

## The Inhibitory Effects of Hypercholesterolemia and Platelet in Fermented and Non-Fermented Preparation of Garlic

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### Abstract

*This Dietary cholesterol augments lipid profile and primes production and activation of platelets, leading to development of atherosclerosis which produce several detrimental effects on cardiovascular health. Ethnomedicine and Mediterranean diet are natural sources and cost effective modes against several ailments including cardiovascular diseases while fermented foods have gained interest due to their increased nutrient profile, enhanced bioavailability and efficacy. Garlic has been known to reduce cholesterol and inhibit platelet activation. We examined whether fermented garlic ameliorates effects of hypercholesterolemia and platelet functions in rats. Methodology: Male SD rats were fed with hypercholesterolemia diet and treated with spirulina, fermented and non-fermented preparations of garlic for one month. Platelet aggregation and granule secretion were assessed to evaluate platelet activation. Liver and kidney weights, lipid and enzymatic profile of serum and whole blood analysis was performed. Expressions of SREBP, ACAT-2 and HMG-CoA were assessed using RT-PCR while liver and adipose tissues were analyzed for histological changes. Both fermented and non-fermented garlic inhibited platelet aggregation and granule secretion while fermented garlic showed greater inhibitor tendency. Fermented garlic significantly reduced liver weight and triglycerides concentrations than non-fermented garlic. Similarly, fermented garlic greatly abrogated the detrimental effects of steatosis on liver and adipose tissues. Fermented garlic significantly improved lipid profile and modulated platelet functions, thereby inhibiting atherosclerosis and platelet related cardiovascular disorders.*

**Keywords:** Hypercholesterolemia, Atherosclerosis, Fermented garlic, Ethnomedicine

## 1. INTRODUCTION

The Cardiovascular diseases (CVD) are among leading causes of death in modern societies. One of the main underlying pathology of CVD is atherosclerosis, for which hyperlipidemia is major known risk factor [1-4]. Hypercholesterolemia augments production and hyper-activation of platelets that primes in response to different agonists, and an underlying pro-coagulant state, consequently leading to hypertension, atherothrombosis, myocardial infarction and stroke [5-7]. Activated platelets can potentially promote

atherosclerosis while pharmacological suppression of platelets has been documented to clinically reduce thrombotic events, thereby inhibiting atherosclerosis and other CVD [8, 9]. Beside the availability of clinical drugs to reduce cholesterol and platelet activation, there is need to develop safer approaches to curtail side effects and complications which may include use of natural products with antithrombotic properties [10]. Recently, researchers have gained interest in ethnomedicine and natural products as potential treatment and prevention for CVD, while various dietary and herbal compounds have shown to reduce atherosclerosis [11, 12]. Mediterranean diet possess anti-atherosclerotic and anti-thrombotic properties due to presence of bioactive compounds which affect platelet function and prevent future risk of developing thrombosis [13].

Garlic has long been used for its potential health benefits and considered as one of the best disease preventive foods as it exhibits anti-platelet, cardio-protective, pro-circulatory, hypo-lipidemic, anti-cancer, chemopreventive and immune booster properties [14]. Enriched flavor and improved nutrient profile are significant commercial and health benefits obtained from fermentation of food, ultimately enhancing quality and efficacy of food or herbal products due to increased bioavailability [15]. We intended to examine and compare whether fermented and non-fermented garlic preparations ameliorate cholesterol and modulate platelet functions in hypercholesterolemic rats.

## **2. Experiment Materials and Methods**

### **2.1 Chemicals**

Collagen (Native collagen fibrils (type I) from equine tendons) and ADP were purchased from Chrono-log (Havertown, PA, USA). ATP assay kit was acquired from Biomedical Research Service Centre (Buffalo, NY, USA) and TRIZOL solution from Invitrogen (Carlsbad, CA, USA). Primers against SREBP, ACAT-2, HMG-CoA and GAPDH were obtained from BIONEER (Daejeon, Republic of Korea). Water was obtained from J. T. Baker (Phillipsburg, NJ, USA). All chemicals were of reagent grade.

### **2.2 Preparation of fermented garlic**

Fermented garlic was prepared by autoclaving and steaming of unpeeled garlic to produce black garlic, followed by addition of lactic acid producing bacteria for the second round of fermentation.

### **2.3 Animals and dosage**

Male Sprague-Dawley (SD) rats (180–200 g) were purchased from Orient Co. (Seoul, Korea) and acclimatized for 1 week prior to experiment in a special animal room with 12/12 light/dark cycle at temperature and humidity of about  $23\pm 2^{\circ}\text{C}$  and  $50\pm 10\%$ , respectively. Rats were randomly divided into five groups ( $n = 5$  in each group) as Normal (normal chow), Control (HCD), Garlic, Fermented garlic (F. garlic) and Spirulina. All the groups fed with hypercholesterolemia diet (HCD) for one week except normal chow group and later orally administered with vehicle, garlic, F. garlic (300 mg/kg) and spirulina (60 mg/kg) once a day along with HCD for one month. One hour after last oral administration, blood and tissues were collected from rats for experimentation and further processing. Experiments were conducted following IACUC guidelines and the protocols were approved by the Ethics Committee of College of Veterinary Medicine, Kyungpook National University, Daegu, Korea (Permit number: 2018-0090).

### **2.4 Preparation of washed platelets**

Whole blood was collected from rats via heart puncture and transferred to a tube containing anticoagulant

acid citrate dextrose solution. Blood was centrifuged at  $170 \times g$  for 7 min to obtain platelet-rich plasma (PRP). The PRP was further centrifuged at  $350 \times g$  for 7 min to isolate washed platelets. The platelet concentration was adjusted at  $3 \times 10^8$  cells/mL using Tyrode's buffer (137 mM NaCl, 12 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 2 mM KCl, and 1 mM MgCl<sub>2</sub> and NaHPO<sub>4</sub>, pH 7.4) and used for platelet aggregation assays. All preparation procedures were performed at room temperature ( $23 \pm 2^\circ\text{C}$ ).

## **2.5 Ex-vivo platelet aggregation and ATP release assay**

Light-transmission aggregometry (Chrono-log, Havertown, PA, USA) was performed to assess platelet aggregation as previously described [17]. Briefly, washed platelets obtained from Normal, Control, Garlic, F. garlic and Spirulina treated rats were incubated at  $37^\circ\text{C}$  with continuous stirring and stimulated with collagen or ADP for 5 min.

To access the ex-vivo effects of given treatments on dense granule secretion, ATP assay was performed as previously described [16]. Briefly, washed platelets obtained from Normal, Control, Garlic, F. garlic and Spirulina treated groups were incubated at  $37^\circ\text{C}$  with continuous stirring and stimulated with collagen or ADP for 5 min. The aggregation reaction was terminated and the platelet mixture centrifuged. The supernatant obtained was used to measure ATP secretion with a luminometer (GloMax 20/20, Promega, Madison, WI, USA) using an ATP assay kit (Biomedical Research Service Center).

## **2.6 Blood biochemical analysis**

Fresh whole blood was taken directly from the heart of animals into EDTA tubes for plasma analysis and hematological parameters. An automatic hematology analyzer (Sysmex XE- 2100D; Sysmex Corporation, Kobe, Japan) was used to perform a complete blood cell count on each blood sample, including hematocrit analysis. Serum samples were collected from blood and Total cholesterol (TC), high-density lipoproteins (HDLs), LDLs, triglycerides, GPT, GOT and creatinine levels were analyzed using the enzymatic method (FUJI DRI-CHEM 4000i, FUJIFILM, Tokyo, Japan).

## **2.7 Histological analysis**

The liver and adipose tissues were collected and fixed overnight in 10% formalin solution, dehydrated, embedded in paraffin, and cut into 5- $\mu\text{m}$  sections. Cross sections of these tissues were stained with hematoxylin and eosin (H&E) and no. of hepatocytes or adipocytes was measured using ImageJ and results presented as a bar graph as percent of normal histology of tissues.

## **2.8 RNA extraction from liver tissue and real-time polymerase chain reaction**

For mRNA expression of SREBP, ACAT-2, HMG-CoA, total RNA was extracted from liver tissue with TRIZOL solution using a homogenizer as previously described [19]. 2 $\mu\text{g}$  of RNA was annealed with OligodT by heating at  $70^\circ\text{C}$  for 10 min and kept in ice for cooling for another 10 min. Reverse transcriptase was carried out using a commercially available pre-mix (Bioneer, South Korea) by heating at  $42^\circ\text{C}$  for 1.5 h and the reaction was stopped by heating at  $95^\circ\text{C}$  for 5 min. The resultant cDNA was added into PCR Premix (Bioneer, South Korea) with the respective target gene primers. The PCR product was run on 1% agarose gel that was stained with ethidium bromide (EtBr) and the gel images were developed using an imager (GE Healthcare, Chicago, IL, US). The band intensities were normalized against GAPDH which was used as the housekeeping gene.

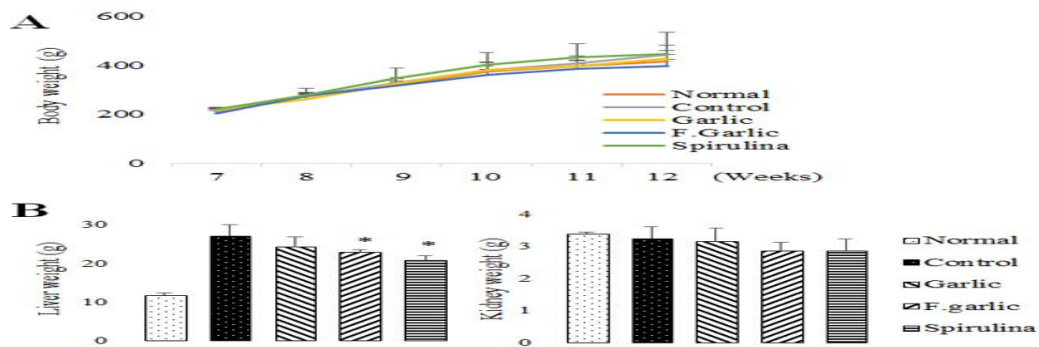
## 2.9 Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test to measure statistical significance of the differences observed (SAS Institute Inc., Cary, NC, USA). All data were presented as means  $\pm$  standard error of the mean (SEM). A p-value of 0.05 or less was considered statistically significant.

## 3. Result and Discussion

### 3.1 Effects of garlic and F. garlic preparations on body and organs weights

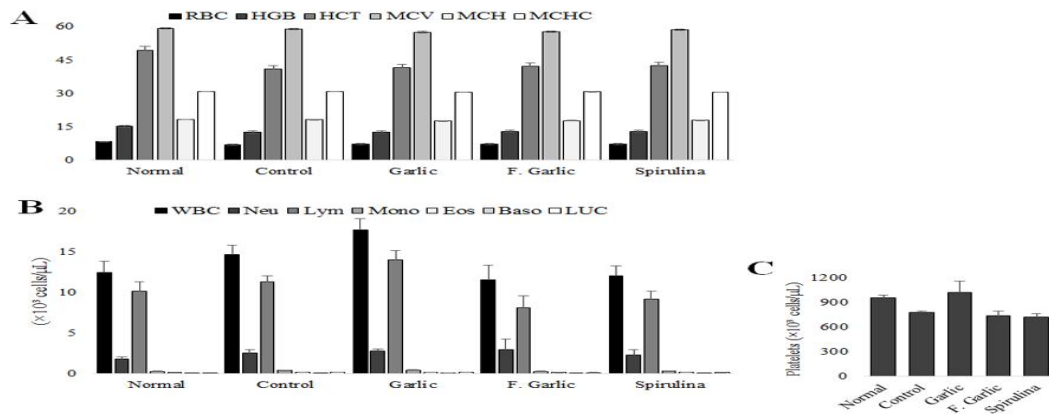
There was no significant body weight difference among groups while high cholesterol diet had significantly increased the liver weight of control group rats compared to normal chow group. There is significant inhibitory trend seen in treatment groups of liver weights, especially in fermented garlic and spirulina group while no difference was observed among kidney weight (Figure 1).



**Figure 1. Effects of garlic and fermented garlic preparations on body and organ weights. Rats weight was observed throughout the experimental period and at the end rats were killed and tissues were weighed. Graph represents the mean  $\pm$  SD (n = 5) \*p < 0.05.**

### 3.2 Blood analysis

As shown in Figure 2, no significant difference was observed among blood cells count, however, there was a little decrease in HCD treated groups compared with normal chow group while no significant difference was observed compared with control group (Figure 2).



**Figure 2. Blood analysis. Rat blood was collected in EDTA tube and analyzed for blood cells count and hematocrit. Graph represents the mean  $\pm$  SD (n = 5).**

### 3.3. Garlic and F. garlic inhibit agonist-induced platelet aggregation and ATP release

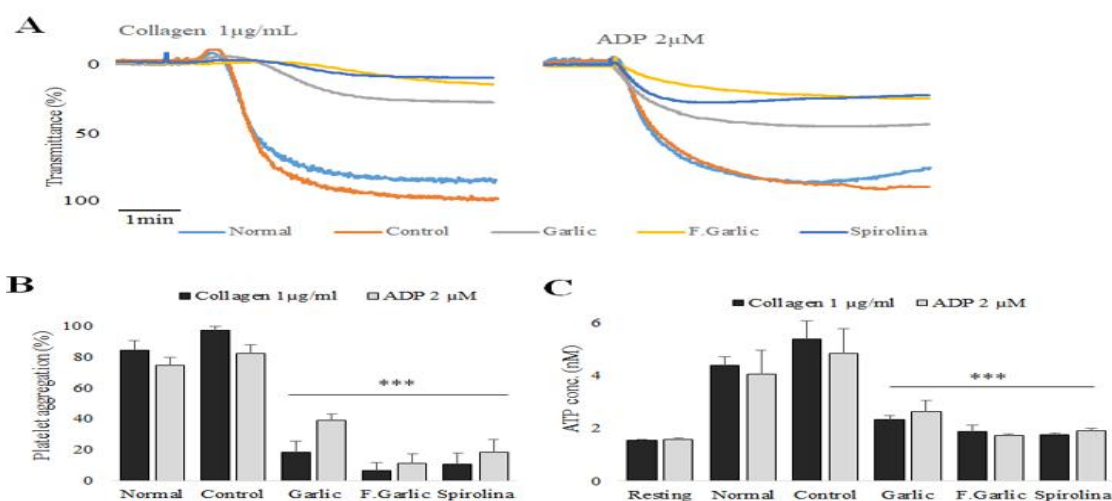
To access the ex-vivo effects of treatments on platelet aggregation, washed platelets were isolated from blood of different groups of rats and platelet aggregation assay was performed. All the treatment groups shown marked inhibition against collagen and ADP-induced platelet aggregation. Interestingly, Control group has shown hyper-activation of platelets than normal chow group. Fermented garlic has shown greater inhibitory tendency toward platelet aggregation among other groups (Figure 3A-B).

Activated platelets release the contents of granules such as alpha and dense-granules and these granules content secretion enhances platelet activation including intracellular signalling pathway.

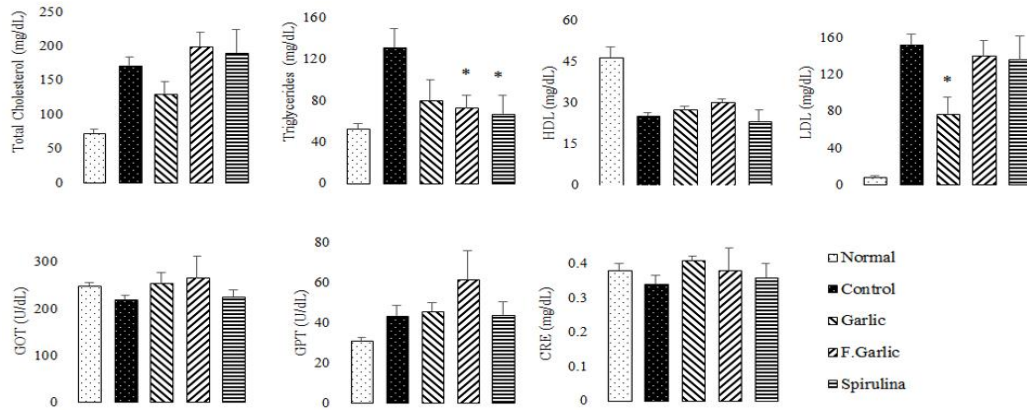
Early phases of platelet activation are characterized with rapid release of ATP [20]. We, therefore, assessed collagen and ADP-induced ATP secretion. As shown in Figure 3C, all the treatment groups significantly inhibited ATP release from dense granules in collagen and ADP-stimulated platelets. Although, all of them showed good inhibition on ATP release, a greater inhibitory trend has been observed among fermented and non-fermented preparations. These data suggest that given treatments exert anti-platelet effects through suppressive effects on platelets granules secretion.

### 3.4. Effects of Garlic and F. garlic on serum cholesterol and enzymes profile

Total cholesterol, LDL and triglycerides are the markers for increased bad cholesterol in the body. Figure 4, showing inhibition of triglycerides among all the treatments groups while significant reduction seen in F. garlic and spirulina group. LDL was greatly reduced in garlic group, however, effect on HDL was insignificant. Figure 4 also showing elevated GPT among HCD groups than normal group but difference was not statistically significant. Overall, no difference observed among serum levels of liver and kidney enzymes.



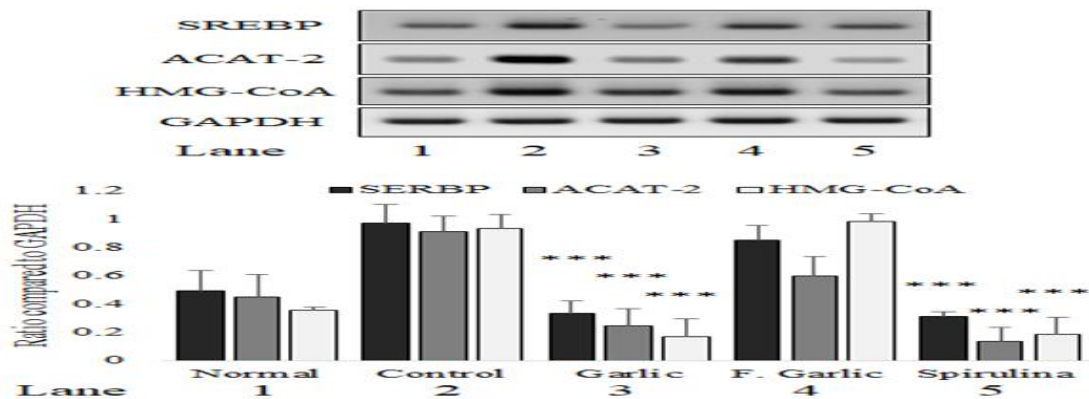
**Figure 3. Garlic and fermented garlic preparations inhibit agonist-stimulated platelet aggregation and granule secretion. (A-B)** Washed platelets obtained from normal chow (Normal), high cholesterol diet (Control), Garlic, F. garlic and Spirulina treated rats were incubated at 37°C with continuous stirring and stimulated with collagen or ADP for 5 min. **(C)** Washed platelets were incubated at 37°C with continuous stirring and stimulated with collagen or ADP for 5 min. Following termination of platelet aggregation reaction, the concentration of ATP was assessed using a luminometer. Results represent the mean  $\pm$  SD of experiments performed (n = 5). \*\*\*p < 0.001 versus vehicle control.



**Figure 4. Effects of garlic and fermented garlic preparations on serum profile. Blood was withdrawn without anticoagulant and collected serum undergone lipid profile and enzyme levels analysis. Graph represents the mean  $\pm$  SD of experiments performed (n = 5). \*p < 0.001 compared with vehicle control.**

### 3.5 Down-regulation of SERBP, ACAT-2 and HMG-CoA expression

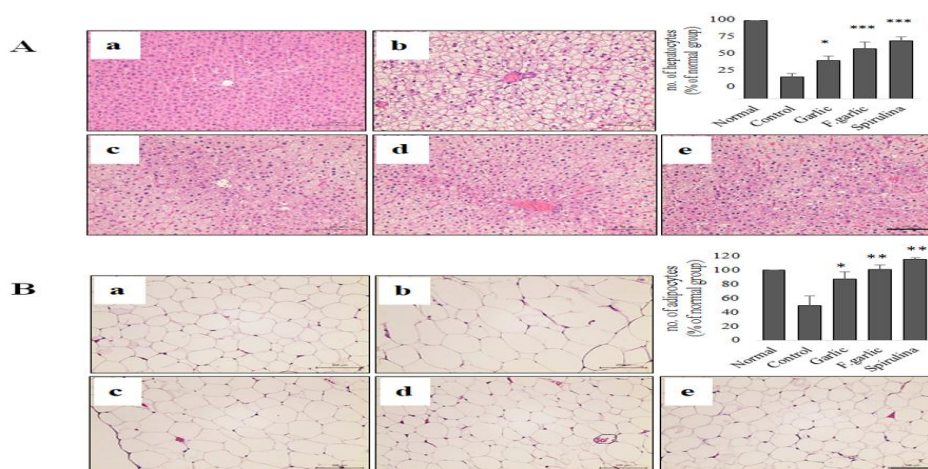
The measurement of sterol regulatory element binding protein (SREBP) is an important determinant of lipogenic gene transcription in the liver while acetyl-CoA acetyltransferase 2 (ACAT-2) and HMG-CoA reductase are related to cholesterol and fatty acid biosynthesis [19, 20]. Spirulina has been known to reduce effects of hypercholesterolemia by regulating SREBP, ACAT-2 and HMG-CoA [20, 21], therefore we used as a positive control. Based on our results, rats fed with only HCD has shown an increase in the mRNA expression of these genes while treatment groups have shown inhibitory trend in HMG-CoA reductase, ACAT-2 and SREBP mRNA expressions. However, only garlic and spirulina have shown statistically significant decrease in expression of HMG-CoA reductase, ACAT-2 and SREBP (Figure 5).



**Figure 5. Reverse-transcriptase polymerase chain reaction (RT-PCR) of liver tissue. The liver tissue from rats treated with Normal, Control, and groups treated with various samples along with high cholesterol diet was excised and RNA was extracted by homogenizing with TRizol solution. cDNA was obtained by reverse- transcriptas and the resultant cDNA underwent polymerase chain reaction with primers of SREBP, ACAT-2, HMG-CoA. GAPDH was used as the housekeeping gene. Resultant product was gel electrophorized and gel images was obtained as shown in (A). Gel electrophoresis was done in triplicate. The intensity of gel images were quantified using Image J software and rationalized against the housekeeping gene, GAPDH as shown in (B). \*\*\*p < 0.001 compared with vehicle control.**

### 3.6 Effects of Garlic and F. garlic on liver and adipose tissues

The H&E staining revealed the regular histology of liver tissue in normal group rats (Figure. 6A(a)) while a clear fatty degeneration (steatosis) of liver tissue was observed in high cholesterol diet (HCD) fed control rats due to microvesicular and macrovesicular fat deposits (Figure. 6A(b)). However, liver tissues of rats recovered the fatty degeneration especially in F. garlic and spirulina (Figure. 6A(c-e)). Similarly, adipose tissues showed the increased size of adipocytes due to fatty deposits in the control group while normal histology was observed in all the treatment groups especially F. garlic and spirulina (Figure. 6B). Hepatocytes and adipocytes were observed to be increased in size in HCD fed control rats while treatment groups significantly inhibited the fat deposition and cell size seem to be close to the normal group. Bar graphs showing the variation of histological changes in liver and adipose tissues among different groups (Figure. 6A-B).



**Figure 6. H&E and staining of liver and adipose tissues. (A-B) At the end of experiment, rats were killed and tissues were collected, fixed and stained. Figure A and B represents tissues from (a) Normal, (b) Control, (c) Garlic, (d) F. garlic and (e) Spirulina. Scale bar represents 100  $\mu$ m. Graph represents the mean  $\pm$  SD (n = 3). \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 compared with normal group taken as percent control.**

## 5. DISCUSSION

Cardiovascular diseases are prevailing at high pace due to modern life style and unhealthy eating habits developed countries especially in western societies. While there are numerous risk factors associated with CVD, a few notable causes include the pathophysiological hyper-activation of platelets and cholesterol, contributing to atherosclerosis and stenosis of blood vessel which lead to ischemia or coronary heart syndrome [3, 11]. Previous studies have reported that chronic high level blood cholesterol is associated with development of atherosclerosis. Generally, dietary cholesterol elicits the production of low density lipoproteins (LDL) by the liver and the intestine, and this change in plasma lipoproteins roots the development of atherosclerotic lesion. In the initial steps of atherogenesis, lipid peroxides injure the vessels which enhance adhesion and aggregation of platelets at the site of injury. Due to complications and high cost of medicines, there is need of alternative strategies for prevention of atherosclerosis e.g., ethnomedicine or dietary therapy. Studies have shown the potential preventive and therapeutic effects of ethnomedicine and natural products including Mediterranean diet in CVD[23].

Garlic has been used in treatment and prevention of CVD and proved to inhibit platelet aggregation [14,

24]. Fermented foods contained improved nutrient profile with enriched flavor, enhanced efficacy and bioavailability [13, 14]. In the present study, we evaluated the effects of fermented and non-fermented garlic in hypercholesterolemic rats. Spirulina has been known to inhibit platelet aggregation and atherosclerosis. Our results show that all the treatment groups inhibited platelet aggregation and granule secretion but fermented garlic shown greater inhibitory tendency. Liver weights increased in rats with alone fed HCD due to cholesterol micro- and macro-vesicular fat deposition while significant reduction observed in F. garlic and spirulina treated rats.

Dietary cholesterol increase lipid profile in HCD fed rats and their cumulative effect lead to atherosclerosis and other cardiovascular ailments. Studies strongly suggest that higher levels of triglycerides-rich lipoproteins or remnant cholesterol are casual risk factors for CVD and all-cause mortality while HDL cholesterol might be the a marker of cardiovascular health but is non-casual [25]. Our results show that alone HCD fed control rats increased the triglycerides concentration while all the treatment groups especially F. garlic and spirulina significantly have inhibited its elevation.

SREBP may promote the transcription of genes that participate in lipid droplet formation while ACAT-2 and HMG-CoA reductase are related to cholesterol and fatty acid biosynthesis [18, 19]. In our results, increased expression of SREBP, ACAT-2 and HMG-CoA reductase in HCD fed rats were observed which indicate that cholesterol synthesis was stimulated and in turn cholesterol accumulated in liver causing increased liver weight, but treatment groups inhibited the expression of these genes especially in garlic and spirulina. The possible reason for the ineffectiveness of F. garlic in some specific parameters may be that compound which is more effective in reducing these genes are degraded during fermentation and non-fermented garlic contained that effective compound to reverse the detrimental effects of hypercholesterolemia. It's very obvious from the histological results HCD fed rats increased the accumulation of micro- and macro-vesicular fat deposition in hepatocytes and adipocytes due to which their size enlarged, but the treatments especially F. garlic and spirulina effectively reduced the damaging effects of steatosis.

## 6. CONCLUSION

This Dietary cholesterol augments lipid profile and primes production and activation of platelets, leading to development of atherosclerosis which produce several detrimental effects on cardiovascular health. Ethnomedicine and Mediterranean diet are natural sources and cost effective modes against several ailments including cardiovascular diseases while fermented foods have gained interest due to their increased nutrient profile, enhanced bioavailability and efficacy. Garlic has been known to reduce cholesterol and inhibit platelet activation. Garlic and fermented garlic have significantly reduced liver steatosis by inhibiting micro- and macro- vesicular fat deposition, triglycerides along with inhibition of platelet aggregation and granule secretion in hypercholesterolemic rats. We data suggests that fermented garlic have therapeutic and preventive effects against atherosclerosis and platelet related cardiovascular disorders.

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