agent, were purchased from LOBA CEMIE PVT. LTD., laboratory reagents and fine chemicals, India. Policosanol represented by 1-Tetracosanol of 97% purity ($C_{24}H_{50}O$, Mw: 354.65) and 1-hexacosanol of 95% purity ($C_{26}H_{54}O$, Mw: 382.71) were supplemented from International Laboratory (IL), USA. Other policosanols which are 1-octacosanol and 1-heptacosanol of 99% and 98% purity, respectively were obtained from Sigma, USA& Germany (Fig. 1).

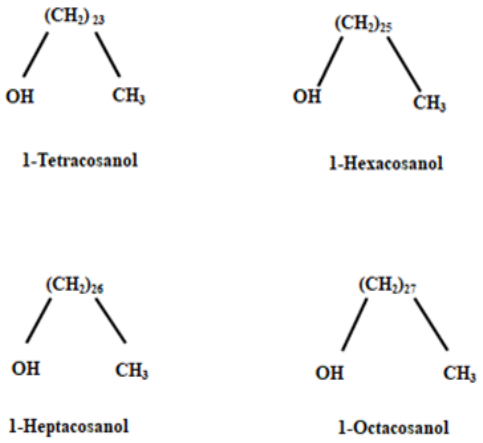

Fig.1 Chemical structure of the studied policosanols

Animals

Male Wistar rats of body weight ranging from 210 to 220g were obtained from the Animal House Unit, National Research Centre, Egypt. Rats were kept in stainless steel cages at ambient temperature, with 12h light/dark cycle. Food and water were supplied *ad-libitum*. Handling and care of animals were carried out according to the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt, and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Preparation of functional food ingredients (nutraceuticals) to be suitable for administeration to rats

Nutraceutical 1 was prepared from tetracosanol in the form of emulsion by adding tween 80 as 10% from tetracosanol weight then diluted with water. Nutraceutical 2 was prepared from policosanol mixture that consisted from tetracosanol, hexacosanol, heptacosanol and octacosanol in the ratio of 3:1:1:1 in the form of emulsion as mentioned in the preparation of tetracosanol. Nutraceuticals 1 and 2 were given to rats in a dose of 10 mg tetracosanol or policosanol mixture/kg rat body weight according to a previous study [18]. A vehicle 1 was prepared consisting from water and tween 80 having the same concentration as in the nutraceuticals to be given to control groups.

Preparation of Triton X-100

Triton X-100 was dissolved in saline (1g triton was completed to 15 ml by saline) and given to rats as 100 mg Triton/ Kg rat body weight according to a previous study [19]. Saline was given intraperitoneal to the normal control rats as vehicle 2.

Composition of the diet

The balanced diet fed to rats throughout the experiment contained 25% protein, 4% fat, 3.71% crude fibers, 1% vitamin mixture, 3.5% minerals′ mixture and 62.79 carbohydrates.

Design of animal experiment

Rats were divided into 4 groups each of eight rats; the first group served as normal control (NC), each rat from the other three groups were treated with one intraperitoneal dose of triton (100mg/kg rat body weight) after an overnight fast from which one group served as dyslipidemic control (DC) the other two groups were the test groups that were given either tetracosanol or policosanol mixture for 15 days prior to triton injection and three days after injection as daily oral dose of 10 mg/kg rat body weight. Rats of the NC and DC groups were given daily oral dose of vehicle 1 (Tween 80 in distilled water). Rats of the NC group were treated with intraperitoneal saline as vehicle 2. All rats were fed on a balanced diet throughout the experiment. Body weights of all rats were measured at the start and the end of the experiment that lasted 18 days. Blood samples were obtained from fasted anesthetized rats, three days after triton injection, and received in heparinized test tubes. Plasma was obtained by blood centrifugation. The determined biochemical parameters were plasma TGs, TC, HDL-C and LDL-C that constitute plasma lipid profile using previously described methods [20-23]. Malondialdehyde (MDA) was assessed as biomarker of lipid peroxidation and oxidative stress adopting the colorimetric method of Satoh [24]. The activities of alanine transaminase (ALT) and aspartate transaminase (AST) were determined as indicator of liver function test by the method described previously by Reitman and Frankel [25]. The ratios of TC/HDL-C and LDL-C/HDL-C were calculated as the elevations of such ratios are considered risk factors for cardiovascular disease. Rats were dissected; livers were separated and kept in 10% formalin for histopathological examination using hematoxylin and eosin [26].

Statistical analysis

Data were expressed as means ± SE. Data were statistically analyzed by one-way ANOVA followed by the Tukey multiple comparison tests using the SPSS statistical program, version 21. Differences were considered significant at $p \leq 0.05$.

Results

Plasma lipids of different experimental groups are shown in table 1. It could be noticed that the DC group showed significant elevation of TG, TC, LDL-C, TC/HDL-C and LDL-C/HDL/C with simultaneous significant reduction of HDL-C compared to the NC group. Policosanol mixture and tetracosanol administrations produced significant reduction in TG, LDL-C, TC/HDL-C and LDL-C/HDL-C compared to DC. Treatment with tetracosanol produced significant reduction in TC while policosanol mixture only induced insignificant reduction compared to DC. Plasma HDL-C demonstrated insignificant increase on administration of either nutraceuticals compared to the DC group. No significant changes in the different lipid parameters were noticed when the group given tetracosanol was compared to that received the policosanol mixture.

Plasma malondialdehyde levels and the activities of ALT and AST of the different experimental groups are demonstrated in table 2. The DC group showed significant increase in MDA with concomitant significant elevation in AST and ALT activities compared to the NC group. Treatment with either policosanol mixture or tetracosanol produced significant reduction in plasma MDA and the activities of ALT and AST compared to the DC group. Plasma MDA showed significant reduction when the group given policosanol mixture was compared with that received tertracosanol while the later produced significant reduction in both ALT and AST activities compared to the group administered policosanol mixture.

Initial body weight, final body weight and body weight gain of different experimental groups were compiled in table 3. It could be observed that triton injection induced significant reduction in final body weight and body weight gain in the DC group and in the groups given either nutraceuticals compared to the NC group.

Figure 2 and table 4 showed the histopathological changes of liver tissue in different experimental groups. The DC group showed severe congestion of blood vessels, severe degeneration and necrosis of hepatocytes, severe vacuolar degeneration and fat deposition in the hepatocytes and severe inflammatory cell infiltration. The group treated with tetracosanol demonstrated mild congestion of blood vessels, moderate vacuolar degeneration and moderate fatty change of hepatocytes and absence of inflammatory cell infiltration. The group given policosanol mixture showed severe congestion of blood vessels, moderate vacuolar degeneration and moderate fatty change of hepatocytes and moderate inflammatory cell infiltration.

Table 1. Plasma lipid profile of different experimental groups

Groups	TG (mg/dL)	TC (mg/dL)	HDL-C (mg/dL)	LDL-C (mg/dL)	LDL-C/ HDL-C	TC/ HDL-C
NC	60.05^{a} ± 3.65	86.41^{a} ± 2.58	52.85^{b} ± 4.3	21.55^{a} ± 4.87	0.408^{a} ±0.14	1.635^{a} ± 0.05
DC	81.39^{b} ± 7.45	111.63^{c} ± 3.27	22.23^{a} ± 3.78	73.122^{c} ± 5.18	3.289^{b} ±0.60	5.021^{c} ± 0.11
Tetracosanol	60.48^{a} ± 2.95	94.14^{ab} ± 3.08	35.6^{ab} ± 8.54	46.444^{b} ± 6.49	1.305^{c} ±0.30	2.644^{b} ± 0.13
Policosanol mixture	58.38^{a} ± 1.98	103.54^{bc} ± 2.28	38.75^{ab} ± 4.65	53.114^{b} ± 4.66	1.37^{c} ±0.32	2.672^{b} ± 0.09

Value: Mean± SE. Different superscript letters mean significant difference while similar letters mean insignificant differences in the same column. NC: Normal control group, DC: The control given Triton X-100 (Dyslipidemic control), TG: triglycerides, TC: Total cholesterol, HDL-C: High density lipoprotein-cholesterol, LDL-C: Low density lipoprotein-cholesterol.

Table 2. Plasma MDA and the activities of ALT and AST of different experimental groups

Groups	MDA (nmol/ml)	ALT (IU/L)	AST (IU/L)
NC	6.15^{a} ± 0.1	41.18^{a} ± 0.36	54.73^{a} ± 0.28
DC	9.94^{d} ± 0.1	74.67^{d} ± 0.46	79.09^{d} ± 0.22
Tetracosanol	8.5^{c} ± 0.2	65.07^{b} ± 0.54	75.13^{b} ± 0.21
Policosanol mixture	7.4^{b} ± 0.1	67.92^{c} ± 0.44	77.6^{c} ± 0.29

Value: Mean± SE. Different superscript letters mean significant difference while similar letters mean insignificant differences in the same column. NC: Normal control group, DC: The control given Triton X-100 (Dyslipidemic control), MDA: Malondialdehyde, ALT: Alanine transaminase, AST: Aspartate transaminase

Table 3. Initial body weight, final body weight and body weight gain of different experimental groups (Mean±SE)

Groups	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
NC	214.0^{a} ± 7.7	288.0^{b} ± 25.02	74.0^{b} ± 17.7
DC	213.6^{a} ± 3.2	245.3^{a} ± 7.70	31.7^{a} ± 5.01
Tetracosanol	213.9^{a} ± 4.2	224.3^{a} ± 8.70	10.4^{a} ± 5.1
Policosanol mixture	214.4^{a} ± 5.5	242.4^{a} ± 11.80	28.0^{a} ± 6.5

Value: Mean± SE. Different superscript letters mean significant difference while similar letters mean insignificant differences in the same column. NC: Normal control group, DC: The control given Triton X-100 (Dyslipidemic control)

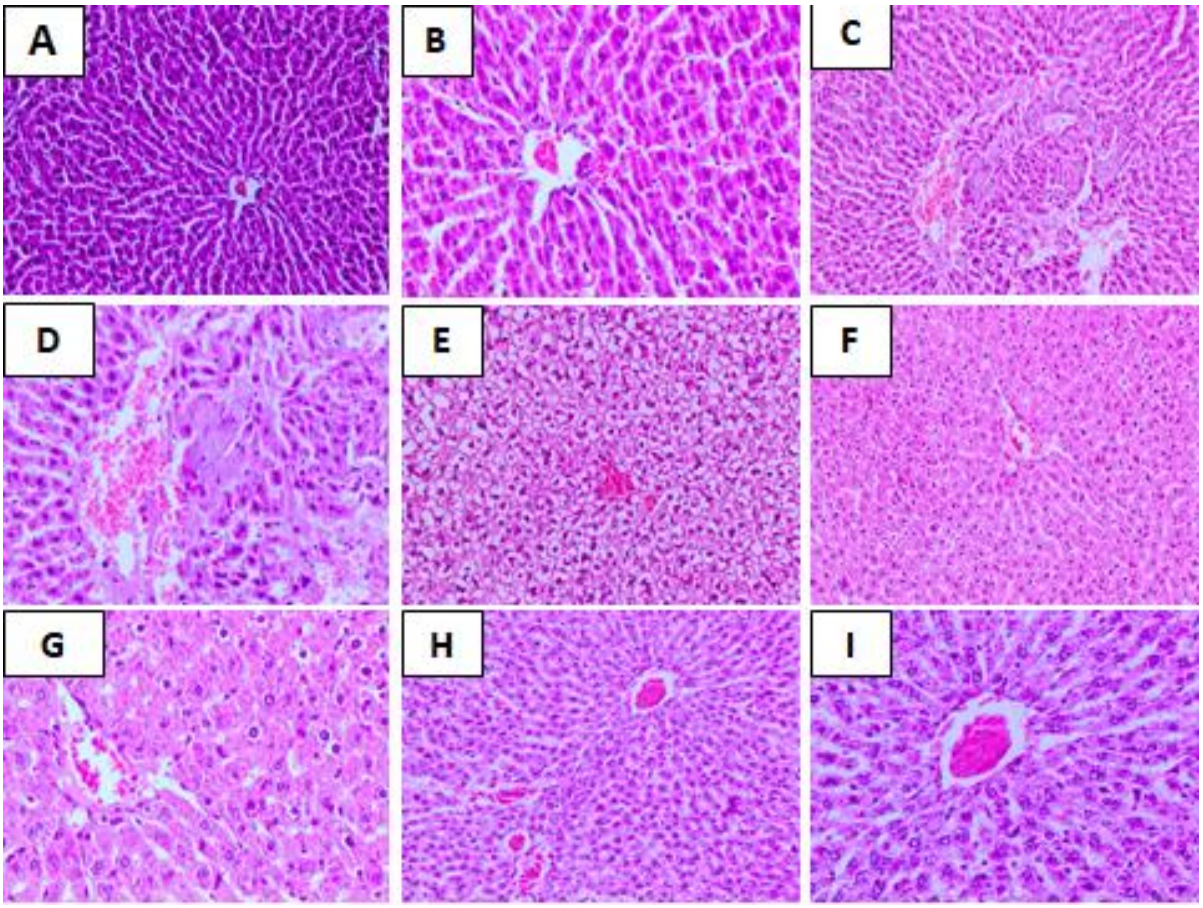

Fig. 2. Histopathology of liver of the different experimental groups using hematoxylin and eosin

A& B: Liver of the NC group (X 100 and X 200, respectively) demonstrated normal appearance, C& D: Liver of the DC group (X 100 and X200, respectively) showed severe congestion of blood vessels, severe degeneration and necrosis of hepatocytes and severe inflammatory cell infilteration, E: Liver of the DC group (X 100) exhibited severe congestion of blood vessels, severe vacuolar degeneration and fatty change of hepatocytes, F& G: Liver of the group treated with tetracosanol (X100 and X200, respectively) demonstrated mild congestion of blood vessels, moderate vacuolar degeneration and moderate fatty change of hepatocytes, H& I: Liver of the group treated with policosanol mixture showed severe congestion of blood vessels, moderate vacuolar degeneration and moderate fatty change of hepatocytes.

NC: Normal control, DC: Dyslipidemic control.

Table 4. Liver histopathological changes in different groups

	NC	DC	Tetracosanol	Policosanol mixture
Degeneration	-	+++	+	+
Vacuolar degeneration	-	+++	+	++
Fatty change	-	+++	+	++
Necrosis	-	+++	+	+
Inflammatory cells infiltration	-	+++	-	++
Congestion	-	+++	++	+++

-: Within normal limits, +: Mild change, ++: Moderate changes, +++: Severe changes.

NC: Normal control, DC: Dyslipidemic control.

Discussion

The disturbance occurring in lipid metabolism leading to dyslipidemia is the main cause of CVDs. Many hypolipidemic drugs exist but they are not fully effective and not absolutely safe. Therefore, it is worthy to find out new effective and safe agents for management of dyslipidemia with consequent reduction of the incidence of CVDs. Functional food components, nutraceuticals, which mainly derived from food, might represent this option due to their expected high safety.

Triton X-100 has been widely used to induce dyslipidemia in experimental animals that mimic human dyslipidemia. The proposed mechanism of action of Triton is blocking the clearance of triglycerides-rich lipoproteins [27]. Triton induced significant increase in plasma TC and TGs as could be seen from the present study which might be due to increased secretion of very low density lipoprotein-cholesterol (VLDL-C) by the liver [28]. Other proposed mechanism of the elevation of blood cholesterol in triton model is the increased hepatic synthesis of cholesterol [29]. Both policosanol mixture and tetracosanol were tested as nutraceuticals in the present study to screen their protective roles towards dyslipidemia in Triton X-100 induced dyslipidemia in rats. In the current study, tetracosanol but not policosanol mixture demonstrated significant reduction in TC while both produced significant reduction in TGs levels. Triton injection produced significant increase in plasma LDL-C which could lead to atherosclerosis through stimulation of plaque formation by oxidized LDL-C induced macrophage foam cells. Generally, it is worthy to mention that the increase in LDL-C might result from consumption of high fat, high sucrose or high fructose diets, lack of physical activity, obesity, diabetes, aging and smoking. Both treatments with either tetracosanol or policosanol mixture were associated with significant reduction of cholesterol in LDL fraction which is considered as a target of numerous hypolipidemic drugs. The LDL-C lowering activity of the tested nutraceuticals might be attributed to the rapid catabolism of LDL-C through liver to be eliminated as bile acids.

The present results showed that Triton induced significant reduction in HDL-C which is a risk factor for developing atherosclerosis, meanwhile treatment by the nutraceuticals prevent such reduction but insignificantly. It is worthy to mention that HDL accelerates the transfer of cholesterol from peripheral tissue like arterial walls to the liver for catabolism. Therefore, atherosclerosis might be inhibited by the elevation in HDL-C a cardio protective lipid. Elevated HDL-C might be attributed to an increase in the activity of lecithin cholesterol acyltransferase that promote the incorporation of free cholesterol into HDL with subsequent back transference to VLDL that taken back by hepatic cell to be catabolized. Hence, the significant reductions in the ratios TC/HDL-C and LDL-C/HDL-C as in the groups treated by both nutraceuticals are good indicators of the reduction of CVDs incidence. Reduction in TC and its LDL fraction, as could be seen from the present results, might be due to increased cholesterol excretion and decreased intestinal cholesterol absorption. It has been shown previously that policosanol could inhibit 3-hydroxy-3- methylglutaryl-CoA (HMG-CoA) reductase activity thereby reduce cholesterol synthesis, this action resulted in reduction of blood cholesterol [10]. The prevention of dyslipidemia on administration of the policosanol mixture and tetracosanol strongly suggest that the activity of such nutraceuticals might be attributed to structure activity relationship of policosanol which resides in its long chain carbon atoms and the terminal hydroxyl group. The linking of the long chain alcohol to fatty acids might enhance beta oxidation of fatty acids in the liver resulting in hepatic fat catabolism. It has been also reported that policosanol administration inhibited the factors of endothelial cell activation represented by P-selectin and circulating vascular cell adhesion molecule-1(sVCAM-1), thereby reduced atherosclerotic wall thickness and was suggested to have protective role against atherosclerotic regions during hypercholesterolemia [30].

It is to be noted that liver synthesizes bile acids through catabolism of cholesterol, thereby eliminates excess cholesterol but the bile acids reabsorbed again from the intestine and inhibit bile acid synthesis and cholesterol catabolism. Blocking of bile acid reabsorption and promotion of bile acid excretion certainly lead to removal of cholesterol [31,32]. It was reported that policosanol accelerated fecal output of cholesterol and bile acids. Therefore, policosanol might stimulate hepatic cholesterol catabolism through conversion of cholesterol into bile acids in hepatic cell causing suppression of cholesterol synthesis by increasing cellular adenosine monophosphate (AMP) levels that produce phosphorylation of AMP-activated protein kinase and inhibition of HMG-CoA reductase [10]. These actions might trigger the uptake of circulating LDL-C into the cells [10], thereby reducing blood LDL-C. It was suggested that policosanol supplementation induces antihypercholesterolemic effect by inhibiting cholesterol biosynthesis, promoting LDL-C uptake and accelerating cholesterol excretion [18]. In this concern, the present study showed tetracosanol, as one of the policosanol compounds, produced significant reduction in plasma TC while policosanol

mixture demonstrated only insignificant reduction meanwhile both treatments induced significant reduction of LDL-C

Elevated Lipid peroxidation as indicated by high MDA on treatment with Triton in the present study could point to increased oxidative stress. An elevated oxidative stress is commonly associated with hypercholesterolemia [33]. As cholesterol is an important structure component of cell membrane, hypercholesterolemia could lead to increase in cholesterol pool producing altered physical activity of cell membrane that might induce leakage of the reactive oxygen species with consequent induction of lipid peroxidation [34, 35]. Elevated lipid peroxidation is believed to be a consequence of dynamic imbalance between antioxidant and oxidant which is a reflection of oxidative stress. Therefore MDA is a marker of lipid peroxidation and is used as a measure of oxidative stress [36,37]. The study of Diniz et al. [38] supported the aforementioned work emphasizing that hypercholesterolemia increases oxidative stress by not only increasing lipid peroxidation but also by increasing production of superoxide. Hence, oxidative stress is defined as dysregulation between reactive oxygen species and the endogenous antioxidant defense mechanism resulting in multiple pathophysiological pathways.

Reactive oxygen species alter intracellular lipids through lipid peroxidation, they also cause protein modification inducing enzymatic alteration [39] as could be seen from liver degeneration from the histopathological examination of the liver of the DC group in the present results that reflected in liver dysfunction manifested by elevation of AST and ALT activities. Liver fat deposition in DC as demonstrated from liver histopathology might also participate in elevating transaminases in DC. Histopathology of liver of the DC group might reflect an induction of steatohepatitis which is a fatty liver with inflammation and hepatocellular degeneration that agreed partially with the work of Gundamaraju et al. [19]. Previously, experimental steatohepatitis was reported to be associated with dyslipidemia and CVDs risks [40].The aforementioned explanation reflecting the status in the DC group that treated by Triton in the present study. The reduced MDA (from biochemical analyses), and the inhibition of liver fat and hepatic cell degeneration (from liver histopathology) on administration of either tetracosanol or policosanol mixture might elucidate the reduced transaminases and the anti-dyslipidemic effect in such groups. Inhibition of hepatic fat accumulation by the studied nutraceuticals might be mediated by inhibiting fat synthesis or elevating beta-oxidation of fatty acids or both. The inhibition of oxidative stress by the present studied nutraceuticals might lead to anti-inflammatory activity that might participate in ameliorating the pathological changes induced by Triton. The antioxidant activity of policosanols might be ascribed to their long carbon chain and they may have an effective therapeutic effect in chronic inflammatory and oxidative stress related diseases as reported previously [41].

Triton injection induced reduction in body weight gain in the DC group compared to the NC group that might reflect a pathological condition due to the disturbed overall picture of the biochemical and histopathological changes in this group. Although the reductions in body weights on treatment with nutraceuticals were insignificant compared to Triton control (DC), however such reduction which was more prominent in case of tetracosanol might be useful in obese subjects that commonly suffer from dyslipidemia and fatty liver, this assumption might be based on the improved biochemical parameters and liver histopathology in such groups.

The superiority of 1-tetracosanol over policosanol mixture in reducing TC, ALT, AST and liver histopathological changes might be linked to the carbon chain length of 1-tetracosanol which is less than that of 1-hexacosanol 1-octacosanol and 1-heptacosanol which is the only variable. Although 1-tetracosanol was present in the policosanol mixture, however its presence was only as 50% of the mixture. The efficiency of policosanol mixture in reducing MDA compared to 1-tetracosanol might be based on the same aforementioned explanation.

Conclusion

Policosanol especially tetracosanol have a protective effect on Triton X-100-induced dyslipidemia in rats. The present results provide strong evidence supporting the beneficial health benefits of policosanol on dyslipidemia and as hepatoprotective. The anti-dyslipidemic activity of policosanol mixture and tetracosanol against Triton X-100 reflected in a significant decrease in TGs and LDL-C, and insignificant increase in HDL-C, while tetracosanol in addition showed significant reduction in TC. Policosanol mixture and tetracosanol produced reduction in oxidative stress and improvement of liver function. Tetracosanol was superior in reducing ALT and AST activities along with improving liver histopathology while policosanol mixture was more efficient in reducing oxidative stress. Also, reductions in TC/HDL-C and LDL-C/HDL-C ratios emphasize the cardiovascular protective effect of policosanol mixture and tetracosanol. Yet further researches are necessary to thoroughly investigate the underlying mechanism of action.

Funding

This research did not receive any fund

Competing interests

No competing interests exist

Acknowledgement

This work was implemented in Nutrition and Food Sciences Department, National Research Centre, Egypt.

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