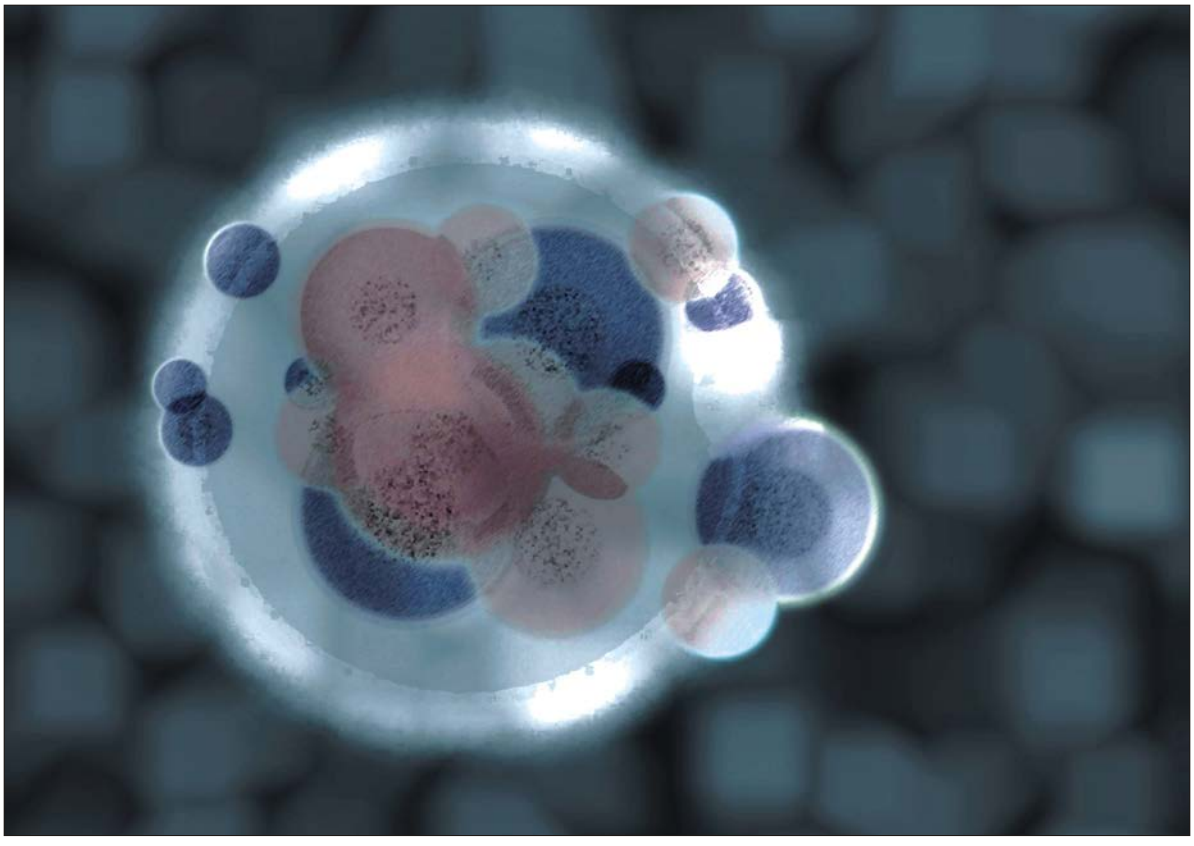

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Research Article
Newly Formulated Antioxidant Rich Dietary Supplement in Jelly Form for Alleviation of Liver Diseases in Rats

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Abstract

Background and Objective: Nonalcoholic fatty liver disease (NAFLD) is the major cause of chronic liver disease worldwide. The present study aimed to prepare a dietary supplement rich in antioxidants that is able to prevent nonalcoholic fatty liver associating metabolic disorders and to limit its progression into steatohepatitis. **Materials and Methods:** This dietary supplement is in jelly form and composed of prunes, dried grapes, carrots, cape gooseberry, pomegranate peel, chicory, sumac, sweet corn, germinated soybeans and vitamins B12, C and D. HPLC analysis and the antioxidant activity for the formulated dietary supplement were carried out. A biological experiment was done on 30 male Wistar rats categorized into 5 groups for 4 weeks after nonalcoholic fatty liver induction by feeding rats high fat and cholesterol (HFC) diet with bile acids for 8 weeks. Biochemical analysis and histopathological examination were done. The one-way ANOVA test was done followed by Duncan test. **Results:** The supplement antioxidant activity was relatively high and HPLC analysis revealed the presence of several polyphenolic compounds. Results revealed an altered lipid profile, an elevation in activity of plasma transaminases, lipid peroxides and tumor necrosis factor alpha (TNF-α), while there was a reduction in total protein, albumin and activity of antioxidant enzymes for HFC fed group. Adding the formula to the diet of the HFC fed rats nearly normalized the aforementioned parameters. Histopathological examination reinforced the biochemical findings. **Conclusion:** The obtained data proved that the prepared dietary supplement managed to restore the metabolic changes accompanying fatty liver and to limit its progression into steatohepatitis.

Key words: Nonalcoholic fatty liver disease, steatohepatitis, dietary supplement, antioxidant activity, lipid profile, antioxidant enzymes, tumor necrosis factor-α

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by abnormal accumulation of lipid in the form of triacylglycerols in hepatocytes (more than 5%). This is known as steatosis and occurs in the absence of alcohol abuse[1]. It is a reversible condition and to some extent non-progressive if the underlying cause is removed. NAFLD is considered as the major cause of chronic liver diseases worldwide and it includes a wide spectrum of hepatic diseases. It is ranging from simple hepatic steatosis (there is only fat accumulation) passing through the nonalcoholic steatohepatitis (NASH). The latter was the more aggressive form of inflammatory fatty liver disease with ballooning and moderate fibrosis. It may proceed to cirrhosis with a high risk of hepatocellular carcinoma (HCC) and hepatic failure[2]. Data obtained from recent surveys suggest that NAFLD is by far the most common liver disease in Europe, United States and in Asia[3]. Generally, the prevalence of NAFLD is estimated as about 30% of adults in developed countries[4] which tends to increase in industrialized countries. From 5-10% of cases develop NASH[5]. In about 20% of patients with NASH, the disease progresses into fibrosis and cirrhosis and potentially to hepatocellular carcinoma over a period of 15-year time[6]. Despite its prevalence, no treatment has been proven to be effective[7]. Some factors contribute directly to the development of NAFLD such as a sedentary lifestyle and increased consumption of high-fat foods and beverages with high concentrations of fructose[8].

Recently, the role of nutrients with therapeutic properties has been highlighted and magnified either in the form of dietary supplements or nutraceuticals or functional foods. Many efforts have been devoted in this respect. Dietary supplements are products that aim to supplement the normal diet with vitamins, minerals, herbs or other botanicals or concentrated sources of nutrients or other substances. They possess nutritional or physiological effect, alone or in combination. They are marketed in dose form and intended to supplement the diet with essential nutrients that are lacking in the regular diet[9]. A dietary supplement may be a plant or part of a plant used for its flavor, scent, or potential therapeutic properties. It may include flowers, leaves, bark, fruits, seeds, stems and roots or their constituents. The presence of many plants with antioxidant properties in one dietary supplement form a network of antioxidants. They possess different phytochemical constituents with certain chemical properties. They are assumed to act in a synergetic way as reported by Blomhoff *et al.*[10] and perform many functions to protect cells from damage, thus ensuring the benefits expected from using this dietary supplement.

Studying of NAFLD/NASH using human materials have so many limitations as the occurrence and progression of NAFLD/NASH proceeds over a long period of time. In addition, there are ethical limitations in administering drugs to patients or collecting liver tissues from patients[11]. Animal models of NAFLD/NASH give the ideal solution for this problem. It provides the facility of both elucidation of the NAFLD/NASH pathogenesis and evaluation of the therapeutic efficacy of various agents. Actually, there are so many animal models for NAFLD/NASH[12,13] among which is the cholesterol, cholate and high fat diet. This model was a well-documented model for inducing NAFLD/NASH as modified from Takahashi *et al.*[14] and it ensures the occurrence of steatosis, steatohepatitis and fibrosis.

Treatments for NASH other than modification of lifestyle by exercise and diet have not been completely established. There was no reported consensus concerning the pharmacological treatment[2,15]. Since there was no safe and proven therapy for fatty liver disease, antioxidant therapies are the solution for preventing fat accumulation in the liver and protecting from the consequent damage implicated by fat accumulation[16]. Hence, the present study aimed to manufacture a palatable dietary supplement. It is composed of a collection of edible antioxidant rich plants each is well known to perform a certain function in treating liver diseases. This dietary supplement includes: Prunes, dried grapes, carrots, cape gooseberry fruit, pomegranate peel, chicory, sumac, sweet corn and germinated soybeans in addition to vitamins B12, C and D. It was also aimed to illustrate the role of this dietary supplement with its high antioxidant potency in amelioration of nonalcoholic fatty liver disease (NAFLD) in rat model and to what extent it may prevent its progression into the more aggressive form of inflammatory steatohepatitis (NASH).

MATERIALS AND METHODS

This study was carried out in May, 2015 at the department of Nutrition and Food Science, National Research Centre, Egypt.

Materials

Composition of the dietary supplement: Most of the constituents of the formula were of natural sources and purchased from the local market. The formula included: Prunes, dried grapes, carrots, cape gooseberry fruit, pomegranate peel, sumac and chicory, it also contains sweet corn and soybean (Giza 82) which were obtained from Agricultural Research Centre, Giza, Egypt. Vitamins B12, C and D were also added to the formula.

Table 1: Composition of diet of control rats as g/100 g diet[23].

Ingredients	Amount (g/100 g diet)
Casein*	21.9
Sucrose	5.0
Cellulose	4.0
Corn oil	8.0
Salt mixture (AIN-93)	3.5
Vitamin mixture (AIN-93)	1.0
Choline bitartrate	0.25
L- Cystine	0.18
Corn starch	56.17

*Protein content of casein was estimated as 54.6%

Materials used for the animal experiment: Most of the constituents of the diet formulated and introduced to the rats (Table 1) were purchased from the local market, while, casein was obtained from Scerma Co., (France). The components of the salt and the vitamin mixtures used for formulation of the diet were of analytical grade and obtained from Fluka (Germany) and BDH (England) Chemical Companies. Cholesterol powder and bile salts used for induction of hypercholesterolemia in rats were obtained from Fine-Chem Limited, (Mumbai, India), while lard was obtained from local market.

Animals used in this experiment were Wistar male albino rats of Sprague Dawly strain obtained from Central Animal House, National Research Centre (NRC), Egypt. Animal experiment was carried out in compliance with the Guidelines for Animal Care and Ethics Committee of the NRC (Egypt) and the study protocol was approved.

Materials used for the chemical analysis of the dietary supplement: Chemicals used for estimation of the antioxidant power of the prepared formula were all of analytical grade, DPPH (1, 1-Diphenyl-2-picrylhydrazyl), ABTS (2, 20-Azinobis (3-Ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt, trolox, TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine) and β-carotene were obtained from Sigma-Aldrich Co., USA. Methanol and Ethanol used for extraction of polyphenols for HPLC determination were of analytical grade and obtained from Fisher Chemicals Co. (UK) and Aldrich Co., (UK), respectively. All the standers used for HPLC analysis were obtained from Sigma-Aldrich Co., (USA).

Materials used for the biochemical analysis: Diagnostic Kit used for estimation of plasma triacylglycerols was obtained from Stanbio Laboratory Company, Texas, (USA). Kits used for determination of total cholesterol, HDL-C total protein, albumin, urea and creatinine in plasma were purchased from Chronolab Systems S.L., (Barcelona, Spain). Kits used for determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were obtained from Salusa Co., (Netherlands). Kit used for evaluation of rat plasma tumor necrosis factor-alpha (TNF-α) based on ELISA technique, catalog no.: "K0331196", was purchased from Koma Biotech Inc., (Seoul, Korea). Kits used for determination of blood hemoglobin, glucose, activity of each of plasma catalase, RBCs SOD and RBCs GPx were obtained from Biodiagnostic Co., (Egypt). Chemicals used for determination of plasma lipid peroxide product (malondialdehyde), tricholoroacetic acid (TCA) and thiobarbituric acid (TBA) were obtained from BDH (England) and Merck (Germany) Companies, respectively.

Methods

Preparation and processing of the dietary supplement: Soybean was germinated for 3 days. All the selected ingredients (prunes, dried grapes, carrots, sweet corn, cape gooseberry fruit, pomegranate peel, chicory, sumac and germinated soybean) were dried in an air ventilated oven at 60°C till complete dryness. Then, the dried ingredients were milled into fine powder in a mechanical grinder (Braun, Germany). The powdered ingredients were mixed together in suitable concentrations assumed to exert bioactive role and according to panel testing (as mentioned in the submitted patent for the Academy of Scientific Research and Technology in Egypt with registering number of 854/2017). A portion of this powder was subjected to chemical analysis for determination of polyphenolic compounds by HPLC and antioxidant power prior to the feeding experiment. Then the powder was used for preparation of a dietary supplement in the form of jelly.

The jelly was prepared according to Ventura *et al.*[17], with some modifications. It was prepared by mixing the water, the formula as powder and sucrose. This combination was heated with continuous stirring and the gelatin was then poured slowly to avoid the formation of lumps. The obtained product was designated as sample (1). Then, the same procedures were carried out with addition of natural color and flavor. The resultant product was designated as sample (2).

Sensory evaluation of the dietary supplement: The dietary supplement (sample 1&2) was subjected to sensory evaluation by volunteers. Each volunteer was asked to give score (out of 20) for taste, flavor, color, appearance, texture and overall acceptability for each one of the tested samples. A sensory score of 10 or above was considered acceptable while, a sensory score below 10 was considered unacceptable.

Extraction of polyphenols: The powdered formula was extracted with either of two solvents, once with 80% methanol and the other with 80% ethanol according to the method of Hayat *et al.*[18]. The moisture content of the sample was determined in order to calculate the concentrations on a dry weight basis.

Determination of polyphenols by HPLC: Individual polyphenols content of the formula was determined in the two extracts by HPLC according to Kim *et al.*[19] as follows:

The HPLC analysis was performed using a Gilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector. The analytical column was an Eclipse XDB-C18 (150×4.6 μm, 5 μm) with a C18 guard column (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile (solvent A) and 2% acetic acid in water (v/v) (solvent B). The flow rate was kept at 0.8 mL min^{-1} for a total run time of 70 min and the gradient program was as follows: 100% B-85% B in 30 min, 85% B-50% B in 20 min, 50% B-0% B in 5 min and 0% B-100% B in 5 min. The injection volume was 50 μL and peaks were monitored simultaneously at 280 and 320 nm. All samples were filtered through a 0.45 μm Acro disc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by congruent retention times and UV spectra and compared with those of the standards.

Determination of antioxidant activity of the dietary supplement: The DPPH radical scavenging activity (DPPH) was determined according to the procedure of Hayat *et al.*[18]. Trolox equivalent antioxidant capacity (TAEC or ABTS) was estimated according to Ventura *et al.*[17]. Ferric reducing power (FRAP or TPTZ) was assessed according to De Moraes Barros *et al.*[20]. β-carotene bleaching test was determined as described by Moulehi *et al.*[21] and lipid peroxidation inhibition activity (LPIA) was estimated according to Srivastava *et al.*[22].

Formulation of the diet: The standard control diet was prepared according to Reeves *et al.*[23] as shown in Table 1. Then the formula in the form of dried powder was added to the diet of the selected groups on the expense of starch.

Animal experiment: Thirty male adult Wistar albino rats of Sprague Dawley strain with a body weight ranging from 190-210 g were used. Animals were maintained in controlled temperature 25°C for a period of one week for accommodation prior to the animal experiment. Food and water were allowed ad libitum to each rat. Animals were divided into 5 groups, each comprising 6 rats. Nonalcoholic fatty liver rat model was induced by feeding a high fat and cholesterol diet containing 200 g lard, 20 g cholesterol and 2.5 g bile salts for each Kg diet as described before in a previous study for our team by Mahmoud *et al.*[24] and as modified from Takahashi *et al.*[14] and Qin *et al.*[25]. The formula was added on the expense of starch to the diet of groups 3, 4 and 5. The feeding experiment started as follows:

Group 1: Given the standard diet and served as negative control

Group 2: Given the high fat and cholesterol (HFC) diet and served as positive control

Group 3: Given the standard diet+the formula as 100 g kg^{-1} diet

Group 4: Given the high fat and cholesterol (HFC) diet+the formula as 100 g kg^{-1} diet

Group 5: Given the high fat and cholesterol (HFC) diet + the formula as 200 g kg^{-1} diet

The experiment lasted for 4 weeks during which body weight and feed intake were followed. At the end of the experiment, body weight gain, feed intake and feed efficiency ratio were calculated and recorded.

Biochemical analysis: After the end of the experimental period, blood samples were collected from suborbital vein. Whole blood was obtained for immediate analysis of hemoglobin according to Betke and Savelsberg[26]. RBCs was separated and washed by saline three times then kept at -70°C until the analysis of the antioxidant enzymes, SOD and GPx according to Nishikimi *et al.*[27] and Paglia and Valentine[28], respectively. Plasma was separated and kept at -70°C until analysis. Plasma glucose was determined according to the method of Trinder[29]. The activity of liver enzymes AST and ALT were measured as described by Henry *et al.*[30]. Total protein and albumin were estimated according to the methods of Koller[31] and Doumas *et al.*[32], respectively. Plasma urea and creatinine each was determined according to Fawcett and Soctt[33] and Murray[34], respectively. The Activity of plasma catalase was estimated by Aebi[35]. Plasma peroxidation product, malondialdehyde (MDA) was detected by the thiobarbituric acid (TBA) assay according to the method of Draper and Hadley[36]. The concentration of plasma TNF-α was determined by an enzyme linked immunosorbent assay (ELISA) technique according to manufacturer's instructions using ELISA Reader (ELISA Reader Model Start F, Awareness Technology, Inc., Palm City, FL, USA).

Lipid parameters were estimated as follows, plasma triacylglycerols (TG) was assessed as described by Scheletter and Nussel[37]. Plasma total cholesterol (TC) was determined as described by Meiattini *et al.*[38]. High density lipoprotein-cholesterol (HDL-C) was determined according to Grove[39]. Low density lipoprotein-cholesterol (LDL-C) was calculated according to Warnick *et al.*[40] as shown in the following equation:

$$\text{LDL-C} = \text{Total cholesterol-(HDL-C+VLDL-C)}$$

where, VLDL-C (very low density lipoprotein-cholesterol) = Triacylglycerols/5

Atherogenic index (A.I.) was calculated as described by Dobiasova[41] according to the Equation:

$$\text{A.I.} = \log \frac{\text{Triacylglycerols}}{\text{HDL-C}}$$

The concentration of all of the previously mentioned biochemical parameters were measured by a colorimetric technique using a spectrophotometer (Shimadzu UV-2401 PC, Australia). Organs including, liver, kidney, spleen and heart were separated and weighed. A portion of the liver was separated and kept in 10% formalin for further histopathological evaluation.

Histopathological examination: Liver tissue was histopathologically examined after being cleared in xylol, embedded in paraffin, sectioned at 4-6 micron thickness and stained with Heamatoxylin and Eosin according to Carleton[42]. Finally, it was examined under microscope. An Olympus (U.TV0.5XC-3) light microscopy with Olympus digital camera for photographing of slides was used.

Statistical analysis: Data were analyzed statistically using the computerized program SPSS software, version "20" for Windows. The one-way ANOVA test was done followed by Duncan test. Data were represented as Mean±SE. The level of significance $p<0.05$ was considered significant, otherwise was non-significant.

RESULTS AND DISCUSSION

Sensory evaluation of the dietary supplement: Results obtained from the sensory evaluation (organoleptic properties) of the dietary supplement for the two samples (Table 2) revealed overall acceptance with nearly the same degree for the two samples. It is thus clear that adding color and flavor to the original formula did not realize much improvement. The obtained results indicate that this formula will have a good chance when being marketed.

Polyphenolic content of the dietary supplement by HPLC: The presence of 9 phenolic compounds detected in the prepared formula as determined by HPLC analysis is shown in Table 3. It is believed that the presence of these compounds contributes to the antioxidant power of the formula[43].

Antioxidant activity of the dietary supplement: The formula possesses high antioxidant power. This was proved by the results obtained from the antioxidant analysis by five different methods (Table 4) to determine the reactivity towards aqueous or organic radicals. As shown in the table, the methanolic extract possesses higher antioxidant power for most of the testes done except for the tests that measured lipid peroxidation and β-carotene, the ethanolic extract showed a higher reactivity than did the methanolic extract. Generally, it can be stated that the prepared formula had a strong antioxidant activity. The exact mechanisms regarding progression of NAFLD into NASH and their pathogenesis are not completely clarified[1]. In a trail to explain these mechanisms, the modern theory "multiple-hit" considers multiple insults acting together to induce NAFLD and explains accurately its pathogenesis and its progression into NASH[5]. The increased oxidative stress and the overproduction of the reactive oxygen species is one of the many factors that act together to induce NAFLD/NASH[44]. Prevention of the increased oxidative stress by counteracting free radical production is expected to participate in treatment of NAFLD and to avoid its transformation into NASH. To fulfill this target, natural antioxidants were obtained from a group of plant sources such as: Prunes, dried grapes, carrots, cape gooseberry

Table 2: Organoleptic properties of the dietary supplement

Formula	Taste (20)	Flavor (20)	Color (20)	Appearance (20)	Texture (20)	Overall acceptability (20)
Sample 1	16.91±0.34[a]	17.86±0.36[a]	17.86±0.36[a]	16.82±0.82[a]	17.09±0.80[a]	16.82±0.50[a]
Sample 2	17.09±0.84[a]	17.00±0.56[a]	17.00±0.43[a]	16.45±0.79[a]	17.45±0.47[a]	16.82±0.64[a]

Data are represented as Mean±S.E. and the mean difference is significant at $p<0.05$. Values that share the same letter at the same column are not significant, while values that share different letters at the same column are significant

fruit, pomegranate peel, chicory, sumac, germinated soybean and sweet corn. They were selected on the basis of their high antioxidant content as documented from previous literature. Prunes are known for their high content of polyphenols and flavonoids with a higher antioxidant power for the dried black plum (prunes) than the fresh ones as reported by Najafabad and Jamei[45]. Rop *et al.*[46] concluded from their study that Cape gooseberry (*Physalis peruviana*) fruit has a high antioxidant activity. From phytochemical screening carried out by Shad *et al.*[47] for the chicory (*Cichorium intybus*), they confirmed the presence of saponins, tannins, terpenoids, flavonoids, anthocyanins and glycosides in each part of the plant rendering this plant to be a potent antioxidant. The major bioactive material of grape is resveratrol which is proved to be a strong antioxidant and anti-inflammatory molecule[48]. Carrot was reported to have potent antioxidant capacity and it reduced bilirubin and urea levels[49] in CCl_4-induced liver injury in Wistar rats and it also reversed the increased levels of acid phosphatase and acid ribonuclease along with glucose-6-phosphatase and cytochrome P-450.

Table 3: Polyphenolic content of the formula (ethanolic and methanolic extracts) as detected by HPLC (mg/ 100g dry weight)

Polyphenols	Ethanolic extract (mg/100 g d.w.)	Methanolic extract (mg/100 g d.w.)
Gallic acid	70	60.33
Protocatchuic acid	318	27.47
Chlorgenic acid	34.1	48.03
Caffeic acid	3.7	-
Ferulic acid	13	-
Coumarin	56.23	18.93
Rosmarinic acid	28.3	122
Cinnamic acid	3.23	-
Chrysin	53.42	285.9

Sweet corn was proved to have antioxidant properties and it reduced nitric oxide production[50]. Soy is a potent antioxidant and it was proved to have antiproliferative and anti-inflammatory characters[51]. Sumac was reported to have high antioxidant activity and hepatoprotective properties[52]. Also, vitamin C was reported to reduce the oxidative stress and inhibit the experimental liver steatosis development in rats induced by a diet deficient in choline[53]. Thus, the present dietary supplement is expected to have a positive impact on rats suffering from fatty liver disorders as it is of high antioxidant potency.

Biological evaluation of the dietary supplement

Body weight gain, feed intake, feed efficiency ratio and hepatosomatic index: Data obtained for body weight gain, feed intake and feed efficiency ratio for all groups are shown in Table 5. Minor changes were observed in the body weight gain and feed intake, but none of them was significant. However, some changes recorded for the feed efficiency ratio (FER) were significant. There was a non-significant change in the FER for the control positive group compared to the control negative group, while a significant increase was found in case of the control group that received the formula compared to either the control negative group or the control positive group. It was noticed that although the control group that received the formula consumed lower food than the control positive positive group, yet the former gained more body weight than the latter reflecting the higher ability of the formula for food utilization.

Table 4: Antioxidant activity for ethanolic and methanolic extracts of the prepared formula represented by DPPH, TPTZ, LPIA, β-carotene & ABTS

	Test				
Extract	DPPH (% scavenging activity)	TPTZ (Mole T E/100g FW)	LPIA (% inhibition)	β-carotene (% inhibition)	ABTS (μmol T/g FW)
Ethanolic extract	34.33±0.37^{a}	793.67±17.32^{a}	0.61±0.008^{a}	37.89±17.31^{a}	0.29±0.001^{a}
Methanolic extract	66.77±3.04^{b}	842.44±19.51^{a}	0.53±0.009^{b}	20.25±0.92^{a}	0.47±0.018^{b}

DPPH (1, 1-diphenyl-2-picrylhydrazyl), TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine), LPIA (lipid peroxidation inhibition activity), ABTS (2, 20-azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid), Values are expressed as Mean±SE and the mean difference is significant at $p<0.05$.Values that share the same letter at the same column are not significant while, values that share different letters at the same column are significant

Table 5: Feed intake, body weight gain, feed efficiency ratio (FER) and liver weight percent of groups

Group	Body weight gain (g)	Feed intake (g)	FER*	Liver wt (%)
Control (-ve)	62.33±15.37^{ab}	613.50±6.08^{a}	0.102±0.027^{a}	3.18±0.32^{a}
Control (+ve)	64.00±4.27^{ab}	586.67±4.48^{a}	0.109±0.008^{a}	3.90±0.08^{b}
Control+formula	74.33±3.83^{a}	595.83±20.84^{a}	0.125±0.009^{b}	5.12±0.09^{c}
HFC+10% formula	64.67±12.48^{ab}	609.00±13.93^{a}	0.103±0.021^{a}	4.88±0.1^{cd}
HFC+20% formula	67.33±5.49^{ab}	631.67±7.19^{a}	0.107±0.009^{a}	4.38±0.08^{bd}

*FER is calculated as body weight gain/feed intake. Values are expressed as Mean±SE and the mean difference is significant at $p<0.05$. Values that share the same letter at the same column are not significant while, values that share different letters at the same column are significant

Table 6: Concentration of serum lipid fractions, TC, TG, LDL-C, HDL-C and A. I. of all studied groups

	Groups				
Parameters	Control (-ve)	Control (+ve)	Control+formula	HFC+ 10%formula	HFC+20%formula
TC (mg dL^{-1})	106.60±6.26^{a}	203.85±22.17^{b}	118.8±15.0^{a}	181.29±18.75^{b}	141.98±18.59^{a}
TG (mg dL^{-1})	73.11±9.45^{ab}	109.28±4.50^{c}	71.36±5.61^{a}	102.59±11.09^{bc}	95.16±12.40^{abc}
LDL-C (mg dL^{-1})	39.51±6.54^{a}	150.08±13.66^{b}	44.60±4.45^{a}	121.68±14.45^{c}	99.07±7.73^{c}
HDL-C (mg dL^{-1})	51.72±5.34^{a}	31.73±1.62^{b}	52.98±6.02^{a}	39.97±4.24^{ab}	41.01±1.43^{ab}
A.I.	0.198±0.06^{a}	0.538±0.03^{b}	0.172±0.06^{a}	0.409±0.05^{bc}	0.347±0.05^{ac}

Total cholesterol (TC), triacylglycerols (TG), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C) and Atherogenic Index (A. I., log [triacylglycerols/ HDL-C]), Values are expressed as Mean±SE and the mean difference is significant at $p<0.05$. Values that share the same letter at the same row are not significant while, values that share different letters at the same row are significant

From the data obtained for the hepatosomatic index Table 5, there was a significant increase in the liver weight percent for the control positive group when compared either to the control negative group or to the control group that received the formula. This index reflects the accumulation of fat in the liver tissue in the form of triacylglycerols as mentioned before by Fassini *et al.*[54]. This accumulated fat was reduced by ingesting the formula (10% concentration). A further reduction of the liver weight percent was noticed in the HFC group that received 20% formula. The formula resulted in a dose dependent improvement in the liver steatosis and this may be attributed to the high antioxidant content of the formula which trap the increased free radicals liberated.

Lipid profile: Lipid metabolism control has been thought as a major factor in NAFLD. Any agent that can inhibit cholesterol synthesis and enhance HDL-C in the body will help in managing the fatty liver disease[16]. Feeding high fat and cholesterol diet altered lipid metabolism as evidenced by the significant increase in the concentration of TC, TG, LDL-C and the atherogenic index together with a reduction in HDL-C for the control positive group compared to the control negative group (Table 6). Mells *et al.*[55] reported similar results when they fed adult male C57BL/6 J mice on a high fat and trans-fat diet in addition to cholesterol in a trail to induce nonalcoholic steatohepatitis. Change in lipid parameters may be a consequence of increased intestinal fat absorption due to increased fat intake. There is a strong evidence correlating Western diet ingestion with its high saturated and trans-fat content to the increasing incidence of NASH[56]. In the present study, the increase in TC, TG, LDL-C and A. I. was reduced and the decrease in HDL-C was counteracted when giving rats the HFC diet and the formula (10%) and it was more or less normalized when feeding rats on the HFC diet+20% formula particularly in case of TC, TG and A. I. meaning that the improvement in the lipid profile was positively correlated with the concentration of the formula. Many constituents of the formula was reported to improve lipid parameters in hypercholesterolemic rats. Pomegranate peel was reported to normalize altered lipid parameters in rats fed on high fat and cholesterol diet[57]. Among the mechanisms that participating in the pathogenesis of NAFLD is the protein misfolding in the stressed endoplasmic reticulum as reported by Sharma[16]. It was added that supplementing NAFLD patients with antioxidants is believed to reduce the protein misfolding that leads to fatty liver and also added that antioxidants reverse or prevent fat from accumulating in the liver. This may be the case in the present study in which an antioxidant rich supplement, particularly when it is of natural sources, helps to reduce fat accumulation in the liver as evidenced from the correction in the aforementioned lipid parameters and supported by the correction in liver hepatosomatic index.

Blood hemoglobin and glucose concentrations, kidney function and liver function: The blood hemoglobin recorded a marked change for the control positive group compared to the control negative group (Table 7). Hemoglobin concentration was reduced as a result of liver malfunction due to fat accumulation in the liver which caused a reduction in the ability of the liver to synthesize protein, hence hemoglobin synthesis was affected. Ahmed and Oates[58] reported similar results when they fed rats on high fat diet to induce NAFLD. Fortification of the diet with either 10 or 20% of the formula did not correct hemoglobin concentration. On the other hand, there was no effect on kidney function. There was a non-significant increase in plasma glucose for the control positive group compared to the control negative group which may be attributed to the increased insulin resistance that is associated with the fatty liver. A slight correction for blood sugar began to occur in the groups that were fed on the formula with the two concentrations reflecting the positive impact of the formula on glucose homeostasis as it may enhance the insulin sensitivity[59].

There was a significant elevation in the activity of both ALT and AST while there was a significant reduction in the

Table 7: Concentration of blood hemoglobin (Hb), plasma glucose and kidney and liver functions of all groups

	Groups				
Parameters	Control (-ve)	Control (+ve)	Control+formula	HFC+ 10% formula	HFC+20% formula
Hb (g dL^{-1})	13.69±0.24^{a}	11.30±0.23^{b}	13.51±0.32^{a}	11.81±0.26^{bc}	11.40±0.25^{b}
Glucose (mg dL^{-1})	71.13±6.53^{ab}	84.10±7.20^{bcd}	68.65±2.67^{a}	81.00±1.49^{abcd}	79.00±2.19^{abc}
Urea (mg dL^{-1})	38.41±5.13^{a}	35.05±1.28^{a}	32.99±1.92^{ab}	34.27±3.06^{a}	31.25±2.07^{ab}
Creatinine (mg dL^{-1})	0.62±0.11^{ab}	0.65±0.07^{abc}	0.58±0.05^{a}	0.56±0.08^{a}	0.60±0.10^{ab}
AST (U L^{-1})	61.67±6.68^{a}	92.00±11.90^{b}	61.00±5.81^{a}	72.33±4.99^{ab}	67.67±8.5^{ab}
ALT(U L^{-1})	16.17±2.96^{a}	39.17±0.70^{ab}	21.17±1.49^{a}	32.5±8.43^{b}	21.67±2.28^{a}
T.P. (g dL^{-1})	8.52±0.29^{a}	5.70±0.31^{c}	8.15±0.31^{a}	7.64±0.46^{ab}	7.49±0.40^{ab}
Alb. (g dL^{-1})	4.52±0.27^{a}	3.19±0.26^{b}	4.25±0.12^{ac}	4.22±0.16^{ac}	4.33±0.18^{ac}

Values are expressed as Mean±SE and the mean difference is significant at p,0.05. Values that share the same letter at the same row are not significant while, values that share different letters at the same row are significant

concentration of both plasma total protein and albumin by feeding rats on high fat and cholesterol diet (the control positive group) compared to the control negative group (Table 7). This is due to fat accumulation in hepatocytes which affects the capacity of the liver to perform its functions. Qin *et al.*[25] have found similar results as they reported a significant increase for ALT and AST when rats were fed on high fat and cholesterol diet for 10 weeks. Also, Tamada *et al.*[60] reported a shooting increase for both ALT and AST in SHRSP5/Dmcr rats which were kept on high fat and cholesterol diet for 2, 8 and 14 weeks. Olorunnisola *et al*[61] also recorded a reduction in serum albumin and total protein when feeding Wistar rats on cholesterol. The previously mentioned altered liver function parameters of the control positive group showed a dose dependent improvement which reached for more or less normalization by using 20% formula. These results highlighted the potency of the formula that was able to restore the liver function. These results may be attributed to the high antioxidant power of this formula, since each component alone was reported previously to have a positive impact on the liver performance. It seems that the combination of these constituents together in one formula reinforce their positive effect. Cape gooseberry fruit (*Physalis peruviana* L.) was previously reported to improve liver function[62]. Also, Al-Olayan *et al.*[63] concluded from their study that *Physalis peruviana* L. restored the elevated liver enzymes AST and ALT to values near that of the control negative group in rats with carbon tetrachloride-induced hepatotoxicity. Moreover, pomegranate peel extract was reported to reduce elevated liver transaminases AST and ALT in hepatic fibrosis induced by bile duct ligation in rats[64].

Malondialdehyde concentration and activity of antioxidant enzymes: High fat and cholesterol diet is well known to induce oxidative stress in different organs of the body such as liver, kidney and heart resulting in the formation of lipid peroxidation products[65]. The oxidative stress may cause steatohepatitis by many mechanisms among which lipid peroxidation[66]. Lipid peroxidation is first initiated by the attack of free radicals on the polyunsaturated fatty acids of the cell membranes leading to their fragmentation into reactive aldehyde compounds[61] represented by the thiobarbituric acid reactive species, the malondialdehyde or MDA (Fig. 1a). MDA showed a significant increase for the control positive group compared to the control negative group. This increase was more or less normalized by feeding the rats with the formula at any of the two levels used. In fact the obtained results confirmed the strong antioxidant power of the formula which was measured by five different methods in this study. This potent antioxidant properties of the formula enabled it to quench and get rid of the increased oxidative stress. The antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase are the natural antioxidant defense mechanisms of the body that prevent the destructive effects of free radicals. An imbalance between reactive free radical formation and scavenging by antioxidants leads to increased oxidative stress and consequently tissue damage[67]. In the present study, a significant decrease in RBCs glutathione peroxidase (Fig. 1d) and a non-significant decrease in both plasma catalase (Fig. 1b) and RBCs superoxide dismutase (Fig. 1c) were found for the control positive group compared to the control negative group. It has been reported previously by Matsuzawa *et al.*[68] that the high-fat diet down-regulated the hepatic gene expression of antioxidant enzymes and might have increased oxidative stress which seems to be in agreement with our results. By adding the formula to the diet with either of the two concentrations used, this decrease was counteracted and reached to nearly the normal values in case of SOD and catalase enzymes but, although improved in case of glutathione peroxidase, yet it is still significantly lower than the control negative group. It has been assumed that a diet rich in antioxidant strengthens the antioxidant defense system of the human body[69].

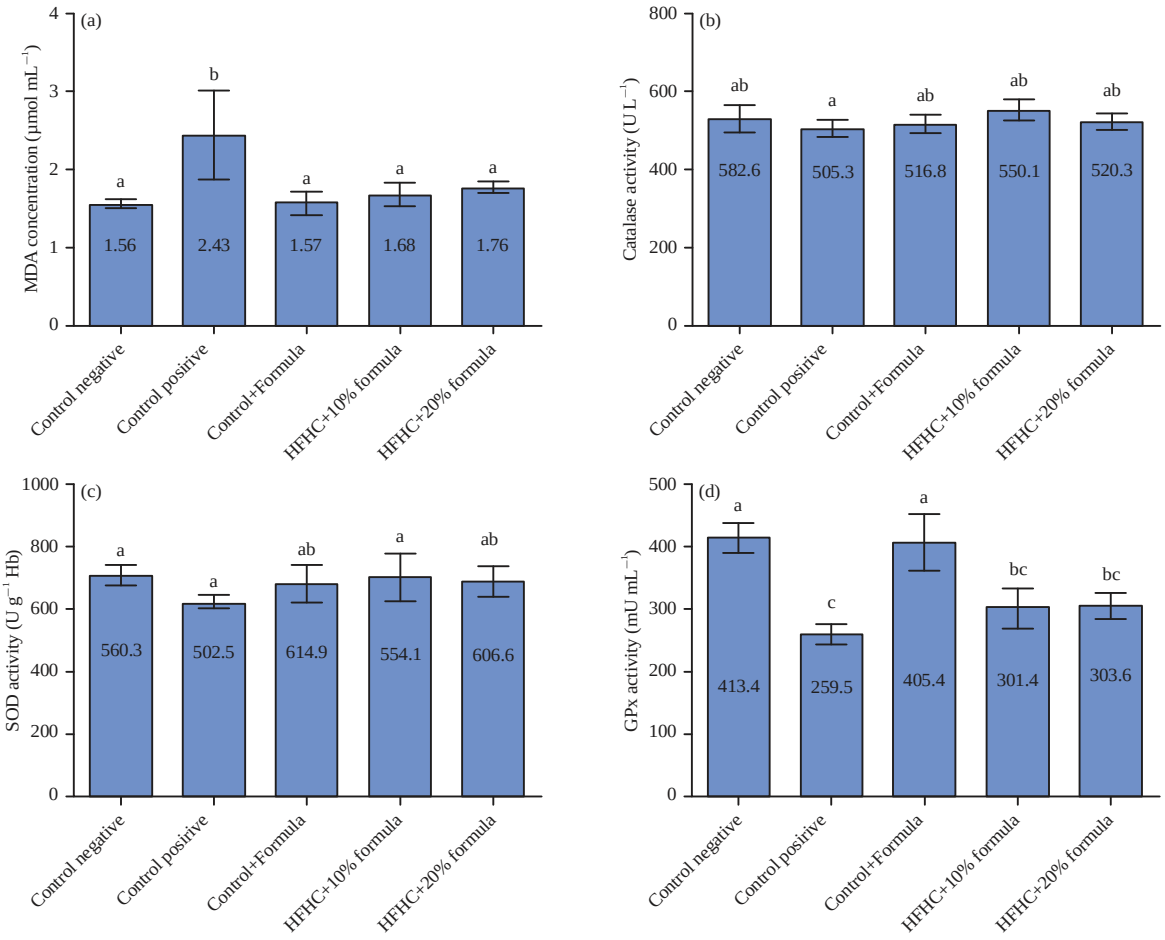

Fig. 1a-d: (a) Concentration of lipid peroxide product, MDA, (b) Catalase activity, (c) Superoxide dismutase (SOD) activity and (d) Glutathione peroxidase (GPx) activity

Bars represent Mean±SE and the mean difference is significant at p<0.05 level. Bars that share the same letter are not significant, while those that share different letters are significant

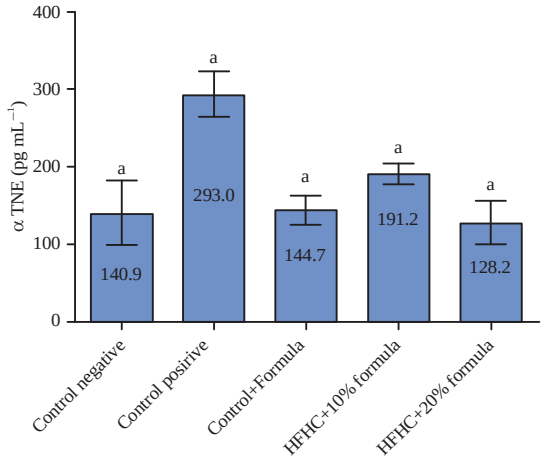

Fig. 2: Tumor necrosis factor alpha (α-TN F) concentration

Bars represent Mean±SE and the mean difference is significant at p<0.05 level. Bars that share the same letter are not significant, while those that share different letters are significant

Concentration of plasma tumor necrosis factor alpha (TNF-α): Tumor necrosis factor alpha (TNF-α) measured in this study (Fig. 2) evaluates the response of pro-inflammatory cytokines to the high fat and cholesterol diet. There was a significant increase (more than two folds increase) in the TNF-α for the control positive group compared to the control negative group. This proves the success of the present protocol to induce steatohepatitis (NASH) in rats. Lieber *et al.*[70] reported similar results of increased TNF-α in the group of rats that were fed on a high fat diet. Moriya *et al.*[71] also reported a similar increase in TNF-α when inducing steatohepatitis by feeding SHRSP5/Dmcr rat high fat and cholesterol diet. Using the formula with its two levels restored the TNF-α in a dose dependent pattern as seen in Fig. 2. These results may be attributed to some of the constituents of the formula which possess anti-inflammatory properties such as grapes, pomegranate and soybeans. Moreover, the improvement in

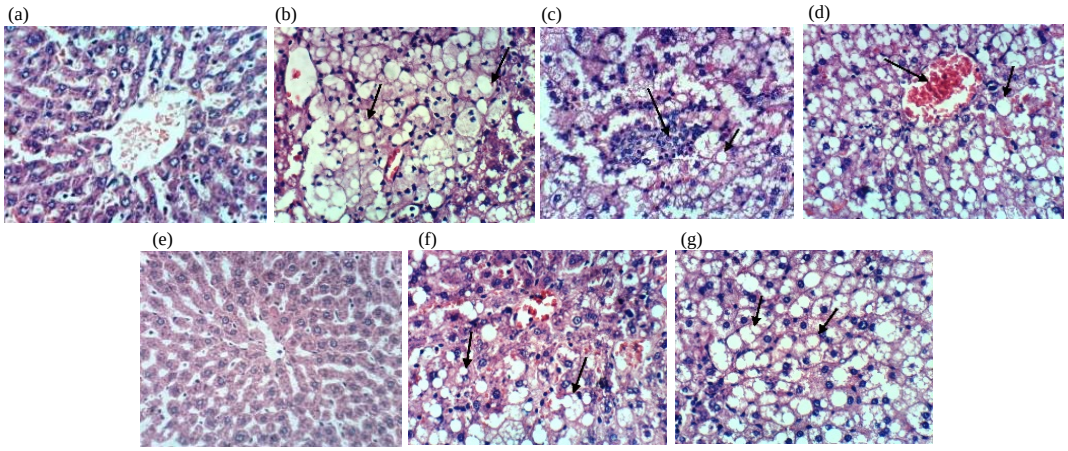

Fig. 3: Light photomicrographs of liver tissue in rats (a-g). (a) Liver of rat from control negative group showing the normal histological structure of hepatic lobule, (b-d) Liver of rat from control positive group showing steatosis of hepatocytes, mononuclear inflammatory cell infiltration and congestion of central vein, (e) Liver of rat from the control group that received the formula showing no histopathological changes, (f) Liver of rat from the group that received HFC diet+10% formula showing steatosis of hepatocytes and (g) Liver of rat from group that received HFC diet+20% formula showing steatosis of hepatocytes (H & E 400X)

the TNF-α may be due to the high antioxidant power of the formula. It was mentioned before that increased oxidative stress due to ingestion of high fat diet may cause steatohepatitis by many mechanisms among which cytokine induction which led to up-regulation of genes encoding pro-inflammatory cytokines such as TNF-α[66]. Using antioxidants counteracts the increased oxidative stress and thus down-regulates genes encoding for the pro-inflammatory cytokines like TNF-α although the high fat and cholesterol diet is still used with the formula. The obtained results indicates that the formula has succeeded to prevent the progression of the nonalcoholic fatty liver disease (NAFLD) into the more aggressive form, the steatohepatitis (NASH).

Histopathological examination of the liver: Histopathological examination of the liver tissue revealed the presence of pronounced hepatic steatosis which reflects fat accumulation in hepatocyte of the control positive group with the presence of mononuclear inflammatory cells (Fig. 3b-d) indicating and confirming the induction of inflammatory steatosis referred to as inflammatory steatohepatitis (NASH) when compared to the normal histological structure of hepatic lobule of the control negative group (Fig. 3a), congestion of central vein was also noticed in the hepatic tissue of the control positive group. Lieber *et al.*[70] reported the presence of mononuclear inflammatory cells along with pronounced hepatic steatosis in the hepatic tissue of the group of rats that was fed on high fat diet which is in agreement with these results. Feeding rats that received HFC diet with the formula with either of its two levels has led to nearly disappearance of the mononuclear inflammatory cells (except for very few mononuclear inflammatory cells that were detected) as illustrated in Fig. 3f, g. These results indicate that the formula with its high antioxidant potency was able to counteract the inflammation induced by the HFC diet, i.e. the formula was able to hinder the progression of the normal steatosis into the inflammatory steatosis or steatohepatitis, still the steatosis present even with ingestion of the formula which may be explained on the basis of that hepatocytes need more time to get rid of the fat accumulation mainly in the form of triglycerides. This is in contrast to the blood lipid parameters which showed improvement due to the ingestion of the formula. This phenomenon was reported before by Fassini *et al.*[54], who mentioned that the triglycerides accumulated in liver tissue need more time to disappear than do that of the plasma. The results of the histopathological examination are in agreement with the results of the hepatosomatic index in this study. There was a significant increase in the liver weight % for the control positive group that although showed a gradual reduction in the groups that were fed HFC diet and the formula with a dose dependent manner, yet still higher than the control negative group significantly.

CONCLUSION

The obtained results proved that NAFLD induced in rats by feeding HFC diet caused drastic changes in some metabolic

functions of the body represented by altered levels of plasma lipid parameters, an increase in the activity of liver transaminases in the circulation, the concentration of plasma lipid peroxides and the level of the anti-inflammatory marker (TNF-a). On the other hand, there was a decrease in the levels of plasma total protein and albumin, the activity of antioxidant enzymes as well as altered histopathological results. The prepared formula, with its powerful antioxidant and anti-inflammatory characters, succeeded to prevent to a great extent these metabolic disorders in a dose dependent manner. This supports the use of the prepared dietary supplement as a complimentary therapy to alleviate nonalcoholic fatty liver disease (NAFLD) and to limit the progression of this disease into the more aggressive form which is the inflammatory steatohepatitis (NASH).

SIGNIFICANCE STATEMENTS

- Analysis of the formulated dietary supplement showed that it possesses potent antioxidant activity and the HPLC analysis revealed the presence of several polyphenolic compounds known with their antioxidant activity
- This study also revealed that dietary supplement was capable of overcoming the metabolic disorders due to the nonalcoholic fatty liver disease in rat model of induced fatty liver as evidenced from the biochemical analysis and histopathological examination

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