WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 14, 952-969. Research Article ISSN 2277– 7105

TO EVALUATE THE EFFICACY OF A HERBAL FORMULATION OF *CLERODENDRUM GLANDULOSUM* (SYN. *COLEBROOKIANUM*) AND *ALLIUM SATIVUM* AS HYPOLIPIDEMIC AGENT

[1]Sinam Ganapati Devi, [2]Puspanjali Khound, [3]Partha Pratim Sarma, [4]Himangshu Sarma, *Rajlakshmi Devi and *Bishnu Prasad Sarma

[1]PG Scholar, Dept. of Kayachikitsa, Govt. Ayurvedic College, Guwahati-14, Assam.

[2,3]Research Scholar, IASST.

[4]JRF, IASST.

*Associate Professor & Head, Life Sciences Division, Institute of Advanced Study in Science & Technology (IASST).

*Principal, Professor & HOD, Dept. of Kayachikitsa, Govt. Ayurvedic College, Guwahati-14, Assam.

Article Received on
15 Sept. 2020,

Revised on 05 Oct. 2020,
Accepted on 25 Oct. 2020

DOI: 10.20959/wjpr202014-19141

***Corresponding Author**
Prof. Dr. Bishnu Prasad Sarma
Principal, Professor & HOD, Dept. of Kayachikitsa, Govt. Ayurvedic College, Guwahati-14, Assam.

ABSTRACT

Clerodendrum glandulosum Lindl. is commonly used in traditional medicine for the management of various diseases including hypertension, dyslipidemia and diabetes without the full understanding of the scientific basis for its use. And, *Allium sativum* has been proven effective in the prevention and treatment of atherosclerosis, which is widely used as a food and medicine by people in daily life. This study sought to evaluate the efficacy of the herbal mixture or formulation of ethanolic extract of *Clerodendrum glandulosum* leaves and garlic cloves on lipid profile of hyperlipidemic wistar rats. The formulation was made in the ratio of CG:AS into 8:1. The rats were pre-treated with the formulation and synthetic hypolipidemic drug atorvastatin. Hyperlipidemia was induced by single intraperitoneal injection of poloxamer- 407(300mg/kg. body weight). Also, acute and sub acute toxicity tests were carried out on wistar rats using standard methods. At the end of the experiment, the animals were subjected to euthanasia and blood samples were separately collected through cardiac puncture. Anti- hyperlipidemic activity was measured in the plasma of rats by estimating the levels of total cholesterol (TC), LDLc, HDLc and Triglycerides (TG). It was observed that the levels of TC, LDLc, TG were

significantly lowered in dose dependent manner and also observed that HDLc level was significantly increased. Results indicate that the formulation was able to positively regulate induced experimental hyperlipidemia by significant alteration in plasma lipid profiles as a prophylactic measure.

KEYWORDS: *Clerodendrum glandulosum, Allium sativum,* poloxamer-407, hyperlipidemia, atherosclerosis, lipid profile, anti-hyperlipidemic activity.

INTRODUCTION

CVD contribute 25-30% of deaths in most of the industrial countries and originated by several risk factors, out of them hyperlipidemia is most important. Hyperlipidemia may be familial or hereditary and due to metabolic consequences associated with changes in diet and lifestyle. The latest statistics from the World Health Organization (WHO) indicate that more people die annually from cardiovascular disease (CVD) than any other ailments. The WHO has predicted that by 2030 almost 23.6 million people will die from CVD related illness worldwide. According to ICMR-INDIAB study, the prevalence of hypercholesterolemia was 13.9%, of hypertriglyceridemia was 29.5%, of low HDLc was 72.3% and high LDLc level was 11.8%. Therefore, it is crucial to maintain the normal body functions by reducing the elevated serum cholesterol to an adequate level.

Over the last two decades, studies have shown that drugs synthesized and derived from herbal medicines contained bioactive principles that are capable of reducing abnormally increased in blood cholesterol or lipids. These natural products are supposed to improve quality of life which can be impaired by western hypolipidemic agents. Accordingly, traditional herbal formulae or medicinal plants would be valuable as new drugs for patients from blood lipid disorders.

On the other hand, most people of North East India use Clerodendrum leaf juice mixed with garlic for treating blood pressure in the folklore medicine. *Clerodendrum* has been reported to possess hypolipidemic and antioxidant activity, *Allium sativum* has been shown to possess a broad spectrum of antitumor, hypoglycemic, hypolipidemic and anti atherosclerotic properties. Thus, it is proposed that CG and AS could be developed in hypolipidemics which guarantees both improving blood lipid levels and reducing complications. Therefore, the present study is aimed to assess the effectiveness of hypolipidemic activity of the herbal formulation of CG and AS.

MATERIALS AND METHODS

Plant material

Clerodendrum glandulosum (Verbenaceae) leaves were collected from botanical garden, Guwahati University and shade dried. Garlic bulbs were collected from local market, Jalukbari. The plants were re-identified and authenticated by Botanical Survey of India, Meghalaya and herbarium stored at Department of Botany, Guwahati University.

Extraction and preparation

Extraction of Clerodendrum leaves was carried out by reflux condensation method using Soxhlet apparatus and that of garlic by cold maceration at State Drug Testing Laboratory, Govt. Ayurvedic College, Guwahati-14. The extracts were concentrated by distillation apparatus till a syrupy consistency was obtained and evaporated at 40-60°C temperature in water bath until semi-solid extract was obtained. Then the formulation was prepared in CG:AS ratio as 8:1.

Chemical and kits

All the chemicals used in the study were of analytical grade and procured from Sigma Aldrich, USA.

Work plan of the study

The experimental design was planned in order to accomplish the hypothesis made in this study including the designing of herbal formulation, in vitro screening of bioactivity and in vivo experiments as represented in figure –

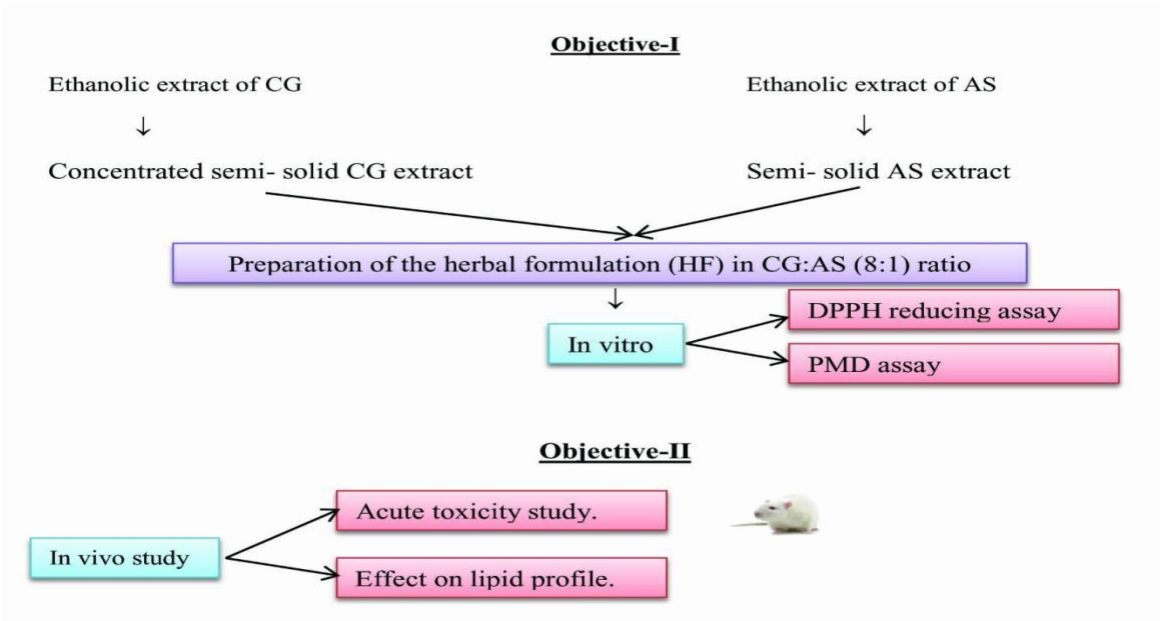


Fig.: Plan of the study

In vitro experiments

a. DPPH radical scavenging assay

The free radical scavenging activity of the prepared extract was determined based on its activity to bleach the stable radical DPPH. Sample at different concentrations were added to DPPH solution followed by thorough vortexing. The preparation without the extract was served as control. The mixture was allowed to remain in dark at room temperature 27°C for 30 minutes and absorbance was measured spectrophotometrically at 515nm against the control. The percentage inhibition (%) of radical scavenging was calculated using the following equation –

Inhibition (%)={(As-Ai)/As}×100

Where, As= Absorbance of control at 515 nm.

Ai= Absorbance of sample at 515 nm.

Ascorbic acid was used as positive antioxidant control.

b. TAC by PMD(Phosphomolybdenum) method

This method is used to analyze the cumulative antioxidant capacity based on the reduction of phosphate- Mo(VI) to phosphate-Mo(V) by the sample and subsequent formation of a bluish green coloured phosphate-Mo (V) complex at acid PH. Working solution of the plant extract was taken as 100microgram/ml. Different concentration of the extracts viz 10, 25, 50, 75 and 100migrogram/ml were taken. Then 250 microliter of PMD reagent was added followed by vortex and incubated for 90minutes at 90°C and cooled at room temperature. Absorbance was measured at 695nm and calculated as follows-

Inhibition (%)={(T-C)/T}×100

Where, T= sample absorbance at 695nm. C= control absorbance at 695nm.

In vivo experiments

a. Acute toxicity study

The acute oral to toxicity study was performed according to the OECD toxicity guidelines.[16] Female wistar rats (n=3) were fasted overnight prior to the experiment, after which the formulation was administered orally in a single dose of 2000mg/kg. The animals were observed continuously for first 2 hours and then occasionally for 4 hours and then for 14 days for any gross change in behavioral, locomotors activity or any other symptoms of toxicity and finally for overnight mortality.

b. Evaluation of anti-hyperlipidemic assay Experimental animals

Male albino wistar rats weighing about 150-200gm were obtained from the animal house of Institute of Advanced Study in Science and Technology (IASST). Experiment on animals were performed in accordance with guidelines of the Institutional Animal Ethical Committee (IAEC) with approval no. 1706/GO/ReBi/S/13/CPCSEA. They were maintained under standard laboratory conditions at room temperature and relative humidity. They were fed with standard pellet diet and water ad libitum so as to adapt to their new environment and to nullify the effect of changes in their general metabolism (acclimatization). At random, the animals were assigned to different groups depending on their weight.

Experimental design

30 rats were divided into 5 groups of 6 animals each (n=6). 12 animals (Group 4 and 5) were pre-treated with the HF (100mg/kg.b.w and 200mg/kg.b.w) suspended in 1% CMC, 6 rats (group 3) with Atorvastatin (10mg/kg.b.w with 1% CMC) by oral administration for 10 days. The rats were induced hyperlipidemia with intraperitoneal injection of single dose of 300mg/kg.b.w poloxamer-407 (Sigma Aldrich, USA) in cold distilled water; then again the rats were orally fed with HF and Atorvastatin respectively once again at 12hours after P-407 injection.

Experimental procedure is summarized as follows-

- Group I- Control group that received normal pellet diet and water.
- Group II- Hyperlipidemic rats induced by intraperitoneal injection of P-407 at a single dose of 300mg/kg.b.w in distilled water.
- Group III- Preventive group orally treated with Atorvastatin at a dose of 10mg/kg.b.w suspended in 1% CMC + P-407 induced hyperlipidemic rats.
- Group IV- Preventive group orally treated with HF at a dose of 100mg/kg.b.w in 1%CMC + P-407 induced hyperlipidemic rats.
- Group V- Treated with HF at a dose of 200mg/kg.b.w in 1% CMC orally + P-407 induced hyperlipidemic rats.

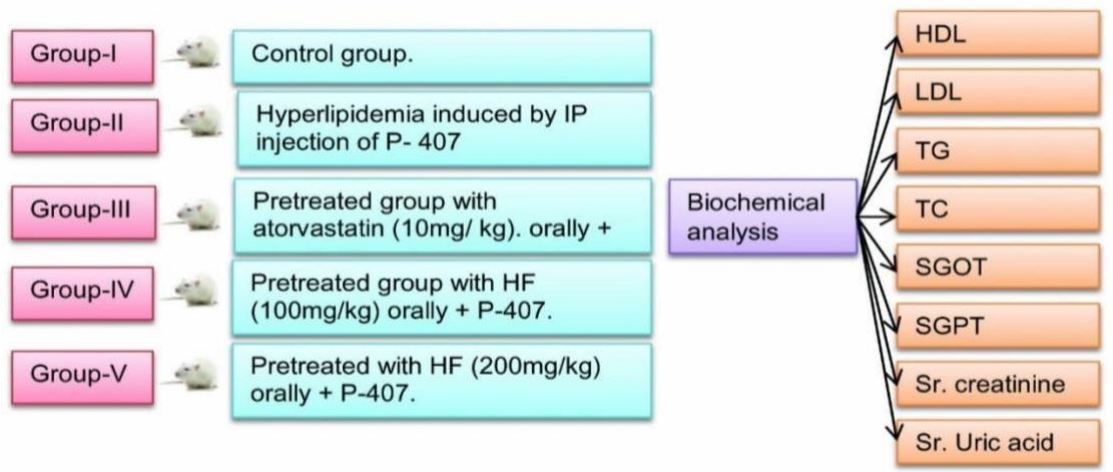
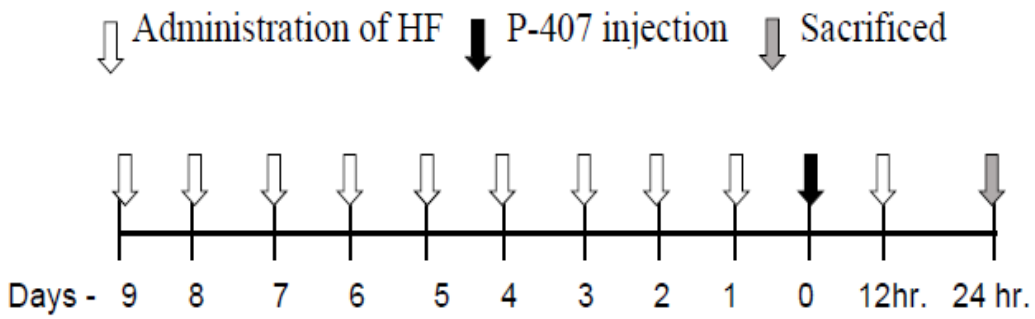


Fig.: Experimental design for evaluation of effect of herbal formulation on hyperlipidemia induced by P-407 in rats.



Collection of blood sample

On 24 hour time point of P-407 injection as a status of 12 hour fasting, animals were subjected to euthanasia. The blood samples were collected through cardiac puncture and the samples collected were centrifuged at 3000rpm/min for 10minutes and serum obtained was used for biochemical analysis.

Biochemical investigation

Serum concentrations of TC, TG, HDL, LDL and serum uric acid were determined by using enzymatic kits (Accurex Biomedical Pvt. Ltd., Thane, India). Serum creatinine, SGOT and SGPT were done by kinetic enzymatic methods.

Statistical analysis

The data obtained in this study were expressed as mean+-SD. All tested samples were statistically analyzed using one way analyses of variance (ANOVA) followed by Post Hoc

Tukey test. The threshold significance was $p<0.01$.

RESULTS

In vitro experiments

a) DPPH radical scavenging activity

The DPPH radical scavenging activity of the formulation was studied by its ability to bleach stable free radical DPPH. Figure illustrates the dose response curve of DPPH radical scavenging activity of the formulation compared with ascorbic acid. As the concentration of formulation go on increases the percentage inhibition goes on increasing which signifies the activity showed by formulation was comparable to the standard antioxidant compound ascorbic acid, although its scavenging activity was significantly lower than that of ascorbic acid.

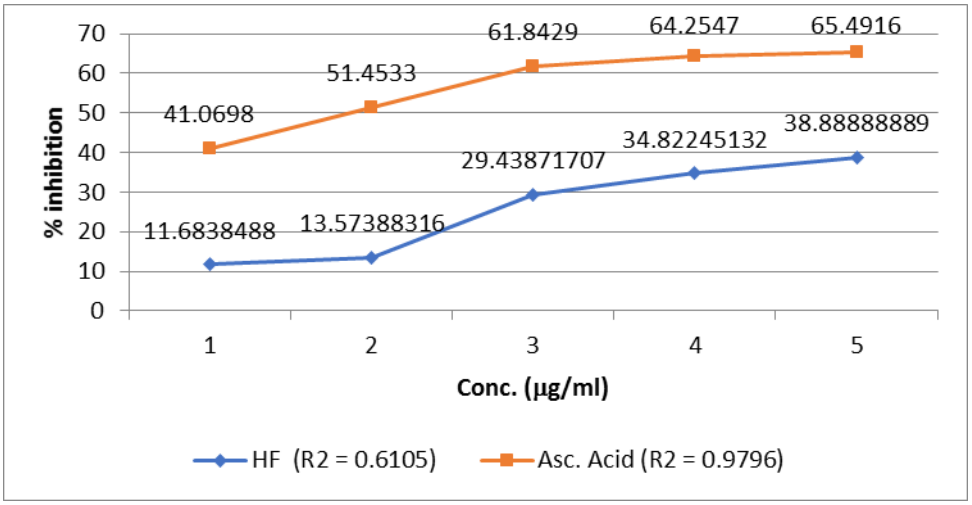


Fig. Comparison of DDPH radicals scavenging activity of the HF to the std. antioxidant ascorbic acid.

b) PMD method

Figure shows the reductive capabilities of the formulation as compared to ascorbic acid. The reducing power of HF was observed to rise as the concentration of HF gradually increased.

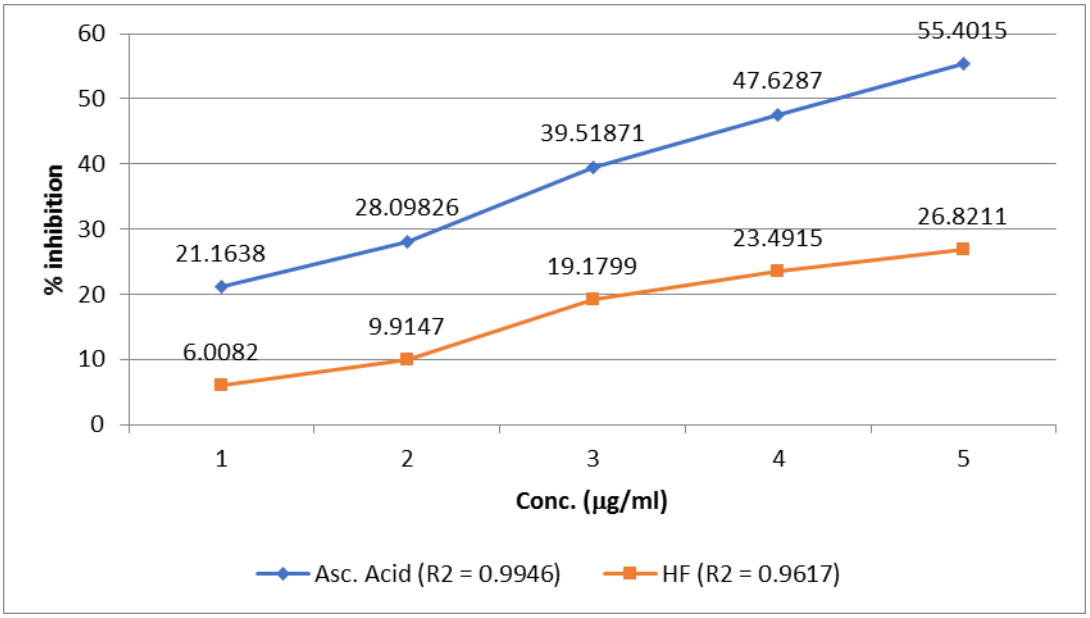


Fig. 2: Comparison of PMD reducing activity of the HF to the std. antioxidant ascorbic acid.

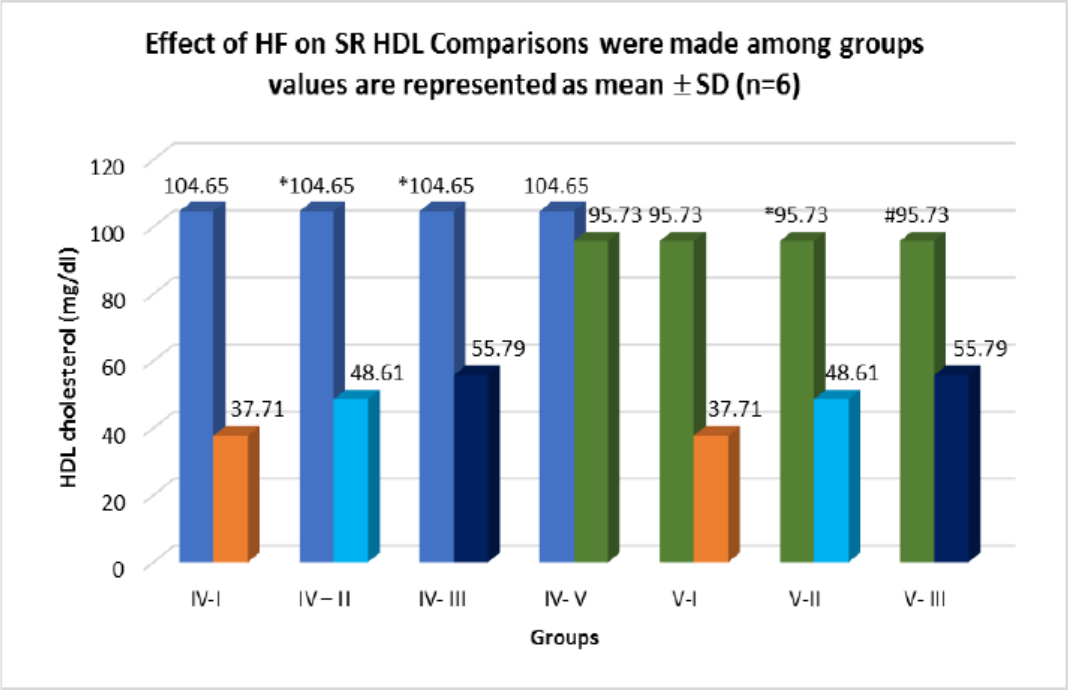
In vivo experiments

I. Acute toxicity study

The acute toxicity study revealed the non toxic nature of the formulation. There was no lethality and untoward reaction on behavioral responses observed. Therefore, usage of an appropriate amount of the formulation should be considered safe.

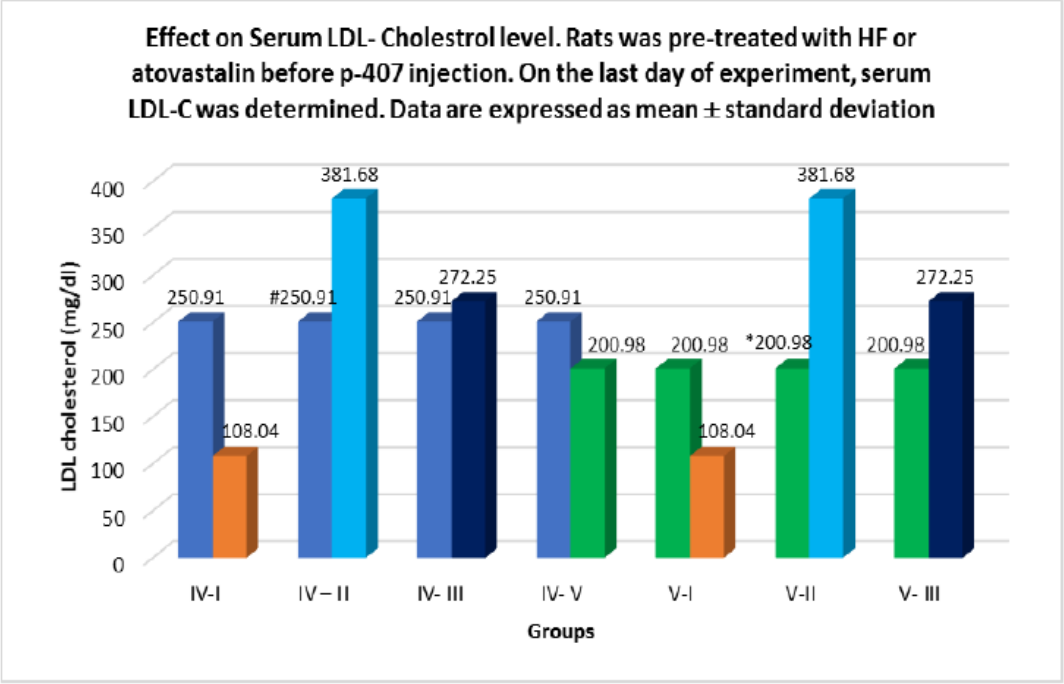
II. Anti hyperlipidemic assay

The rats were pre-treated with the formulation (100mg/kg or 200mg/kg) or atorvastatin (10mg/kg) before P-407 injection. On the last day of experiment, biological parameters were determined and compared among groups. The serum lipid profile for each group is reported as follows –



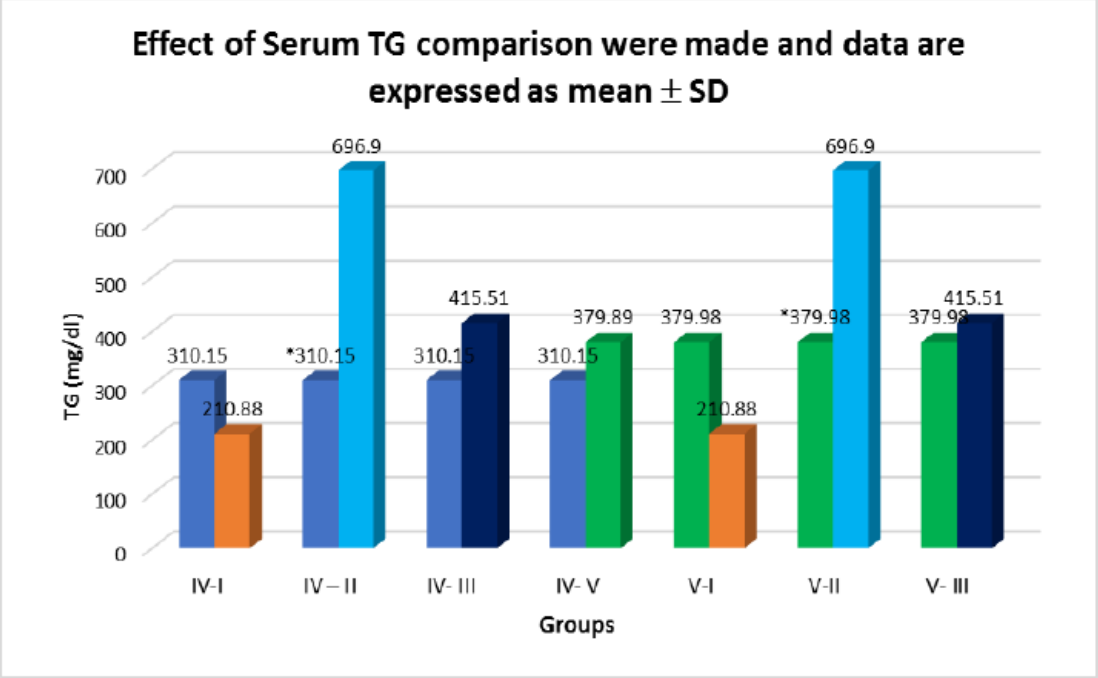
*p<0.001 Group I Group II Group III Group IV

# p<0.01 Group V

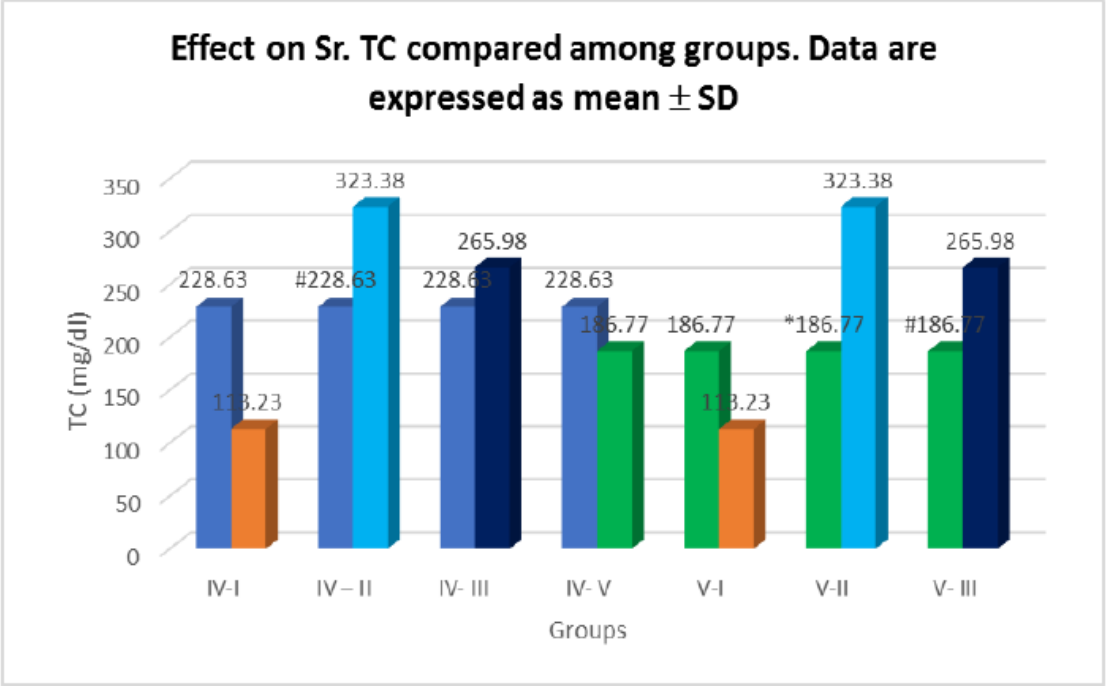


*p<0.001 Group I Group II Group III Group IV

# p<0.01 Group V

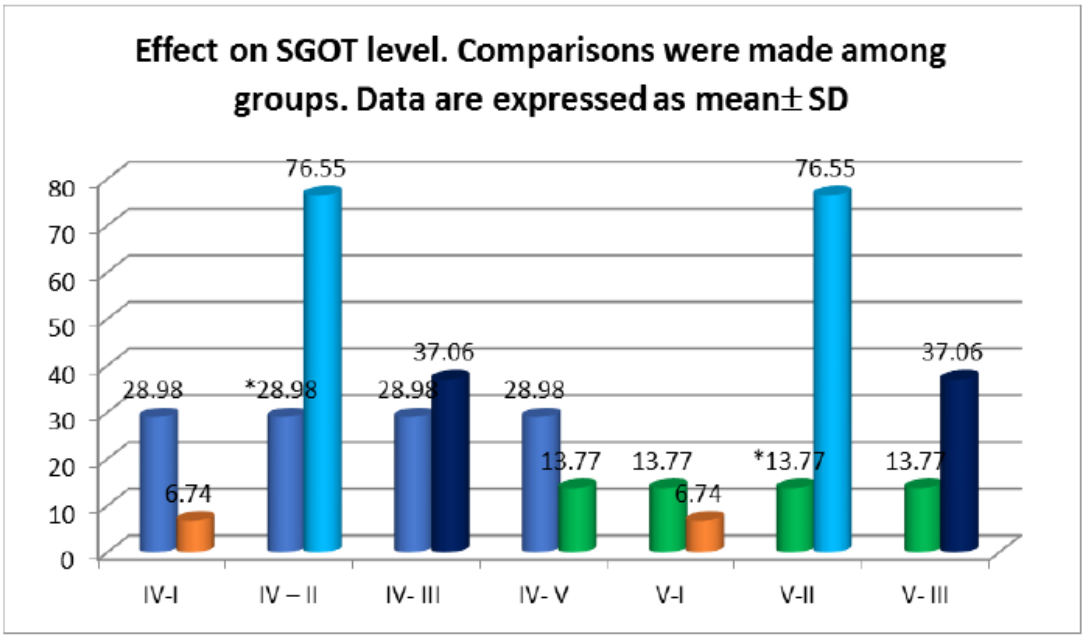


*p<0.001 Group I Group II Group III Group IV Group V

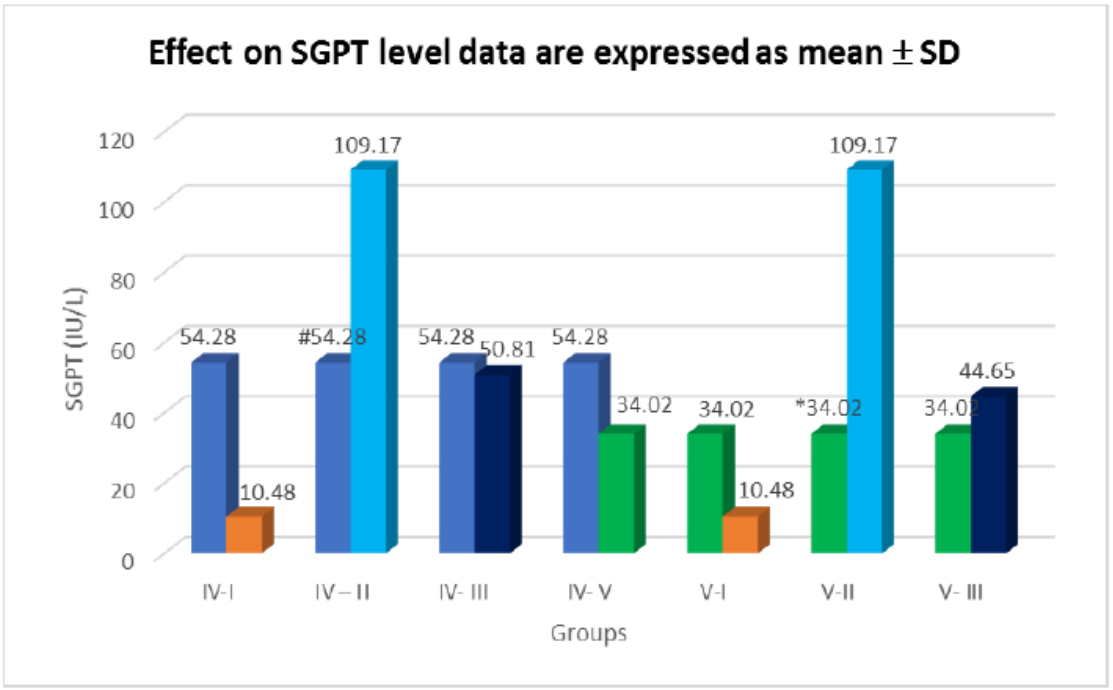


*p<0.001 Group I Group II Group III Group IV

#p<0.01 Group V

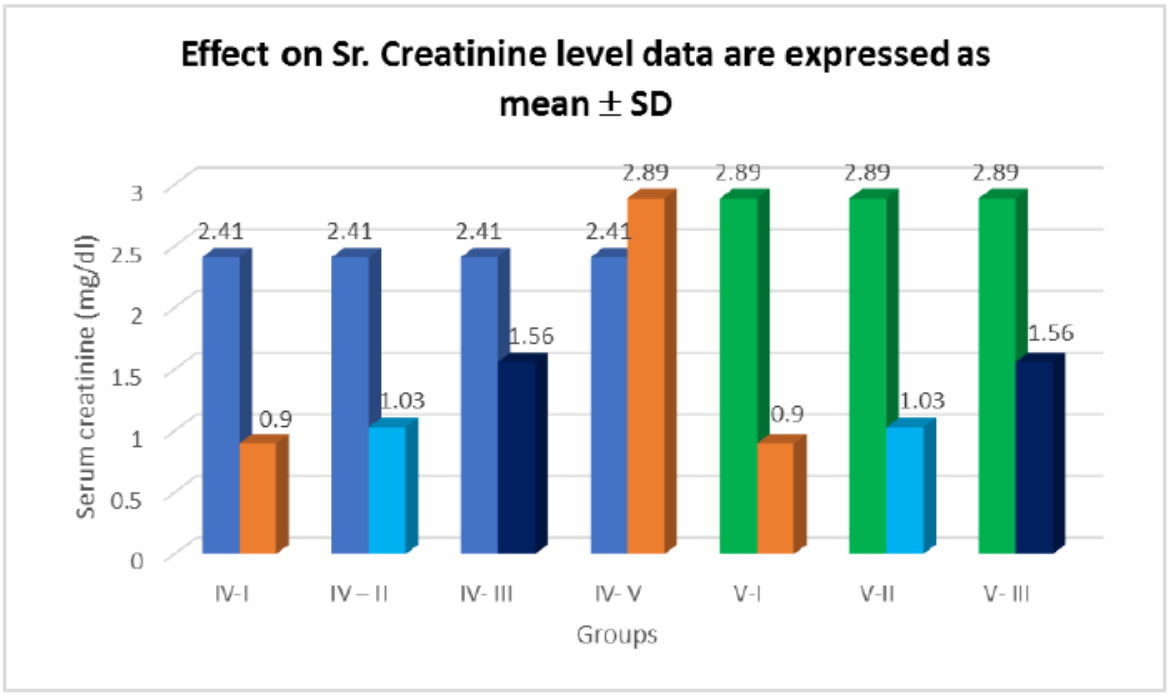


*p<0.001 Group I Group II Group III Group IV Group V

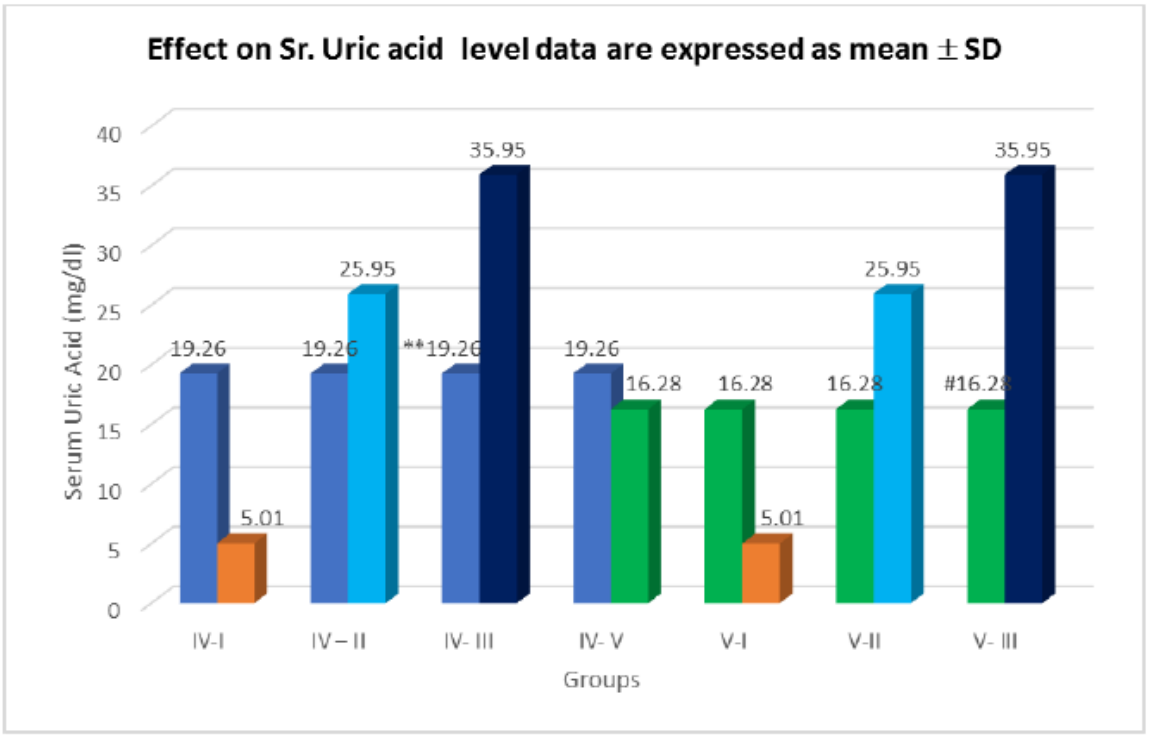


*p<0.001 Group I Group II Group III Group IV

#p<0.01 Group V



Group I Group II Group III Group IV Group V



**p<0.05 Group I Group II Group III Group IV

#p<0.01 Group V

Table: Effect of HF on lipid profile & liver enzymes.

Group	HDL	LDL	TG	TC	SGOT	SGPT	Sr. Creatinine	Sr. Uric acid
I	37.71 ± 2.96	108.04 ± 28.02	210.88 ± 152.62	113.23 ± 17.39	6.74 ± 2.84	10.48 ± 2.36	0.9 ± 0.21	5.01 ± 0.51
II	48.61 ± 6.36	381.68 ± 132.49	696.90 ± 116.48	323.38 ± 71.64	76.55 ± 24.45	109.17± 41.55	1.03 ± 0.34	25.95 ± 13.02
III	55.79 ± 7.87	272.25 ± 48.35	415.51 ± 134.35	265.98 ± 53.12	37.06 ± 35.16	50.81 ± 22.84	1.56 ± 0.59	35.95 ± 15.68
IV	###***^^^104.65± 35.89	**250.91 ± 42.54	***310.15± 89.86	**228.63± 54.20	***28.78± 24.50	*54.28± 47.27	2.41 ± 3.33	19.26 ± 11.86
V	###***^^95.73± 35.40	***^^200.98± 22.81	***379.98± 159.17	***^^186.77± 50.18	***13.77± 6.99	***34.02± 39.47	2.89 ± 2.01	16.28 ± 6.33

The values are expressed as mean+-SD.

###p<0.001, ##p<0.01 and #p<0.05 when compared with normal control.

***p<0.001, **p<0.01 and *p<0.05 when compared with hyperlipidemic group.

^^^p<0.001, ^^p<0.01 and ^p<0.05 when compared with atorvastatin 10mg/kg.

It is observed that the herbal formulation used in the study has characteristically reduced TC, TG and LDL(p<0.001) as well as increased HDL level(p<0.001). The HF also showed significant lowering of SGOT and SGPT levels. It was also noted that LDL and TC significantly decreased in the HF group V (200mg/kg) at p<0.01 and increased HDL(p<0.01) compared to the standard drug atorvastatin 10mg/kg.

DISCUSSION

Hyperlipidemia has become epidemic today and it is essential to understand the consequences of it. It is one of the disorders of non-communicable disease, which laid down foundation stone of diabetes mellitus, metabolic syndrome, hypertension and cardiovascular complications.

To assist in decreasing the pervasiveness of this disease, plant based therapies are recognized for their therapeutic applications as they either have minimal or no side effects. Current lipid lowering drugs don't satisfy the clinical goal, reduction of cardiovascular and cerebrovascular disorders. Moreover, those drugs often cause adverse effects associated with impaired quality of life. Despite the progress in conventional chemistry and pharmacology in producing effective drugs, the plant kingdom might yet provide useful sources of new medicines. Over the past 20 years or so, interest in traditional medicines has increased considerably in many

parts of the world. Traditional medicines all over the world are being re-evaluated by extensive research on different plant species with regard to their therapeutic principles and potential. From this point of view, it is believed that traditional herbal drugs would be good resource for hypolipidemia.

This study investigated the possibility of (HF) herbal formulation as a candidate for hypolipidemics, based on its traditional applications. This study was carried out with a herbal based formulation based on the ethnic practice of NE India tribes who are using leaves of Clerodendrum glandulosum (CG) and cloves of Allium sativum (AS) as folkloric remedy to treat Hypertension and cardiovascular diseases. Earlier studies clearly provide the evidence that the combination on being formulated in a CG:AS ratio of 8:1 exhibit health beneficial phytosynergy, granting scientific credibility to the documented folklore use of this herbal formulation. Phytochemical investigation revealed the possible existence of synergism in the developed formulation.[3]

In order to induce hyperlipidemia animal model, P-407 (Poloxamer-407) was injected Intraperitoneal to wistar rats. P-407 is known as a general lipase inhibitor by altering the normal elimination of lipids in bile. In this experiment, a single dose injection with P-407 (300mg/kg b wt.) drastically elevated lipid levels, LDL-C and triglycerides. However, pre-treatment with the HF significantly attenuated the abnormality of those lipid parameters. A low dose of HF(100mg/kg) significantly lowered TG and raised HDL-C while high dose (200mg/dl) specifically lowered LDL-C, TC and liver enzymes SGOT and SGPT. However, the HF does not show any improvement in the serum creatinine and serum uric acid level. The result showed better efficacy than the standard drug atorvastatin 10mg/kg in lowering LDL and TC($p<0.01$) and increasing HDL ($p<0.01$).

Acute Toxicity Study was done on rats as per OECD guidelines. A dose of 2000mg/kg b wt. of HF was given orally and observed the rats for any unwanted symptoms. The result showed no toxicity with any untoward reactions, which indicates the HF is totally safe up to a dose of 2000mg/kg b wt.

As per earlier reports, plants produce an amazing variety of metabolites such as isoflavones, phytosterols, saponins, fibers, polyphenols, flavonoids and ascorbic and these have aroused much interest for their role in lipid and antioxidant metabolism. Flavonoids consists of a large group of these polyphenolic compounds and are found to be useful in various disease

conditions. It has been confirmed that consumption of phenolic compound rich foods or beverages prevents heart diseases. In present study, it is investigated that the HF (CG:AS in 8:1 ratio) shows dose dependent percentage inhibition of DPPH as well as PMD in antioxidant assay as compared to the standard antioxidant compound Ascorbic acid although it is significantly lower than ascorbic acid. In DPPH Assay the IC50 of HF is 34.09 while that of Ascorbic acid is 19.27; and the IC50 value in PMD Assay for HF and ascorbic acid is 190.25 and 21.61 respectively. This indicates that HF shows Antioxidant Capacity in concentration dependent manner.

Currently, the prevention of hyperlipidemia, its associated complications and improving quality of life are the main goals of management of patients with lipid disorders and drug developments. This study explored the activity of HF in an animal model. Taken together, this study produced a scientific basis for clinical application and anti-lipidemic drug development using this formulation HF (CG:AS in 8:1 ratio).

CONCLUSION

The major findings are

- P-407 injection elevated the serum lipid levels and then pre-treatment with HF (100mg/kg. b. wt. and 200mg/kg. b. wt.) showed significant reduction in the LDL-C, TG, and TC levels at $p<0.001$ and significant increase in HDL-C level($p<0.001$). Also noted the more effectiveness as compared to the standard drug treated group ($p<0.01$).
- The decrease in the levels of hepatic enzymes, ALT and AST indicate the hepatoprotective nature of the HF.
- The HF showed antioxidant properties in DPPH scavenging and PMD reducing capacity in a dose dependent manner. It can be concluded that the HF possesses the potent antioxidant substances which may be responsible for its anti-inflammatory and chemo protective mechanism as well as justify the basis of using this formulation as folkloric remedies.
- HF was tested for toxicity issues before its investigation for in vivo efficacy. The results showed that oral administration of HF did not produce any toxic effects in wistar rats at dose of 2000mg/kg. b. wt. Therefore, usage of an appropriate amount of the HF for its traditional use should be considered safe.

From these findings, it is clearly indicated that the HF of the selected medicinal plants possess good antihyperlipidemic activity in hyperlipidemic rats. The observations established

the traditional claim and thus the HF could be a potent anti hyperlipidemic agent for use in future.

REFERENCES

1. Gilles Dumortier et al, A Review of P-407 Pharmaceutical and Pharmacological characteristics, Pharm Res. (2006).
2. Vidyadhar et al., Hypolipidemic activity of simvastatin in triton x-100 induced hyperlipidemic wistar rats, World J pharm Sci., 2014; 2(10): 1310-1315.
3. Hazarika, Ankita; Chandra Boruah, Dulal; Kumar Sharma, Dhirenda; Devi, Rajlakshmi; In Vitro Antioxidant and In Vivo Antihyperlipidemic Activity of a Diherbal Formulation of Clerodendrum colebrookianum Walp. And Allium sativum in hyperlipidemic rats, Journal of Pharmacy Research, 2012; 5(6): 3343.
4. Singh et al., IJPSR, 2017; 8(8): 3591-3598.
5. Zjumira G. M. Wout, Edward A. Pec, Jack A. Maggiore, Robert H. Williams, Prema Palicharla, Thomas P. Johnston; Poloxamer 407 – Mediated changes in plasma cholesterol and triglycerides following Intraperitoneal injection to rats; PDA Journal of Pharmaceutical science and technology, 1992; 46(6): 192-200.
6. Subasini et al, Phytochemical Analysis and Hyperlipidemia activity of Nelumbo nucifera in male wistar rats. International Journal of Pharmacy teaching and practices, 2014; 5(1): 935-940.
7. Jang, Woo Seok; Kim, Yoon-Sik; Seol, In-Chan; Antioxidant and lipid lowering effects of Artemisia capillaris on a rat model of hyperlipidemia, The journal of Korean oriental medicine, 2012; 33(2): 11-24.
8. Noor Nailis Sa'adah et al., Analysis of lipid profile and atherogenic index in hyperlipidemic rat that gives methanolic extract of parijata, AIP conference proceedings, 2017; 1854.
9. Jadeja RN et al., Clerodendrum glandulosum ameliorates high fat diet induced alteration in lipid and cholesterol metabolism in rats, Rev. bras. 2010.
10. MC Thounaojam et al., A preliminary study on hypolipidemic effect of aqueous leaf extract of Clerodendrum glandulosum Coleb, IJGP, 2009; 3(4).
11. DK Patel et al., Clerodendrum glandulosum methanolic extract ameliorates high fat diet induced hyperlipidemia in male rats, Pharmacologyonline, 2009; 2: 323-331.
12. Ravi raj Singh N Jadeja et al., Anti obesity potential of Clerodendrum glandulosum. Coleb aqueous leaf extract, Journal of Ethno pharmacology, 2011; 135(2): 338-343.

13. Khound, Puspanjali; Devi, Rajlakshmi; Anti glycation and anti oxidant potential of Clerodendrum glandulosum Lindl. In the leaf extract and it's fractions, proceedings of International conference on drug delivery (ICDD), 2020.
14. Kalita, Janmoni; Singh, S. Suresh Kumar; Khan, Mohammad Latif; Clerodendrum colebrookianum Walp. A potential folk medicinal plant of North East India, AJPBR, 2012; 2(4): 256-261.6p.
15. Menaka C Thounaojam et al, Toxicological evaluation and hepatoprotective potential of Clerodendrum glandulosum Coleb. Leaf extract, Human and experimental toxicology, 2011; 30(1): 63-70.
16. OECD (Organization for Economic and Co-operation and Development). OECD guidelines for the testing of chemicals/session 4, Health effects Test no. 423: Acute oral toxicity-Acute Toxic Class method, OECD, Paris, 2002.
17. Hitesh R Patel et al, Int. J. PharmTech Res., 2009; 1(2).
18. K. Prasad, Atherosclerosis, 2008; 197: 34.
19. R. Devi and D. K. Sharma, J. Ethnopharmacol., 2004; 90: 63-68.
20. R. Devi, et al., Life Sci., 2005; 77: 2999.
21. Harnafi, et al., Food Chem., 2008; 108: 205.
22. Institute for Laboratory Animal Research, Guide for the care and use of laboratory animals: 8th Ed., (2011).
23. Hetal R Chaudhary et al., The single dose Poloxamer 407 model of hyperlipidemia, J pharm Sci., (2013).
24. Katsube T, Tabata H et al., Screening for Antioxidant in edible plant products: comparison of LDL oxidation assay, DPPH radical scavenging assay and Folin- Ciocalteu assay; Journal of the Agricultural and Food Chemistry, 2004; 52: 2391-2396.
25. http://en.wikipedia.org/wiki/hyperlipidemia
26. http://images.Google.com.
27. Chiavarini M, Minelli L, Fabiani R; Garlic consumption and colorectal cancer risk in man: a systematic review and meta-analysis. Public Health Nutr., 19(2): 308-317. [Google Scholar]
28. World Health Organization http://www.who.int/mediacentre/factsheets/fs317/en/[accessed31.01.11]
29. N. Jadid, D. Hidayati, S. R. Hartanti, B. A. Arraniry, R. Y. Rachman, Wiwi Wikanta; Antioxidant Activities of Different Solvent Extracts of Piper retrofractum Vahl. Using DPPH Assay; AIP Conference proceedings 1854, 020019 (2017)

30. http://doi.org/10.1063/1.4985410 .
31. Institute for Laboratory Animal Research, Guide for the care and use of laboratory animals: 8th Ed., (2011).