ABSTRACT
Abnormal lipid metabolism is a main cause of dyslipidaemia, which is a major risk factor for cardiovascular disease, obesity, cholestasis, and overall mortality. The aim of the present study is to evaluate the previously prepared functional milk beverage fortified with kiwi pulp and sesame oil as hypolipidemic and antioxidant agent in hypercholesterolemic rats. The evaluation was down through measuring total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), aspartate and alanine aminotransferases (AST & ALT), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT) and total protein content. The evaluation was extended to observe the histopathological changes in rats liver after treatment. The results revealed that treatment with the milk beverage significantly (p<0.0001) decreased TC, LDL-C, TG, SOD, MDA, AST, ALT, ALP and GGT levels, while GSH, HDL-C and total protein contents were significantly increased. The noticeable improvement in the liver architecture confirmed our results. In conclusion, the functional milk beverage is considered to be a hypolipidemic and antioxidant agent due to its enrichment with phenolic compounds, vitamin C and E. Further studies are needed to explore its role for human health.
KEYWORDS: Functional milk beverage; hypercholesterolemia; antioxidants; liver histopathology.

1. INTRODUCTION
Milk beverage is an attractive, nutritious, healthy and relatively low-cost thirst satisfying drink consumed by all types of people. Functional beverages containing fruit and milk not only intended to satisfy hunger, deliver humans with required nutrients but also have a role in health promotion and disease prevention.\(^1\) These health benefits are owing to antioxidant compounds present in fruit as well as in milk and reduced the risk of many diseases. Some of which are cardiovascular diseases, cancer, Diabetes mellitus, Alzheimer, cataracts and age-related functional decline.\(^2\)

Hypercholesterolemia is a serious problem faced by many countries. It is a major concern for health professionals, because it is one of the primary risk factors for the development and growth of cardiovascular diseases such as atherosclerosis, hypertension and cerebral infarctions.\(^3\) Diet related chronic diseases such as type 2 diabetes, cardiovascular diseases, several types of cancer, and increased morbidity and mortality can be attributed to obesity and overweight.\(^4\) In general, obesity occurs due to an imbalance between energy intake and energy output. An imbalance in this ratio results in accumulation of fat inside adipose tissues, elevation low density lipoproteins inside blood and other fat-accumulation organs\(^5\) and elevation of reactive oxygen species (ROS). The body has a complex defense to minimize the damaging effect of various oxidants; central of this defense are the antioxidant enzymes.\(^6\)

Although synthetic drugs provide a quick relief and show rapid effects for mitigating lipids anomalies, their serious side effects cannot be ignored. Therefore, increasing attention is paid to utilizing natural products for alleviation of these chronic diseases.\(^4\)

The aim of this work was to evaluate the functional milk beverage fortified with kiwi pulp and sesame oil as a hypolipidemic, antioxidant and hepatoprotective agent in high fat diet induced hypercholesterolemia in rats.

2. MATERIALS AND METHODS
2.1. Materials
Fresh skim milk (protein 3.5%, fat 0.5% and T.S. 11.2%) was obtained from the Faculty of Agriculture Cairo Univ. Giza, Egypt. High quality Kiwi fruits were obtained from local
market. Sesame oil was obtained from the unit of pressing and extracting natural oil, National Research Center, El-Dokki, Egypt. Emulsifier mono and diglyceride 60% was obtained from Misr for Food Additives (MISAD), Giza, Egypt. Pectin was obtained from Sisco Research Laboratories (SRL) Mumbai, India. Sodium ascorbate was obtained from El Nasr Pharmaceutical Chemicals Co. (Cairo, Egypt). All chemicals used for biological study were of high analytical grade, product of Sigma-USA, Aldrich Chemie-Germany and Biomedicals-France.

2.2. Methods

2.2.1. Preparation of Kiwi Pulp
Kiwi fruits were washed with water to remove any external dirt’s. Both ends and the skin of each fruit were removed, cut to pieces and then homogenized in a blender, the resultant pulp was packaged in polyethylene bags and stored at -18°C until used.

2.2.2. Preparation of milk beverage
Fresh skimmed milk was heated to 45°C mixed with the stabilizer-emulsifier mixture (pectin 0.1% and monoglyceride 0.1% w/w), and then blended. Sesame oil was added at the levels of 0.5, 1.5 and 3% w/w, respectively to the fresh skimmed milk and then homogenized using high speed blander. Twenty percent of the Kiwi pulp was blended in filled milk containing sodium ascorbate (0.1%) to avoid discoloration before thermal processing. Sucrose (10% w/w) was added to the prepared flavored milk mixed well, the mixture was mixed again, heated up to 60-70°C, homogenized using high speed blender at 18,000/min. for 5 min. The prepared beverage was pasteurized at 72°C for 15 Sec. and immediately cooled down to 10°C, filled into dark glass bottles, and stored at 5°C until analyzed.

2.3. Biological study

2.3.1. Animals &Ethics
Male Wistar albino rats (120: 140g) were selected for this study. They were obtained from the animal house, National Research Center, Egypt. All animals were kept in a control environment of air and temp with access of water and diet ad libitum. Anesthetic procedures and handling with animals were complied with the ethical guidelines of Medical Ethical Committee of the National Research Centre in Egypt and performed for being sure that the animals do not suffer at any stage of the experiment.
2.3.2. Animal feeding
Control groups were fed with standard diet contained 16.1% protein, 13.83% fibers, 11.38% water, 8.12% ashes, 47.00% carbohydrate and 3.4% fats (El- Kahira Co. for Oil and Soap). Hypercholesterolemic groups were fed with standard diet containing 150g lard/kg diet. The modified diet was taken along with oral administration of cholesterol to get a condition of high fat and cholesterol level and to ensure triglycerides elevation.

2.3.3. Administration regimen
Administration regimens were five times per week for nine consecutive weeks. The milk beverage dose was selected according to our previous work by El-Sayed et al. who mentioned that the most effective concentration was 1.50g of sesame oil/100 ml. If we will consider this dose to be given to human (60kg b.wt.) in our future work, then the selected dose for rats is 0.025g/kg body weight. Cholesterol was orally given at a dose 30 mg/animal. Lipanthyl drug (Mina Pharm., Egypt) was orally given at a dose 50 mg/kg body weight. The dose of lipanthyl drug was calibrated to exactly contain 50mg of fenofibrate/kg body weight.

2.3.4. Experimental design
Forty male rats were divided into five groups (eight rats each) as follows: Group 1: normal healthy control rats. Group 2: normal healthy rats orally administered with the milk beverage. Group3: hypercholesterolemic rats. Group 4: rats forced at the same time and for the same duration with cholesterol and milk beverage. Group 5: rats forced with cholesterol and lipanthyl drug.

2.3.5. Sample preparation
Blood was collected from each animal by puncture of the sub-lingual vein, left for 10 min to clot and centrifuged at 3000 rpm for serum separation The separated serum was stored at -80°C for further determinations of liver function enzymes (AST, ALST, ALP, GGT), lipid profile (TC, HDL-C, LDL-C, TG) and serum total protein content.

Liver tissue was homogenized in normal physiological saline solution (0.9% NaCl) (1:5 w/v). The homogenate was centrifuged at 4°C for 5 min at 3000 rpm and the supernatant was stored at -80°C for further estimation of hepatic oxidative stress markers; glutathione (GSH), malondialdehyde (MDA) and superoxide dismutase (SOD).
2.4. Biochemical determinations
Total cholesterol (TC) was determined by the method of Meiattini et al. [14], HDL-C by Bustein et al. [15], LDL-C assay method by Assmann et al. [16] and triglycerides by Fossati and Prencipe. Malondialdehyde was assayed according to the method of Buege and Aust, glutathione (GSH) by Moron et al. [19], total superoxide dismutase by Nishikimi et al. [20], aspartate and alanine aminotransferases by the method of Gella et al. [21], alkaline phosphatase by the method of Rosalki et al. [22], GGT was estimated by the method of Szasz [23] and total protein was assayed by the method of Bradford [24].

2.5. Histopathological study
Representative slices of liver tissues were taken from the eviscerated animals and fixed in buffer formalin (10%). After fixation, the paraffin-embedded sections in 4 µm thickness were stained by haematoxylin and eosin (H&E) and Masson's trichrome.

2.6. Statistical analysis
All data were expressed as mean ± SD of eight rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program accompanied with least significance difference between groups (LSD) at $P < 0.05$.

3. RESULTS
With respect to the lipid profile in normal control rats, a decrease in TC (10.61%), LDL-C (19.24%) and TG (14.53%) levels were observed, while HDL-C recorded significant increase by 12.32%. These observations revealed hypolipidemic effect of the milk beverage used.

Hypercholesterolemic rats recorded significant elevation in TC (42.53%), LDL-C (80.83%) and TG (77.77%) levels, while HDL-C showed significant decrease by 35.68% as compared with the normal control group. Treatment of hypercholesterolemic rats with the milk beverage significantly reduced TC, LDL-C and TG by 18.14, 37.36 and 29.25%, respectively, while HDL-C recorded significant increase by 47.91% as compared with the hyperlipidemic group. Lipanthyl drug attenuated the TC, LDL-C and TG level by 22.86, 23.24 and 32.74%, respectively, while it increased HDL-C by 32.67% as compared with the hyperlipidemic rats. Hence, we noticed that treatment with the milk beverage improved TC, HDL-C, LDL-C and TG levels by 25.86, 30.72, 67.41 and 52.00%, respectively. Lipanthyl drug improved the TC, HDL-C, LDL-C and TG by 32.59, 21.41, 41.93 and 58.21%, respectively (Fig. 1).
Fig. 1: Effect of treatment with milk beverage on lipid profile in hypercholesterolemic rats.

- Data are mean ± SD of eight rats in each group.
- Values are expressed as mg/dL.
- Statistical analysis are done using one way analysis of variance (ANOVA) using Co Stat Computer program accompanied with least significance level (LSD) between groups at $p<0.05$.
- Letters are significant values between groups at $p<0.0001$.

Regarding to the liver function indices in normal control rats, insignificant changes were observed in AST, ALT, ALP and GGT enzyme activities revealed extract safety. Hypercholesterolemic rats revealed significant increase in AST, ALT, ALP and GGT levels by 36.93, 96.07, 114.18 and 91.90%, respectively as compared with the control group. Hypercholesterolemic rats treated with the milk beverage recorded reduction in AST, ALT, ALP and GGT levels by 22.69, 39.00, 40.58 and 59.18%, respectively. Lipanthyl treatment attenuated the level of AST, ALP and GGT by 13.15, 20.50, 43.76 and 42.79%, respectively as compared with the hypercholesterolemic group. We noticed that treatment with the milk beverage improved the AST, ALT, ALP and GGT by 136.26, 76.47, 86.93 and 71.35%,
respectively, while lipanthyl drug improved the liver function indices by 18.01, 40.19, 93.74 and 82.12%, respectively (Fig. 2).

Fig. 2: Effect of treatment with milk beverage on liver function indices in hypercholesterolemic rats.

- Data are mean ± SD of eight rats in each group.
- Values are expressed as U/L.
- Statistical analysis are done using one way analysis of variance (ANOVA) using Co Stat Computer program accompanied with least significance level (LSD) between groups at \( p < 0.05 \).
- Letters are significant values between groups at \( p < 0.0001 \).

With respect to oxidative stress markers in normal rats, the results revealed insignificant changes in GSH, MDA and SOD levels as well as the protein content as compared with the control group. Hypercholesterolemic rats showed significant increase in MDA and SOD by 95.45 and 42.94%, respectively, while GSH and total protein recorded significant decrease by 48.71 and 28.09%, respectively as compared with the control group. Treatment with the milk beverage showed significant decrease in MDA and SOD levels by 34.88 and 22.93%, respectively as compared with the hypercholesterolemic rats, while GSH and total protein
levels showed significant increase by 68.82 and 31.18%, respectively after treatment. Hypercholesterolemic rats treated with lipanthyl recorded significant decrease in MDA and SOD levels by 41.96 and 26.89%, respectively comparing with the hyperlipidemic group, while GSH and total protein content recorded significant increase by 59.16 and 29.74%. Therefore, we noticed that treatment of hypercholesterolemic rats with the milk beverage improved GSH, MDA, SOD and total protein levels by 35.29, 68.18, 32.78 and 22.77%, respectively. Lipanthyl treatment improved GSH, MDA, SOD and total protein by 30.34, 81.81, 38.44 and 21.38%, respectively (Fig.3).

Fig. 3: Effect of treatment with milk beverage on serum total protein content and oxidative stress markers in hypercholesterolemic rats.

- Data are mean ± SD of eight rats in each group.
- Serum protein is expressed as mg/ml, GSH and MDA as µg/mg protein, SOD as µmol/mg protein.
- Statistical analysis are done using one way analysis of variance (ANOVA) using Co Stat Computer program accompanied with least significance level (LSD) between groups at \( p<0.05 \).
- Letters are significant values between groups at \( p<0.0001 \).

Regarding to the histopathological finding of control rats and control rats treated with the milk beverage, the results showed intact lobular hepatic architecture and normal hepatocytes
distribution without any pathological changes (Fig. 4 a-d). This indicated that the milk beverage had no side effect of the hepatic architecture.

Hypercholesterolemic rats liver showed distorted lobular hepatic architecture with moderate hydropic degeneration (black arrows), severe micro and macrosteatotic changes (red arrow), severe interlobular inflammation (yellow arrow) and congested blood vessels (green arrow) (Fig. 4 e, f). Treatment of hypercholesterolemic rats with the milk beverage showed intact lobular hepatic architecture and normal hepatocytes with mild sinusoidal congestion and dilatation (black arrows) (Fig. 4 g, h).

Hypercholesterolemic rats treated with lipanthyl drug showed intact lobular hepatic architecture and normal hepatocytes with fibrotic improvement. Mild sinusoidal and blood vessels congestion and dilatation was also seen (black arrow) (Fig. 4 i, j).

**Fig. 4:** Photomicrograph of hematoxylin and eosin (H&E) and Masson's trichrome stained sections (100x) of normal rat liver (a, b), normal rat liver treated with the milk beverage (c, d), hypercholesterolemic rat (e, f), hypercholesterolemic rat treated with milk beverage (g, h), and hypercholesterolemic rat treated with lipanthyl (i, j).
4. DISCUSSIONS
The risk of accumulation of excessive cholesterol inside the body and cardiovascular diseases is a serious concern for health professionals all over the world in the 21st century. Several studies have indicated that diet treatment or drug therapy to regulate cholesterol can reduce subsequent cardiovascular disease (CVD)-associated mortality and morbidity.

Concerning to the lipid profile in normal rats, normal healthy rats treated with the milk beverage recorded significant decrease in lipid profile parameters except HDL-C that recorded significant increase. On the basis of this observation, the milk beverage used in the present study plays as an anti-atherogenic role through the inhibition of lipids oxidation, elevation of HDL-C (good cholesterol) and reduction in LDL-C (bad cholesterol) that can be deposited in the blood vessel walls and becomes a major component of atherosclerotic plaque lesions.

Hypercholesterolemic rats in the present study showed significant increase in total cholesterol, LDL-C and TG in hypercholesterolemic rats as compared to the control group, while HDL-C level showed significant decrease. These results were in accordance with the results of Awad et al. and Hamed who found alternation in lipid profile after administration of rats with cholesterol or fed with a high fat diet.

A main hepatic antihyperlipidemic mechanism works by suppressing fatty acid synthesis and by activating fatty acid oxidation through regulation of certain genes. Suggesting that the alteration induced by the HFD is partly due to oxidative damage, therefore the antioxidants may have a significant protective effect on the pathways leading to transcription of these genes by scavenging ROSs. Treatment with the milk beverage and lipanthyl recorded improvement in total cholesterol, HDL-C, LDL-C and TG. Adaramoye et al. attributed the attenuated of cholesterol and triglyceride levels to the effect of treatment that may be reduce the hepatic triglyceride biosynthesis and favor the redistribution of cholesterol among the lipoprotein molecules. Afonso et al. added that treatment with phenolic compounds reduced TC and HDL-C levels. They attributed the decrease in cholesterol to a decrease in the micellar solubilization of cholesterol in the digestive tract, to an increase in bile flow, bile cholesterol and bile acid concentration and to a subsequent increase in the fecal excretion of steroids.
The reduction of total cholesterol by the milk beverage was associated with a decrease of its LDL fraction, which is the target of several hypolipidaemic drugs. This result suggests that cholesterol-lowering activity of the milk beverage can be result from the rapid catabolism of LDL-cholesterol through its hepatic receptors for final elimination in the form of bile acids, therefore LDL-cholesterol level may be used for monitoring the treatment of patients with elevated blood cholesterol levels. Treatment also led to improve the level of serum HDL-cholesterol, indicating its promising protective role against CVD. Yokozawa et al. \(^{[34]}\) explained the phenomenon of CVD according to the role of HDL that exerts part of its anti-atherogenic effect by counteracting LDL oxidation or HDL that may promotes the reverse cholesterol transport pathway, by inducing an efflux of excess accumulated cellular cholesterol and prevents the generation of an oxidatively modified LDL. On the basis of this explanation, the milk beverage under investigation may probably plays as an anti-atherogenic role through the inhibition of lipids oxidation as well as the elevation of HDL cholesterol.

Treatments with lipanthyl drug (fenofibrate), decreased triglycerides and HDL levels. This agrees with the mechanism of action of fibrates \(^{[35]}\) which their LDL-cholesterol lowering activity is not strongly marked, but the triglycerides decreasing effect of them is very spectacular especially by both stimulation of the gene expression of lipoprotein lipase leading to enhanced catabolism of VLDL, synthesis of fatty acids and reduced VLDL secretion.

In the present study, treated rats with the milk beverage recorded significant reduction in SOD and MDA, while GSH recorded significant increase. This amelioration of the antioxidant levels after treatment was attributed to the constituents present in the milk beverage that may decrease the levels of lipid peroxidation products by scavenging free radicals. This was confirmed by the previous work of El-Sayed et al. \(^{[11]}\) who postulated the role of this beverage as antioxidant through the \textit{in vitro} inhibition of DPPH free radicals and its constituent of vitamin C and E as antioxidants. Afonso et al. \(^{[33]}\) added that treatment with phenolic compounds may have reduced the concentration of superoxide anion and therefore attenuating the oxidative stress state.

In the present study, hypercholesterolemia also caused significant elevation in liver function enzymes. Kim et al. \(^{[8]}\) and Prasad \(^{[36]}\) attributed this increase in enzyme activities to the increase in ROSs and lipid peroxidation process involved in hyperlipidaemia which affected membrane permeability and led to leakage of enzymes into circulation. Due to the role of the milk beverage as antioxidant, ameliorations of the liver function indices; AST, ALT, ALP,
GGT and total protein were also recorded. Lipanthyl drug also ameliorated the level of the liver enzyme activities as a result of attenuation of the hypercholesterolemic state and the elaborated free radicals, which in turn reflected to the amelioration of liver functions. These results were in accordance with the results of Awad et al. [28, 29], and Hamed [30] who confirmed the role of lipanthyl drug in treatment of hypercholesterolemia and attenuation of free radicals elevation secondary to hypercholesterolemia. The observed decrease in serum protein was confirmed by the results of Awad et al. [37] Mühlfeld et al. [38] attributed the decrease in serum protein to a condition of proteinuria where the protein was excreted in the urine due to the renal damage as a result of hypercholesterolemia.

In the present study, the light microscopic examination of the liver sections of hypercholesterolemic rat livers revealed a large accumulation of macrovesicular and microvesicular fat in the livers as well as dark inflammatory infiltrates in the high fat/cholesterol fed liver. This was in agreement with Zheng et al. [39]; Bailey et al. [40] who observed the same structure of liver after high fat diet. This was attributed to the presences of fatty liver as a result of hypercholesterolemia and accumulation of large vacuoles of fat. This microscopic examination also confirmed the present biochemical determinations, where cholesterol and triglycerides were increased. Treatment with milk beverage and lipanthyl drug, showed only focal area of fatty droplets and less degenerative changes in the liver, which was also confirmed by the reduction observed in the level of cholesterol and triglycerides after treatment with the milk beverage.

4. CONCLUSIONS
In conclusion, our results suggest that the prepared functional milk beverage recorded hypolibidemic and antioxidant activities. It had the ability to improve the lipid profile (TC, HDL-C, LDL-C and TG) and the oxidative stress markers (GSH, MDA and SOD) as well as the liver function indices (AST, ALT, ALP, GGT and total protein). The presence of phenols, vitamin C, E and its ability to in vitro scavenge free radicals confirmed our results. Future analysis involving the bioavailability of the milk beverage contents may help to clarify the role of these antioxidant compounds in cholesterol homeostasis and oxidative stress parameters.

CONFLICT OF INTEREST
The authors declared that no conflict of interest.
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