December 30, 2018

PharmacologyOnLine

Archives • 2018 • vol.3 • 261-271

THE ROLE OF CRUDE METHANOL LEAF EXTRACT OF *MORINGA OLEIFERA* IN PROTECTION AGAINST HYPERLIPIDAEMIA AND CARDIOMYOPATHY IN ALBINO RAT FED A HIGH CHOLESTEROL DIET AND CARBIMAZOLE.

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Abstract

Cholesterol-enriched diet has been reported to adversely affect the health of humans and animal species. The prevention of cardiovascular disease through alternative medicine is rapidly gaining the attention of researchers all over the world. The antihyperlipidaemic and cardioprotective effects of crude methanol leaf extract of *Moringa oleifera* (MEMO) in male albino wister rats fed a high cholesterol diet (HCD) and carbimazole (CBM) was investigated. Twenty (20) rats were randomly grouped into four groups: A, B, C and D of five animals per group. Rats in groups A-C were coadministered HCD (2000mg/kg) and CBM (60mg/kg) once daily for six weeks. Group D served as normal control. Groups C received daily dose of crude MEMO (200mg/kg) for six weeks. Group B (positive control) received a standard lipid lowering drug (Atorvastatin, 20mg/kg) for six weeks. Lipid profiles: Total cholesterol (TC), High density lipoprotein (HDL-C), Low density lipoprotein (LDL-C), Very low density lipoprotein (VLDL), and Triglycerides (TG) were assayed or calculated; and CK, LDH and AST were assayed. The levels of TC, LDL, TG and VLDL were highly elevated significantly in the affected group (HCD+CBM alone) when compared with normal control ($p<0.05$). Administration of MEMO (200mg/kg) and atorvastatin (20mg/kg) separately in the presence of HCD+CBM challenge significantly lowered the elevated levels of TC ($p<0.01$ or $p<0.05$), LDL ($p<0.01$ or $p<0.05$), TG ($p<0.05$) and VLDL ($p<0.05$) when compared to the affected group. Whereas, HDL-cholesterol level was significantly elevated ($p<0.05$) by the extract in comparison with the affected group. The methanol extract showed a significant protective action on elevated creatine kinase, LDH and AST activities in the hypercholesterolaemic and carbimazole- overdosed rats. Histopathological results showed a concomitant association with the biochemical findings. This study shows that the extract has antihyperlipidaemic and cardioprotective effects, and can be used to prevent hypercholesterolaemia-induced cardiomyopathy or atherosclerosis.

Keywords: *ethnopharmacology, Moringa oleifera, antihyperlipidaemia, cardioprotection*

Introduction

Moringa oleifera is a ubiquitous and exceptionally nutritious vegetable tree with a variety of potential uses as ayuvedic herb. *Moringa oleifera*, popularly called the "miracle tree", is a monogeneric plant in the family Moringaceae. It has long been cultivated and all its parts being consumed and used for a variety of purposes across the tropics. This is because of its impressive range of nutritional and medicinal values (1). *Moringa oleifera* has gained usefulness in pharmacological activities as revealed in some studies (2-5)

Hyperlipidemia can also be defined as concentration of lipid in the blood of a fasted (12 h) patient that exceeds the upper range of normal for that species. Lipids are transported in a protein capsule. The size of that capsule, or lipoprotein, determines its density. The lipoprotein density and type of apolipoproteins it contains determines the fate of the particle and its influence on metabolism (6, 7).

Hyperlipidaemia contributes significantly in the manifestation and development of atherosclerosis and coronary heart diseases (CHD). Cardiovascular diseases, including atherosclerosis, are the most common cause of mortality and morbidity worldwide (6). Although several factors, such as diet rich in saturated fats and cholesterol, age, family history, hypertension, hypothyroidism and life style etc play a significant role in causing heart failure. Hypothyroidism has widely been established to cause hypercholesterolaemia, which will raise the risk for CHD by many times. This can cause CHD, which is the precursor to heart failure. Excessive use of the anti-thyroid hormone drug, carbimazole could not only lead to hypercholesterolaemia but also cause oxidative stress on organs and tissues, including the heart muscles (8-12).

Thus investigation into the prevention and treatment of abnormal cholesterol levels and cardiac muscle injury, with a simple and common medicinal plant like *Moringa oleifera*, is an important step in maintaining optimum health. The aims of this research were to evaluate the phytochemical constituents of *Moringa oleifera* and the antihyperlipidaemic and cardioprotective effects of methanol leaf extract of *Moringa oleifera* in rats fed on high cholesterol diet.

Materials and Methods

Experimental animals

Twenty (20) apparently healthy adult male albino rats about three (3) months old were used for the research. They were obtained from the animal house of the college of medicine, University of Nigeria Teaching Hospital (UNTH) Enugu. The rats were divided into four (4) groups. Groups: A, B, C and D, of five (5) rats per group according to their body weights (200±30g) and each group housed separately in clean steel gauzed cages. They were housed under standard condition of temperature (28±3°C) and a 12hours light/ 12hours dark cycle at the animal house at Anatomy department, College of medicine, University of Nigeria, Enugu Campus. They were allowed to acclimatize for a period of two (2weeks). The rats were fed with standard pellets (Top feed, Nigeria) and clean water *ad libitum.* The cages were cleaned daily and food and water changed daily. All the animals used in this study were handled according to Institutional guidelines describing the use of rats and in accordance with the American Physiological Society guiding principles for research involving animals and human beings (13). In addition, proper care was taken as per the ethical rule and regulation of the concerned committee of the University of Nigeria, Nsukka, Enugu State, Nigeria.

Collection and authentication of M. oleifera

Fresh leaf samples of M. *oleifera* were obtained from Adazi Nnukwu in Aniocha LGA of Anambra, a State in South Eastern part of Nigeria, in the month of April, 2018. The leaves were authenticated by a consultant taxonomist in the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka with voucher number (UNN/0042). The leaves were carefully picked and dried at room temperature prior to extraction.

Preparation of crude methanol extract

The completely dried leaves of *Moringa oleifera* were ground into powder using a hammer mill (500# grinder/Fuyu metal, Linyi fuyu metal product, co, Ltd, China) and thereafter, passed through 52 mm sieve

(turgens and co, Germany). The powdered leaf (400g) of *Moringa oleifera* was weighed out and macerated in 2.5liters of absolute methanol in a gallon and left for 48hours. The mixture was intermittently agitated during the extraction process. After 48hours, the mixture was sieved using muslin cloth and filtered with Whatman No.1filter paper and the filtrate was concentrated by evaporating in an oven (Gallenkamp, UK) at 60°C. The yield of the syrupy methanol extract was 10.6 % (w/w). The appropriate dose for the experiment was reconstituted from the concentrate (20 g) using physiological saline as the solvent or diluent (200 mg/ml). This was labeled the methanol extract of *Moringa oleifera* (MEMO).

Preliminary Phytochemical screening

Preliminary phytochemical screening of leaf of M. *oleifera* for the presence of glycosides, flavonoids, saponins, steroids, tannins, carbohydrates, proteins and terpenoids was carried out at Department of Pharmacognosy, Faculty of Pharmaceutical Science, University of Nigeria Nsukka. Procedures outlined by Trease and Evans (14) were employed for the analyses.

Preparation of high-cholesterol diet (HCD)

A mixture of 75g of commercially available cholesterol powder and 9g of sodium deoxycholate (bile salt added to increase bioavailability) was dissolved in coconut oil and made up with the same solvent to 300ml to give 250mg/ml.

Preparation of carbimazole solution

Hundred tablets of 5mg (i.e. 500mg) carbimazole obtained from *hovid*® Inc., Malaysia were ground to powder, dissolved in distilled water and made up to 250ml in a measuring cylinder to give a stock concentration of 2mg/ml.

Preparation of atorvastatin solution

Ten tablets of 10mg (i.e. 100mg) atorvastatin obtained from *pfizer*® Inc., New York, USA were ground to powder, dissolved in distilled water and made up to 50ml mark in a volumetric flask to give a stock concentration of 2mg/ml.

Induction of hyperlipidaemia and cardiac injury

Each experimental rat was co-administered with high cholesterol diet (HCD) at the dose of 2000mg/kg and high dose of 60mg/kg carbimazole solution through an oral gauge every morning for six weeks.

Experimental design

The rats were divided into four (4) groups:
Group A received 60mg/kg of carbimazole, 2000mg/kg of HCD with neither extract nor drug for 6 weeks. This served as the negative control group.
Group B received carbimazole, HCD and 20mg/kg of Atorvastatin for 6 weeks. This served as positive control group.
Group C received carbimazole, HCD and 200mg/kg of crude MEMO
Group D was given neither HCD nor the extract; therefore no treatment. This group served as the normal control

Sacrificing of Animal and Sample Collection

At the expiration of six weeks, fasting blood samples were collected from the axillary vein under chloroform anesthesia. The blood samples collected into plain tubes were centrifuged to obtain serum for estimation of lipid profile (Total Cholesterol, HDL cholesterol, LDL Cholesterol, VLDL Cholesterol and triglyceride), CK-MB, LDH and AST.

Biochemical analysis

Measurement of serum lipid profile

Total cholesterol was estimated using cholesterol oxidase method as described by Fredrickson *et al.* (15). HDL was determined using Precipitation method as described by Albers *et al.* (16). Triglyceride (TG) was estimated using glycerol phosphate oxidase method as described by Fossati and Prencipe (17). VLDL determination –(Calculated: VLDL = TG/2.2). LDL was determined using the Friedewald's equation:

LDL Cholesterol = Total cholesterol—(VLDL + HDL) Cholesterol

Measurement of Cardiac biomarkers

The serum activity of the creatine kinase isoenzyme, CK-MB was measured using the kinetic colorimetric method according to Gerhardt *et al.* (18). LDH was measured using ENZOPAK LDH kit. AST was

by colorimetric method as described by Reitman and Frankel (19).

Histopathological studies

The excised heart was fixed in 10% formal saline for 24 hr and further processed using the conventional paraffin wax embedding technique for light microscopic examination. The paraffin-embedded testicular tissues were sectioned at 5 microns and stained using the Haematoxylin and Eosin [H and E] Staining procedure (20). The histological sections were examined using an Olympus ^{TM} light microscope.

Statistical analysis

Data analysis was done using GraphPad prism version 6.0 (GraphPad, San Diego, CA, USA). The results of the biochemical assays were reported as mean±SEM (standard error of mean). The level of significance was tested using one way analysis of variance (ANOVA), followed by the Tukey post hoc analysis. Probability levels of less than 0.05 were considered significant.

Results

Phytochemical results

Phytochemical analysis indicated the presence ofcarbohydrate, reducing sugar, alkaloids, glycosides, flavonoids, tannins, saponins, terpenoids, proteins, acidic compounds and steroids in the methanol leaf extract (Table 1).

Effects of crude methanol leaf extract of Moringa oleifera on body weight.

The effect of crude methanol leaf extract of *Moringa oleifera* on body weight of albino rats fed a high cholesterol diet and carbimazole is represented in figure 1. It was observed that rats in the (HCD+CBM) group showed symptoms of hyperlipidaemia and heart failure. The mean increase in body weight was highest in the (HCD+CBM) group (negative control) in comparison with other groups.

Biochemical results

Table 2 shows the results of serum lipid profile parameters: total cholesterol, HDL, LDL, Triglyceride (TG) and VLDL levels in four (4) groups of five (5) animals which received oral administration of high cholesterol diet (2000mg/kg body weight) and high dose of carbimazole (60mg/kg) and/or crude methanol leaf extract of *Moringa oleifera* (MEMO; 200mg/kg) or atorvastatin (20mg/kg) for 6 weeks. From the results, MEMO showed significant antihyperlipidaemic potentials ($P<0.05$) in comparison with negative control (HCD+CBM). Furthemore, it was observed that the standard drug (atorvastatin) showed much better antihyperlipidaemic effects in the rats than the extract.

In Figure 2, albino Wister rats which received (HCD+CBM) alone (negative control) for 6 weeks showed the highest serum CK-MB, LDH and AST levels (cardiac markers of injury) [Figure 2A, 2B and 2C; respectively]. Data show that coadministration of high cholesterol diet and high dose carbimazole for 6 weeks induced a significant increase in the level of serum CK-MB, LDH and AST in the rats ($p<0.01$ or $p<0.05$) in comparison with normal control. The positive control, atorvastatin, a standard lipid-lowering drug was shown to induce a significant decrease in CK-MB, LDH and AST levels in the rats compared with (HCD+CBM) alone ($p<0.01$ or $p<0.05$). Interestingly, MEMO also significantly decreased CK-MB, LDH and AST levels when compared with (HCD+CBM) alone ($p<0.01$ or $p<0.05$).

Histopathological results

In figure 3, microscopic examination of the hearts isolated from the rats at sacrifice revealed no histopathological alteration in the normal control rats (Figure 3D). Presence of significant distortion and necrosis of the myocardial fibres were observed in the heart of rats treated with oral administration of high cholesterol diet and high dose carbimazole (Figure 3A); however non-significant degenerations were observed in rats administered with atorvastatin (20mg/kg) and MEMO (200mg/kg) separately (Figure 3B and 3C respectively). The hearts of rats in group B and C showed no significant histological alterations when compared with the normal control group.

Discussion

Cardiomyopathy is a disease of the heart muscle that makes it difficult for the heart to pump required blood volume. It can lead to heart failure. It may be due to heart valve problems, myocardial infarction or other cardiac disorders; such as atherosclerosis,

which in itself is as a result of prolongation of abnormally high blood lipid levels. Symptoms includes breathlessness with exertion or even at rest, chest discomfort and pressure, fatigue, swelling of legs, ankles and feet, bloating of the abdomen due to fluid buildup etc. Antilipidaemic drugs have been considered among the main therapeutic drugs in the treatment of cardiomyopathy.

Furthermore, the use of the anti-thyroid drug, carbimazole has been implicated in potentiating cardiac injury. Cholesterol-enriched diet has been reported to adversely affect the health of humans and animal species. However, the treatment or prevention of cardiovascular disease through alternative medicine is rapidly gaining the attention of researchers all over the world.

In hyperlipidaemic rat models, cholesterol aggravates myocardial ischaemia reperfussion injury via activating endoplasmic reticulum stress-mediated apoptosis (21). Carbimazole, a potent anti-thyroid drug for treatment of thyrotoxicosis, has been extensively reported to have toxic effects on delicate organs, such as the testes and other tissues, as extensively reported by Saber *et al.* (22-24) and Orji *et al*. (25). Furthermore, hypothyroidism has been reported to alter antioxidant defense system in rat (23, 26). Hence, carbimazole has been scientifically proven to cause oxidative stress, at toxic levels, in the system. Based on this scientific evidence, in this present study, we administered an overdose of carbimazole (60mg/kg, oral), over 40 times the dosage Saber *et al.* administered, for 6 weeks.

Aside enhancing the buildup of cholesterol in the blood, the mechanism of cardiac injury by carbimazole is not well understood. Although the mechanism of testicular injury by carbimazole has been reported to be due to increase in oxidative stress, we believe that the coadministration of high cholesterol diet and the high dose carbimazole to the rats for 6 weeks, synergistically induced the chronic cardiac side effects like cardiomyopathy or congestive heart failure observed in this study. Therefore, the myocardial changes could be explained on the basis of increased oxidative stress. An imbalance between free radical production and endogenous myocardial oxidants has been suggested to play major role in pathogenesis of congestive heart failure (27).

In the last few decades, there has been the growing use of herbal medicines because most plants are believed to have medicinal value, *Moringa oleifera* being one of them (28). *M. oleifera* is one of the numerous plant adjuvants for the treatment of hyperlipidaemia. The aim of this study was to evaluate the antihyperlipidaemic effect of methanol leaf extract of *M. oleifera* in hypercholesterolaemic rats; using atorvastatin as a reference drug. The methanol extract of *M. oleifera* in this study prevented hyperlipidaemia and cardiotoxicity after the 6weeks treatment period. Treatment with atorvastatin caused significant antihyperlipidaemic effect. Our histopathological results showed concomitance with our biochemical results. Our observations agree with the works by Ganatra *et al*. (4) and Fikriansya *et al*. (5)

MEMO showed ameliorating effects in the hypercholesteraemic albino wistar rats comparable to the reference drug (atorvastatin). The comparable effect of *M. oleifera* with atorvastatin in reducing serum lipid profile levels and reducing the elevated cardiac markers in the rats suggests similar mode of action. Since the result of the study revealed that MEMO has beneficial effect on the lipid profile and is cardioprotective, its mechanism of action could be deduced from our research.

Cholesterol homeostasis is regulated by the two mechanisms: cholesterol biosynthesis in which HMG-Co-A reductase catalyses rate limiting process and cholesterol absorption of both dietary cholesterol and cholesterol clearance from the liver through biliary secretion (29). Furthermore, by targeting hepatocytes and inhibiting HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor, they alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure.

Phytochemical result revealed the presence of some bioactive constituents such as: carbohydrate, reducing sugar, steroids, saponins, tannins, terpenoids, flavonoids, alkaloids, and glycosides. This finding agrees with the findings of Bamishaiye *et al*. (30). Alkaloids, tannins, flavonoids, saponins and terpenoids are reported to have bioactive anti-hyperlipidaemic principles (31). The presence of saponins, tannins, flavonoids, alkaloids and

terpenoids may explain why *M. oleifera* is used for hypertension treatment (3); because the constituents are used pharmacologically to treat hypercholesterolaemia. Hyperlipidaemia and resultant oxidative stress could be prevented by endogenous and exogenous antioxidants (32). Flavonoids are strong antioxidants, and could be used to ameliorate oxidative stress.

Acknowledgments

The authors express deep sense of gratitude to Mr. Chris Ireoba, The head of department of the Laboratory Division at Eastern Nigeria Medical Centre, Enugu, and all the technical staff for their kind cooperation.

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Table 1: Preliminary phytochemical analysis of crude methanol extract of *Moringa oleifera* leaf.

CONSTITUENT	INDICATION
Carbohydrate	++
Reducing Sugar	+
Alkaloids	+++
Glycosides	++
Saponins	++
Tannins	+++
Flavonoids	+++
Resins	++
Proteins	+
Oils	–
Acidic Compounds	–
Terpenoids	++
Steroids	+

Key: +++ = More intensely present; ++ = Present; + = Present (in trace amount); – = Absent

Table 2: Comparison of serum lipid profile parameters of treated groups with negative controls.

Groups	Serum Total Cholesterol (mmol/L)	Serum HDL (mmol/L)	Serum LDL (mmol/L)	Serum Triglyceride (mmol/L)	Serum VLDL (mmol/L)
HCD+CBM (Negative Control)	5.747 ± 0.245	1.203 ± 0.199	3.570 ± 0.374	2.130 ± 0.364	0.970 ±0.165
HCD+CBM+ATOR (Positive Control)	4.237 ± 0.118**	2.117 ±0.107*	1.220 ± 0.009**	1.270±0.050*	0.907 ± 0.003
HCD+CBM+MEMO (Test)	4.516± 0.204*	2.097 ± 0.104*	1.715 ± 0.087*	1.549 ± 0.029	0.704± 0.013*
Normal Control	4.450 ± 0.126*	2.225 ± 0.063*	1.618 ± 0.046*	1.336 ±0.038*	0.607 ± 0.017*

Values given as Mean ± SEM. **P<0.01 or *P<0.05 is significant when HCD+CBM (negative control) is compared with all other groups.

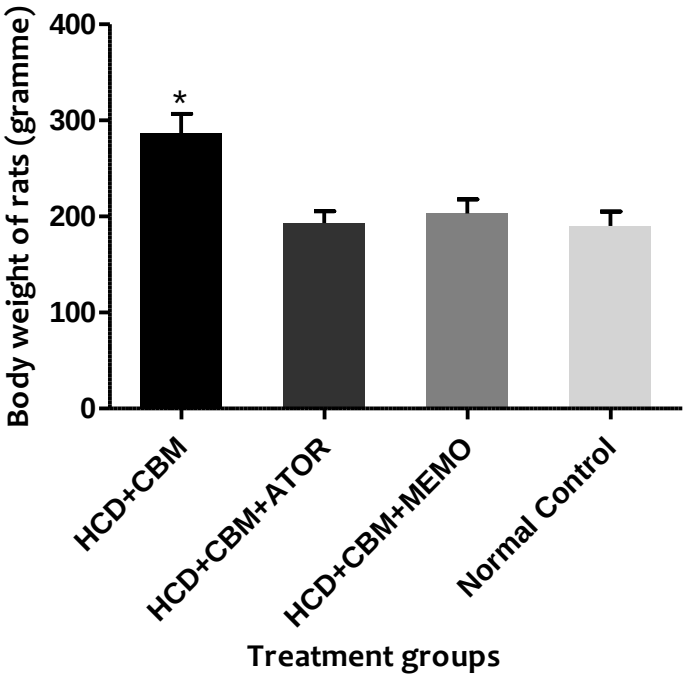

Figure 1: Effects of crude methanol leaf extract of *Moringa oleifera* on body weight**.** Histogram show the body weight of rats in the experimental groups. The preliminary data show coadministration of HCD+CBM induced a significant weight gain. However, oral administration of atorvastatin or MEMO significantly induced lower body weight when compared with (HCD+CBM) (negative control). Albino whister rats (n=5) were administered with Atorvastatin or MEMO once a day for 6 weeks in the presence of HCD+CBM challenge. The data are presented as mean±SEM of body weight (gramme) for individual treatment. See *Materials and Methods* for experimental details. Statistical analyses were performed using ANOVA (* $p < 0.05$).

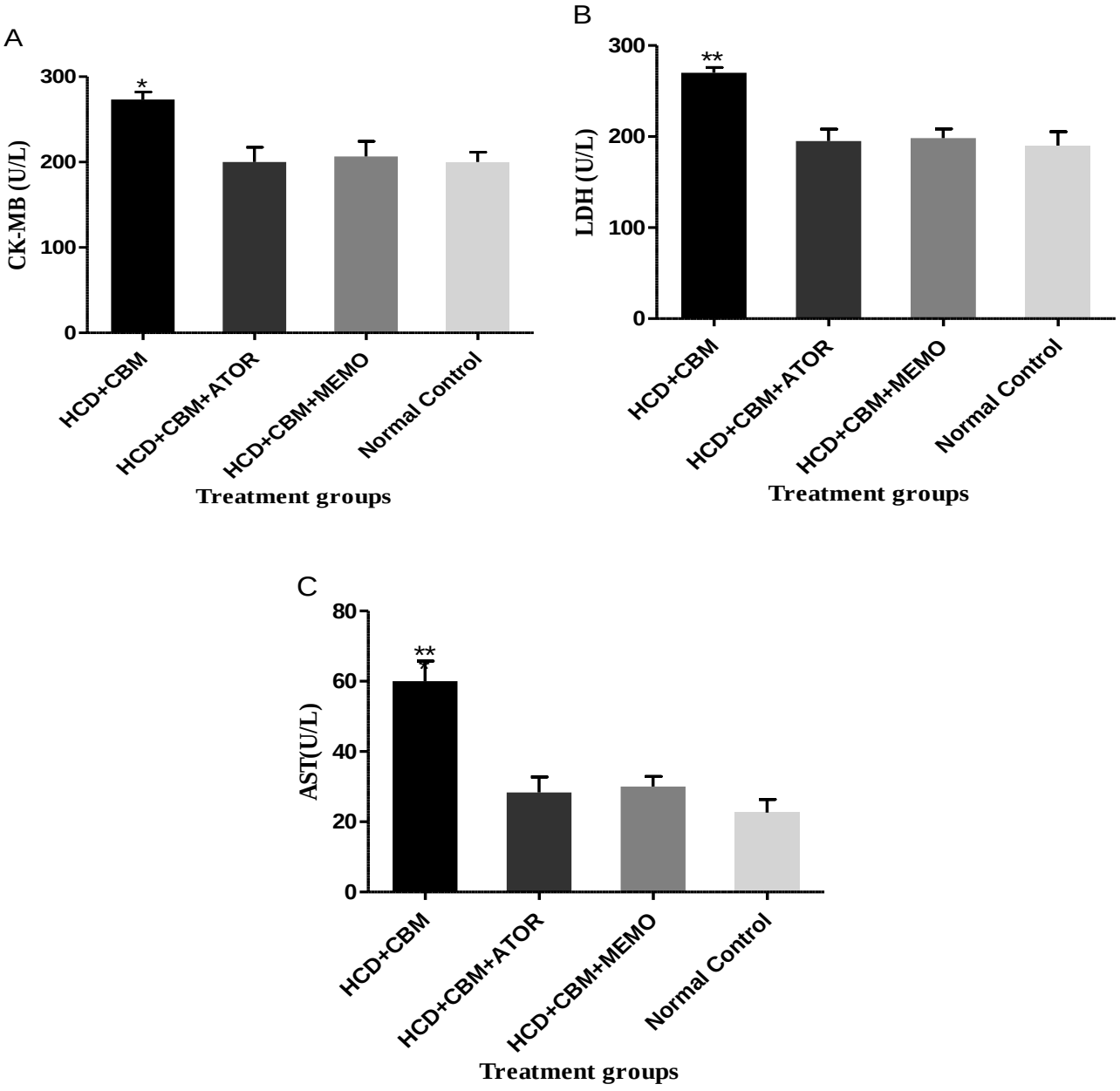

Figure 2. Serum CK-MB, LDH and AST levels after intervention with Atorvastatin (20mg/kg, oral) or MEMO (200mg/kg, oral). The histograms A, B and C show serum CK-MB, LDH and AST levels respectively following treatment with (HCD+CBM) alone or in combination with Atorvastatin or MEMO. The preliminary data show Atorvastatin or MEMO significantly induced lower CK-MB, LDH and AST levels when compared with (HCD+CBM) (negative control). Albino whister rats (n = 5) were administered with Atorvastatin or MEMO once a day for 6 weeks in the presence of HCD+CBM challenge. The data are presented as mean±SEM of serum CK-MB, LDH and AST levels (U/L) for individual treatment. See *Materials and Methods* for experimental details. Statistical analyses were performed using ANOVA. (** $p < 0.01$ or * $p < 0.05$).

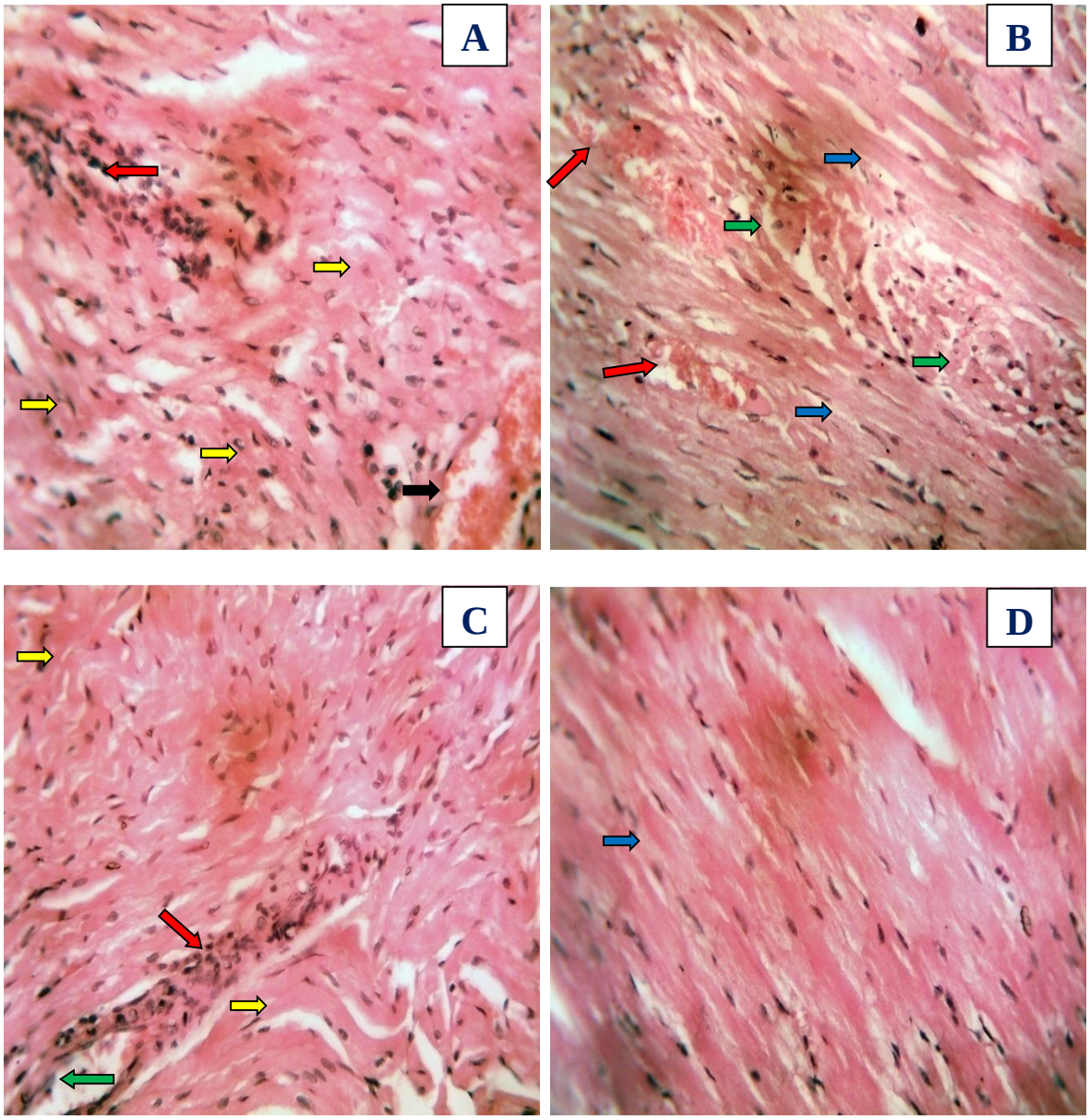

Figure 3: Histopathology and photomicrograph of heart. (A) Co-administration of high cholesterol diet (HCD) and carbimazole (CBM)-treated rats. Myocardial fibres are distorted (yellow arrows), some have undergone necrosis with infiltration by leucocytes (red arrow). There is congestion of the blood vessel (black arrow). **(B)** HCD + CBM + Atorvastatin-treated rats. Myocardial fibres are normal –longitudinal (blue arrows) and oblique (green arrows) sections. The blood vessels seen are congested (red arrows). **(C)** HCD + CBM + MEMO- treated rats. Myocardial fibres appear wavy (yellow arrows – early sign of myocardial necrosis). Some fibres around a blood vessel (green arrow) are necrotic with infiltration by leucocytes (red arrow). **(D)** Normal control rats. No pathological lesions in the myocardial fibres (blue arrow). [Stain: H and E; ×400].