ijmps

Vol 04 issue 06
Section: Healthcare
Category: Research
Received on: 16/01/14
Revised on: 11/02/14
Accepted on: 03/03/14

THE HEPATOPROTECTIVE EFFECT OF *HIBISCUS ROSA SINENSIS* FLOWER EXTRACT ON DIET - INDUCED HYPERCHOLESTEROLEMIA IN MALE ALBINO WISTAR RATS

Anupam Biswas[1], Urban J.A. D'Souza[1], Shankar Bhat[2], Damodar D.[1]

[1]Department of Physiology, KVG Medical College, Sullia, Karnataka, India
[2]Department of Physiology, Yenepoya Medical College, Yenepoya University, Mangalore, KA, India

E-mail of Corresponding Author: anupambiswas111@yahoo.co.in

ABSTRACT

Background: Hypercholesterolemia is associated with to coronary heart disease (CHD) which is the principal cause of morbidity and mortality worldwide. Over the last few decades the reputation of herbal remedies has increased globally due to its therapeutic efficacy and safety.

Aim: This study was carried out to investigate the hepatoprotective potential of locally grown *Hibiscus rosa sinensis* flower extracts in diet induced hypercholesterolaemic rat hepatocytes.

Methodology: Male Wistar rats (180-230gm) were divided into seven groups of six animals each (n=6). The first group served as the control. All the animals were induced hypercholesterolemia by feeding pure cholesterol and cholic acid orally mixing with coconut oil with 2:1 ratio for 4 weeks. Three groups were given HRS flower extracts orally, at doses of 80mg / kg, 160mg / kg and 240 mg / kg body weight once a day for 5 days (acute) and another three groups were given same doses of HRS flower extracts for 30 days (chronic). At the end of treatment duration all animals were sacrificed by cervical dislocation. Laparotomy was performed. Blood was collected by cardiac puncture and allowed to clot. Serum was separated for the estimation of lipid profile, liver enzymes, total protein, albumin levels and plasma were used to estimate the MDA levels. Atherogenic Index (AI) was calculated.

Results: There was increase in body weight in cholesterol fed experimental animals which was reversed with HRS fed groups. There was a dose dependent increase in serum hepatic marker enzymes and total protein levels significantly ($p>0.001$) in the cholesterol fed groups and reversed with HRS flower extract fed acute ($p>0.005$) and chronic ($p>0.001$) groups. Increase in blood MDA level were seen in hypercholesterolaemic groups and significantly reduced ($p>0.05$) in HRS flower extract treated animals.

Conclusion: The present study suggests that HRS flower extract could play a hepatoprotective role against hypercholesterolemia through the regulation of cholesterol levels and inhibition of lipid peroxidation.

Keywords: Hibiscus rosa sinensis, cholesterol, atherogenic index, coronary heart disease, hepatic markers, lipid per oxidation.

INTRODUCTION

Liver diseases are major worldwide health problem, with high endemicity in developing countries. They are mainly caused by chemicals and drugs when taken in very high doses. The plant kingdom is undoubtedly valuable as a source of new medicinal agents. Over the last few decades the reputation of herbal remedies has

increased globally due to its therapeutic efficacy and safety. In recent years numerous traditional medicinal plants were tested for their antidiabetic, hypolipidemic and hepatoprotective potential in the experimental animals.[1] Indian traditional medicines and prescriptions with beneficial effects against various pathological conditions have recently attracted a great deal of attention as alternative therapies. Hyperlipidemia related liver diseases are the current medical as well social problem leading to increasing morbidity and mortality. The major risk factors of hyperlipidemia are associated with atherosclerosis which predisposes ischemic heart disease and cerebrovascular disease.[2] Indian traditional herbs or medicines have recently attracted a great deal of attention as alternative therapies for various pathological conditions. Those with beneficial effects against human diseases have been developed as a result of clinical experience accumulated over time and have been used widely for the treatment of a variety of inflammatory conditions, cardiovascular disorders and other ailments[3,4] Hibiscus rosa sinensis (HRS) flower extract is commonly used to treat symptoms related to blood circulation deficiencies, and is well known to reduce blood and plasma viscosity and thus improve microcirculation. [5] In addition, it has received much attention due to its numerous biological activities, such as inhibition of platelet aggregation, suppression of hypertension and anti-aging. [6-10] Liver is an important organ actively involved in many metabolic functions and is the frequent target for a number of toxicants. [11] Hepatic damage is associated with distortion of the several metabolic functions. [12]

Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver disease are inadequate and sometimes can have serious side effects. [13] In the absence of a reliable liver protective drug in modern medicine, there are a number of medicinal herbs recommended for the treatment of liver disorders. There is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity. Therefore an attempt had made to developed using indigenous medicinal plants, with proper pharmacological experiments and chemical trials.

The present work intends to study of natural products useful in the treatment of liver diseases. Despite advances in modern medicine, there is no effective drug available that stimulates liver function, offer protection to the liver from damage or help to regenerate hepatic cells. There is urgent need, therefore for effective drugs to replace / supplements those in current use. It is necessary to search for alternative drugs for the treatment of liver diseases to replace currently used drugs of doubtful efficacy and safety. Therefore, the present study was aimed to investigate the hepatoprotective effect of HRS flower extract on liver enzymes and blood lipids in rats fed cholesterol enriched diet.

MATERIALS AND METHODS

Plant material –Fresh flowers of *Hibiscus rosa sinensis* were collected from Kurunjibag locality, Sullia, D.K, Karnataka. A voucher specimen of the flower was identified and authenticated. A voucher herbarium specimen was also preserved for future reference. The flower petals were air dried completely under shade and powdered and stored until subjected to solvent extraction.

Preparation of extract: The grounded powder was extracted in soxhlet apparatus using distilled water (1:10 ratio)[14]. The extract was filtered and concentrated in a rotatory evaporator, at 30–40 ◦C under reduced pressure to obtain a thick dark brown extract. The extract yield was 14% w/w.

Phytochemical screening: Preliminary screening of the flower extract for various phytochemical classes was carried out based on the reported methods.[15] The crude extract was screened for the

presence of saponins, flavonoids, tannins, phenols, sterols, alkaloids, and anthocyanins.

Chemicals: Pure cholesterol purchased from Merck Specialities Private Ltd, Mumbai, cholic acid, trichloro acetic acid and thiobarbituric acid purchased from Loba Chemie Private Ltd, Mumbai. All other reagents and chemicals used in the present study were of analytical grade (AR). Edible quality coconut oil was purchased from local market. Only double distilled water was employed for preparing the reagents.

Animals: Wistar strain male albino mature (12-15 weeks old) rats of approximately same age group, having body weight 170–220 grams were used from animal house of K.V.G. Medical College, Sullia, D.K.

All animals were housed in polypropylene cages in groups of 6 to provide sufficient space and allowed to acclimatize for 2 weeks before study at laboratory conditions. Rats were maintained as per the standard condition with pellet diet (Gold Mohur-Lipton India Ltd, Mumbai) and water ad libitum. All experimental procedures and animal maintenance were done under the guidelines of Institutional Ethics Committee for the use of animals in the experiment.

Induction of experimental hypercholesterolemia

In order to induced hypercholesterolemia the method reported by Bopanna el al was followed.[16] Hypercholesterolemia has been induced by feeding pure cholesterol and cholic acid orally mixing with coconut oil with the dose (i,e 1% pure cholesterol and 0.5%cholic acid, the ratio between cholesterol and cholic acid was 2:1) for 4 weeks.

Experimental Groups Design and Treatment with HRS extract

After the acclimatization period 48 Male Wistar rats (180-230gm) were randomly divided into two groups. Experimental group with 42 rats and control group with 6 rats.

Only hypercholesterolemia induced animals are considered for experimental groups. Out of these one group made for hypercholesterolaemic control (n=6) and other experimental animals further divided into three acute groups and three chronic groups (n=6). The experimental acute and chronic groups animals were given HRS flower extracts orally with the help of oral gavage. Each group received HRS flower extract at dose level 80mg, 160mg and 240mg /kg body weight daily once for 5 and 30 days as acute and chronic groups respectively. First treatment day with HRS was considered treatment day 1. The exact dosage for each rat was corrected every day for individual body weight by appropriate volume adjustment. Positive control group of animals received normal diet and water only. After 24 hrs of last HRS treatment, on 6th day (for acute groups) and on 31st day (for chronic groups), the animals were sacrificed by cervical dislocation. Thorax was opened and heart exposed and blood sample was collected by cardiac puncture. A portion of blood sample was centrifuged and plasma were separated and used for determination of TBARS level. The remaining blood was allowed to clot in a test tube and serum was collected. The serum was used to determine the total cholesterol (TC), triglyceride (TG) and high density lipoprotein (HDL) levels enzymatically, using commercially available kits E-Coline (Merck). Very low density lipoprotein (VLDL) and low density lipoprotein (LDL) levels were estimated indirectly by using the formula: VLDL=TG/5, LDL=TC-HDL-VLDL. Atherogenic Index was calculated as follows: atherogenic index = (Total cholesterol-HDL cholesterol) / HDL cholesterol. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined according to the method described by Reitman and Frankel[17], whereas alkaline phosphatase (ALP) activity was estimated by Belfield method.[18] Total protein, albumin and globulin levels were determined using commercially available Agappe Diagnostic Kit. Estimation of Lipid peroxidation

marker- Thiobarbituric acid reactive substances (TBARS) levels in the plasma were determined by the method described by Okhawa et al.[19]

Statistical Analysis

All the data was expressed as Mean ±SEM. Analyzed by using one - way ANOVA, followed by Bonferoni post – hoc test. The statistical tests were obtained from statistical package of social sciences (SPSS) version 19, 2007. Values of $P<0.05$ were considered as statistically significant.

RESULTS

Phytochemical analysis of the crude extract of Hibiscus rosa sinensis (HRS) showed the presence of alkaloids, saponins, tannins, phenolics and flavonoids. These entire tests were done at Bangalore Test House, Bangalore.

Changes of Body Weights

As shown in the table:- 1, feeding normal rats on basal diet supplemented with 1% cholesterol and 0.5 % cholic acid for four weeks, significantly($p<0.001$) increased body weight. Feeding hypercholesterolemic rats on diet supplemented with HRS flower extract with different doses in acute groups animals showed significantly ($p<0.05$) decreased body weight as compared to hypercholesterolaemic control group rats. Similarly, in the chronic group animals body weight decreased highly significantly.($p<0.001$)

Serum Lipid Profiles and Atherogenic Index (AI)

As demonstrated in the fig-1, hypercholesterolaemic rats fed with HRS flower extracts in different doses for acute group animals showed decreased in total cholesterol level significantly ($p<0.05$) In chronic group hypercholesterolaemic rats (fig-2) showed significantly ($p<0.05$) decreased level of triglyceride and VLDL with HRS fed group at the doses of 80mg/kg BW. In the 160mg/kg BW fed HRS flower extract group showed significant decrease ($p<0.05$) in total cholesterol and triglyceride level and high significantly decreased in VLDL level ($p<0.001$). In the 240mg/kg BW HRS fed group showed decline in serum cholesterol, triglyceride and VLDL levels highly significantly ($p<0.001$). A dose dependent reduction in atherogenic index was observed. For acute group rats at the highest doses of HRS, AI reduced significantly ($p<0.05$) as compared to control hypercholesterolaemic groups (fig-3). In the chronic hypercholesterolaemic animals fed with HRS flower extract at the dose level of 80mg/kg BW showed significant decrease ($p<0.05$) of AI and it reduces (fig-3) highly significantly ($p<0.001$) with the remaining doses.

Activities of Liver enzymes and Lipid peroxidation

It is clear from fig-4, that experimentally produced hypercholesterolaemic groups showed significant ($p<0.001$) rise in SGPT and ALP levels in serum, feeding of HRS flower extract for four weeks to hypercholesterolaemic rats significantly ($p<0.001$) decreased the levels of AST, ALT, ALP enzymes in the serum and significantly ($p<0.001$) increased in Total Protein (TP) levels compared to the control hypercholesterolaemic group (fig-5). The lipid peroxidation markers were high in hypercholesterolemic group but significantly ($p<0.001$) reversed with chronic HRS fed groups (fig-6).

DISCUSSION

The results of the present study about the hepatoprotective effects of HRS flower extract demonstrated that the lipid lowering action of this natural product may be mediated through inhibition of hepatic cholesterol biosynthesis, increased fecal bile acids excretion and enhanced plasma lecithin: cholesterol acyl transferase activity and reduction of lipid absorption in the intestine.[20] Liver protective plant contains a variety of chemical constituents like phenols, coumarins, glycosides, alkaloids, tannins and flavonoids.[21]

Hyperlipidemia is known to enhance the risk of coronary heart disease, fatty liver diseases and

carcinogenesis which is associated with reactive oxygen species formation. In recent years many studies have focused on the bioavailability of phenolic compounds in the prevention and treatment of obesity. It has also promising effects on the body weight and fat mass development. In particular increases in the amount of the fat in the diet, have been shown to be associated with the risk of obesity and hyperlipidemia in human and rodents by altering cholesterol and triglyceride levels in plasma and tissues.[22] A number of scientific reports indicate that certain flavonoids, steroids have protective effect on liver due to its antioxidant properties. Phytochemically flavonoids and alkaloids might play role in hepatoprotective activity. Phenolic compounds and flavonoids have pharmacological properties such as antioxidant, antimutagenic, antithrombotic, anti inflammatory, anticancer and antihyperlipidemic. The flavonoids are well documented as hepatoprotective activities. Hence it has been reported to be effective in liver diseases. They are widely distributed in plants and form a part of the human diet.[23,24,25,26] Ni and Dom(2009) is suggested that the rich presence of tannins in HRS flower can keep the level of LDL(harmful) cholesterol in check. This can keep our heart healthy.

Hepatic dysfunction due to inhalation of hepatotoxin is increasing World Wide. Among the various mechanisms involved in the Hepatotoxicity by hepatotoxin, one is oxidative damage through free radical generation.[27] Management of liver disease is still a challenge to the modern medicine. Conventional medicine is now pursuing the use of natural products such as herbs to provide the support that the liver needs on a daily basis. In addition high content of soluble fiber can reduce strain on liver by removing extra water and toxin.[28] Feeding HRS flower extract 240mg/Kg BW was more effective on decreasing the elevated level of liver enzymes (AST,ALT, ALP) due to hyperlipidemia. AST predominantly found in mitochondria of hepatocytes. ALT is more specific to liver and thus is a better parameter of detecting liver injury. ALP and Total Protein (TP) are also associated with liver cell damage. Administration of high cholesterol diet caused a significant elevation of liver enzyme such as AST, ALT and ALP level in blood attributed to the damages structural integrity of liver because they are released into circulation after cellular damages, which indicates development of Hepatotoxicity.[29,30] The co administration of HRS flower extract have prevented the increased serum marker enzymes. This is an agreement with the commonly accepted view that serum levels of AST, ALT and ALP return to normal with healing of hepatic parenchyma and regeneration of hepatocytes.

The levels of TBARS as an index of lipid peroxidation a degardative process of membranous lipid in liver tissue of hypercholesterolemic rats was significantly elevated when compared to control animals. Lipid peroxidation level was restored towards their normal value by treated with HRS flower extract indicates prevention of accumulation of lipid peroxidation. The increase in TBARS level in liver induced by hypercholesterolemia suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism.[31] A massive decrease in lipid peroxidation in liver tissue of HRS flower extract treated groups indicates that the extract posses antioxidative properties. The flower extract contain antioxidant and hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress. Reduction of serum albumin in hypercholesterolemic group may be due to formation of protein adduct, leads to covalent modification of cellular target protein, cell death and organ damage. [32]

CONCLUSION

A possible mechanism of the HRS flower extracts as hepatoprotective may be due to its antioxidant effect or inhibition of cytochromeP_{450}. This might be due to the higher contents of flavonoids

presents in the extract, which could have reduced the accumulation of unwanted hypercholesterolemia derived metabolites. This study recommends that dietary intake of HRS flower extract can be beneficial to patients suffering from hypercholesterolemia and liver diseases. Hepatoprotective plant species, seeking vast multidimensional future research work up to the molecular level to establish new up to date scientific data about this plant species and to elucidate its exact mechanism of protective effect. Future studies may be aimed at hepatoprotective study in other chronic models of hepatopathies, antioxidants and free radical scavenging potentials, toxicological studies and other pharmacological activities as well.

ACKNOWLEDGEMENT

We are grateful to the KVG Ayurveda Medical College, Sullia for generously providing the facilities for HRS flower extract preparation. Grateful thanks to KVG Medical College management, Prof. Dr. Chidananda, Medical Director, Prof. Dr. Sheela G Nayak, Principal and late Prof. Ramakrishna, Administrator, for their kind support and facilities for conducting this research.

REFERENCES

1. Girish C, Konar BC, Jayanthi S, Rao KR, Hepatoprotective activity of formulations in CCL_4 induced liver toxicity in mice. Ind J Exp. Biol, 2009,47:257-263.
2. Karin Conde, Marcela Vergara-Jimenez, Brian R. Krause, Roger S, Newton, Maria Luz Fernandez, Hypocholesterolemic actions of atorvastatin are associated with alterations on hepatic cholesterol metabolism and lipoprotein composition in the guinea pig, Journal of Lipid research, 1996; 37,2372-2382.
3. Gupta N.K, Dixit V.K (2009), Evaluation of hepatoprotective activity of Cleome viscosa Linn. Extract. Indian journal of pharmacology, 41, pp 3640.
4. Gupta SS (1994), prospects and perspectives of natural plant products in medicine. Indian J. Pharmacol., 26, pp 112.
5. Sachdewa A, Nigam R, Khemani L.D, Hypoglycemic effect of *Hibiscus rosa sinensis* L. leaf extract in glucose and streptozotocin induced hyperglycemic rats. Indian Journal of Experimental Biology. 2001; 39, 284–286.
6. Sachdewa and L. D. Khemani, Effect of *Hibiscus rosa sinensis* Linn. ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *Journal of Ethno pharmacology*. 2003; 89, 61–66.
7. S. D. Kholkute and K. N. Udupa, Antiestrogenic activity of *Hibiscus rosa sinensis* Linn. flowers, *Indian Journal of Experimental Biology*.1976; 14, 175–176.
8. N. Vasudeva and S. K. Sharma, Post-coital antifertility activity of *Hibiscus rosa-sinensis* Linn. roots, *Evidence-Based Complementary and Alternative Medicine*. 2008; 5, 91– 94.
9. B. Shivananda Nayak, S. Sivachandra Raju, F. A. Orette, and A. V. Chalapathi Rao, Effects of *Hibiscus rosa sinensis L (Malvaceae)* on wound healing activity: a preclinical study in a Sprague Dawley rat. *International Journal of Lower Extremity Wounds*. 2007; 6, 76–81.
10. V. S. Kasture, C. T. Chopde, and V. K. Deshmukh, Anticonvulsive activity of *Albizzia lebbeck*, *Hibiscus rosa sinesis* and *Butea monosperma* in experimental animals. *Journal of Ethnopharmacology*.2000; 71, 65–75.
11. Gupta, A.K. and N. Misra, (2006), Hepatoprotective Activity of Aqueous Ethanolic Extract of Chamomile capitula in paracetamol intoxicated albino rats. A m .J. Pharm.Toxicol., 1, pp 17- 20.
12. Handa S.S and Sharma A (1990), Hepatoprotective activity of andrographolide from Andrographis paniculata against carbontetrachloride. Indian J Med Res., 92, pp 27683.

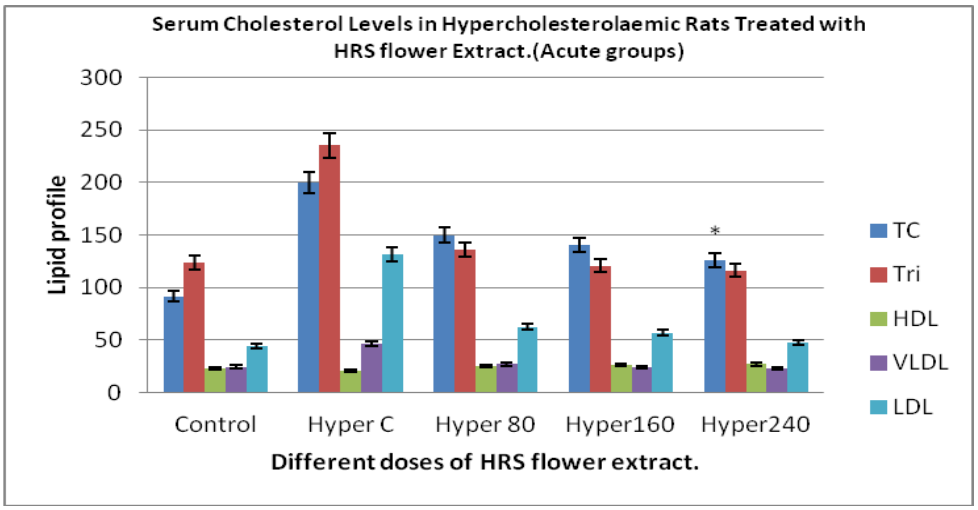
13. Sultana S, Perwaiz M and Ather M. Crude extracts of hepatoprotective plants, Solanum nigrum and Cichorium intybus inhibit free radical mediated DNA damage. J. Ehanolpharmacol.,1994; 47:189-92.
14. Soxhlet, F. Die gewichtsanalytische Bestimmung des Milchfettes, Polytechnisches J. Dingler's. 1879; 232, 461.
15. Evans W C: Phytochemistry. Trease and Evans Pharmacognosy. 5th edition Delhi: Elsevier;2006,135-150.
16. Bopanna KN, Bhagyalakhsmi N, Rathod SP, Balaraman R, Kannan J. Cell culture derived Hemidesmus indicus in the prevention of hypercholesterolemia in normal and hyperlipidemic rats. Ind J Pharmacol 1997;19:105-6.
17. Reitman S and Frankel S. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Am J Clin 1957; 28:56-63.
18. Belfield A, Goldberg DM. Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme 1971;12:561-73.
19. Okhawa H, Qohishi N, Yagi K. Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.* 1979;95:351–358.
20. Yazdanparast R and Bahramika S, Evaluation of the effect of Anethum graveolens L crude extracts on serum lipids and lipoproteins profiles in hypercholesterolaemic rats, DARU.2006; 6, 88.
21. Pengelly Andrew, Triterpenoids and saponins, The constituents of medicinal plants (CABI Publishing, USA), 2004,74.
22. Ghule B V, M H Ghante, A N Saoji and P G Yeole, Hypolipidemic and antihyperlipidemic effects of Lagenaria siceraria (Mol.) fruit extracts, Indian J Exp Biol. 2006;44, 905-909.
23. Lanksy P S, Plants that lower cholesterol, Acta Horticulture. 1993;332, 131.
24. Sidhu G S and Oakenful D G, A mechanism for hypocholesterolaemic activity of saponins, Br J Nutrition.1986; 55, 643.
25. Sidhu G S and Oakenful D G, Could saponins be a useful treatment for hypercholesterolaemia? Eur J Clin Nutr. 1990; 44, 79.
26. Cleghorm CL, Skeaff C M, Mann J and Chisholm A, Plant-sterol enriched spread enhances the cholesterol lowering potential of fat-reduced diet, Eur J Clin Nutrition. 2003;57,70.
27. Regisusan Daniel, Mathew Biju C, Devi K S and Augusti K T, Antioxidant effect of two flavonoids from the bark of Ficus bengalensis Linn in hyperlipidaemic rats, Indian J Exp Biol, 1998;36, 902.
28. Mona M, Rashad, Sorial AC, Moharib: Effect of type and level of dietary fibre suppliments in rats, Grasas Y Aceites, 2003;54(3):277-284.
29. Guntupalli M, Chandana V, Palpu Pushpangadan and Annie Shirwaikar, Hepatoprotective effects of rubiadin, a major constituent of Rubia cordifolia Linn. J Enhnopharmacol, 2006,103,484-490.
30. Girish C, Konar BC, Jayanthi S, Rao KR, Hepatoprotective activity of formulations in CCL_4 induced liver toxicity in mice. Ind J Exp. Biol, 2009,47:257-263.
31. Shankar N L, Manaralan R, Venkappaya D, Raj C. Hepatoprotective and antioxidant effects of Camiphora berryi (Arn) Engl bark extract against CCl_4 induced oxidative damage in rats. Food and Chemical Toxicology.,2008;46: 3182-85.
32. Janbaz K H, Saeed S A and Gilani A H. An assessment of the potential of protopine to inhibit microsomal drug metabolizing enzymes and prevent chemical induced hepatotoxicity in rodents. Pharmacological Research.,1998; 38: 215-18

Table 1:- Body Weights in hypercholesterolaemic rats treated with *Hibiscus rosa sinensis* flower extract.

Group	Doses (mg/kgBW)	5Days(Acute)			30Days(Chronic)		
		Initial(g)	Final(g)	Gain(g)	Initial(g)	Final(g)	Gain/lost(g)
Control	-	191.33 ±5.6	200.33 ±4.80	9.0	194.66 ±5.16	215 ±2.75	20.34
Hyperlipidemia control	1%	191 ±4.33	300** ±2.82	110	191 ±4.33	300** ±2.82	109
Hyperlipidemia+HRS	80	300** ±2.82	292.33 ±3.2	8.67	298.66** ±5.88	282.33** ±3.44	16.33
Hyperlipidemia+HRS	160	302** ±4.56	290* ±3.34	12	304.33** ±4.08	282.66** ±3.72	21.67
Hyperlipidemia+HRS	240	304.33** ±6.37	287.66* ±7.52	16.67	305** ±5.47	273.33** ±4.32	31.67

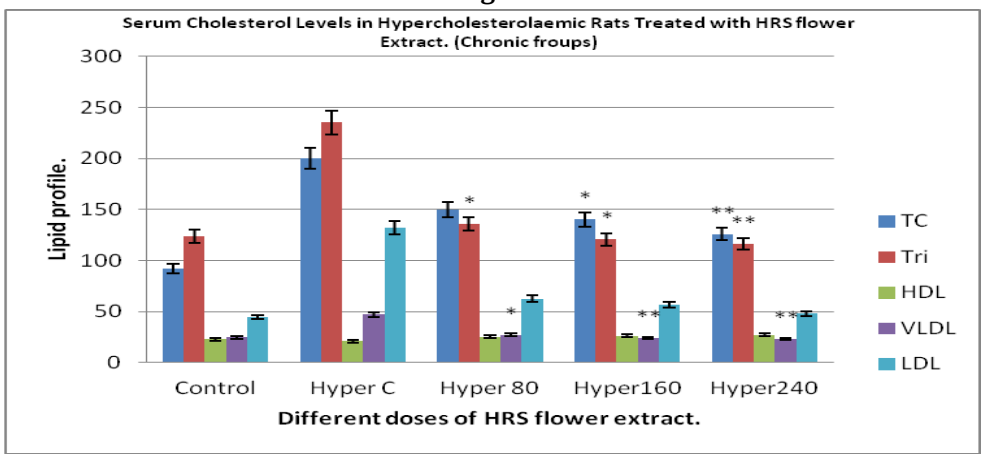
* The mean difference is significant at the (p< 0.05) level, ** The mean difference is significant at the (p <0.001).

Fig:- 1



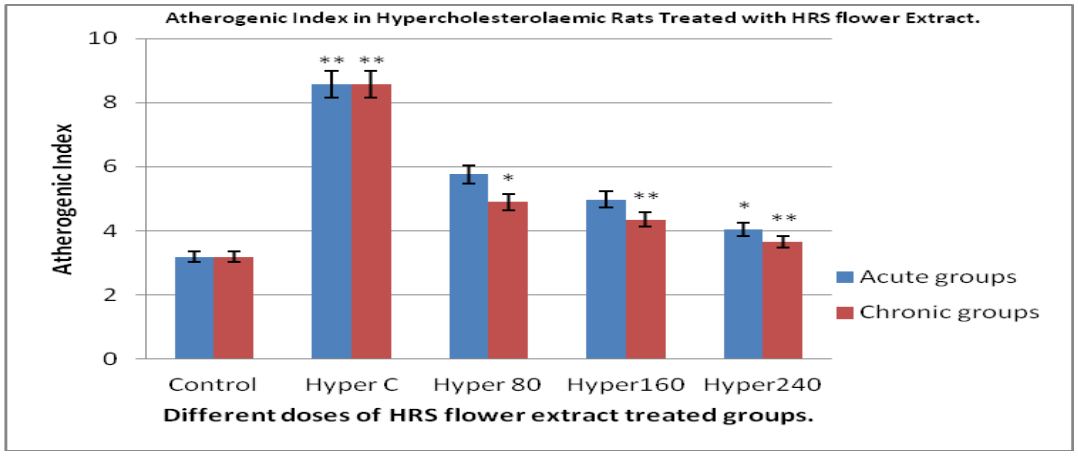
* The mean difference is significant at the (p< 0.05)level.

Fig:-2



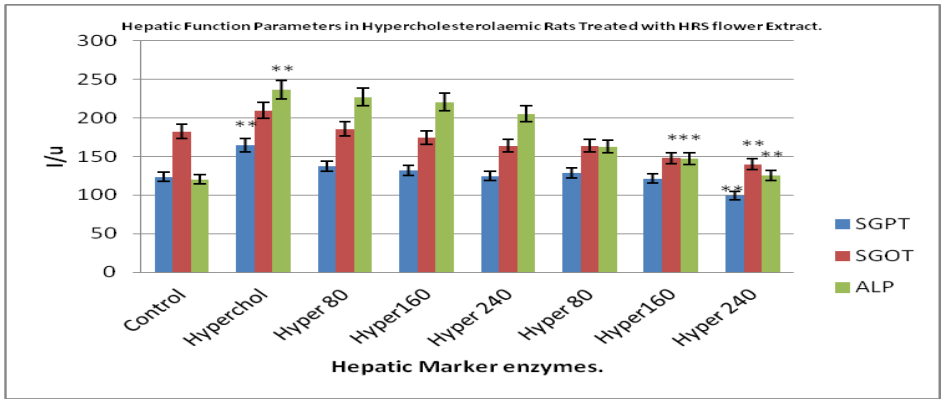
* The mean difference is significant at the (p< 0.05) level, ** The mean difference is highly significant at the (p< 0.001) level.

Fig:- 3



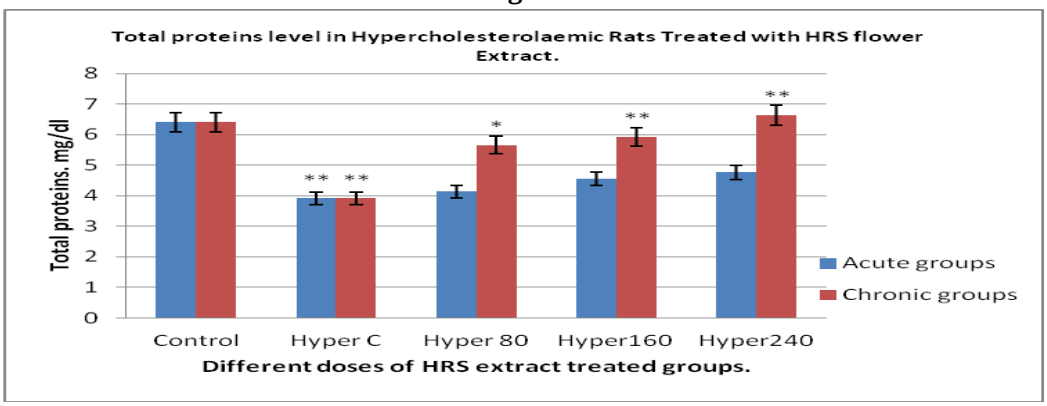
* The mean difference is significant at the (p< 0.05)level, ** The mean difference is highly significant at the (p< 0.001) level.

Fig:- 4



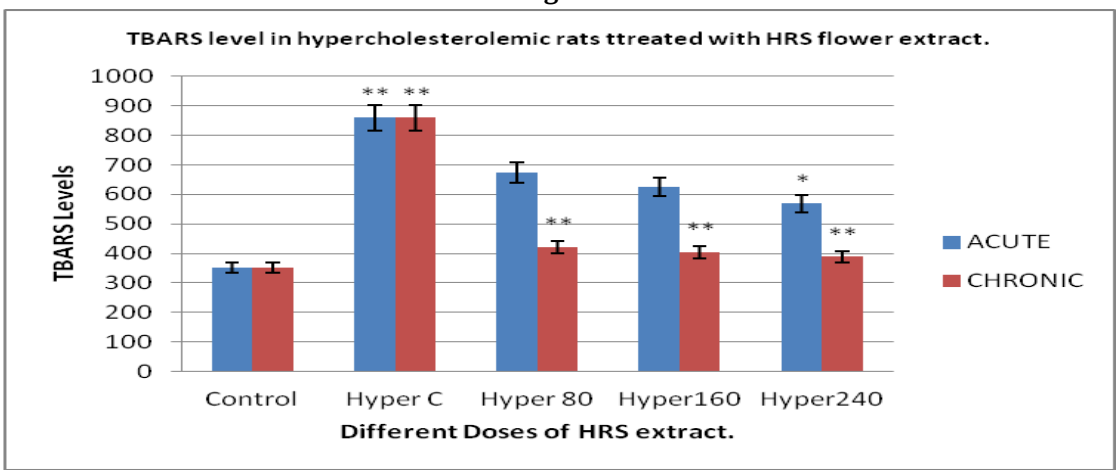
* The mean difference is significant at the (p< 0.05)level, ** The mean difference is highly significant at the (p< 0.001) level.

Fig:- 5



* The mean difference is significant at the (p< 0.05)level, ** The mean difference is highly significant at the (p< 0.001) level.

Fig:- 6



* The mean difference is significant at the (p< 0.05)level, ** The mean difference is highly significant at the (p< 0.001) level.