CHAPTER - 2

Screening Of Medicinal Plants for Anti-Hyperlipidemic Activity in Triton Induced Hyperlipidemic Animal Model
## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pg. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Introduction</td>
<td>81</td>
</tr>
<tr>
<td>2.2 Materials and Methods</td>
<td>82</td>
</tr>
<tr>
<td>2.2.1 Collection of plants material and extraction</td>
<td>82</td>
</tr>
<tr>
<td>2.2.2 Chemicals</td>
<td>83</td>
</tr>
<tr>
<td>2.2.3 Animals</td>
<td>83</td>
</tr>
<tr>
<td>2.2.4 Induction of hyperlipidemia</td>
<td>84</td>
</tr>
<tr>
<td>2.2.5 Pharmacological evaluation</td>
<td>84</td>
</tr>
<tr>
<td>2.2.6 Statistical Analysis</td>
<td>84</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>84</td>
</tr>
<tr>
<td>2.4 Materials and Methods</td>
<td>85</td>
</tr>
<tr>
<td>2.4.1 Collection of plant material and extraction</td>
<td>85</td>
</tr>
<tr>
<td>2.4.2 Chemicals</td>
<td>86</td>
</tr>
<tr>
<td>2.4.3 Animals</td>
<td>86</td>
</tr>
<tr>
<td>2.4.4 Experimental design for dose dependent activity of MEHR and META</td>
<td>86</td>
</tr>
<tr>
<td>2.4.5 Statistical Analysis</td>
<td>87</td>
</tr>
<tr>
<td>2.5 Results</td>
<td>87</td>
</tr>
<tr>
<td>2.5.1 Effect of MEHR on serum lipid profile</td>
<td>87</td>
</tr>
<tr>
<td>2.5.2 Effect of META on serum lipid profile</td>
<td>89</td>
</tr>
<tr>
<td>2.6 Discussion</td>
<td>91</td>
</tr>
<tr>
<td>2.7 Bibliography</td>
<td>93</td>
</tr>
</tbody>
</table>
2.1 INTRODUCTION

Elevated level of plasma concentration of total cholesterol (TC), low density lipoproteins cholesterol (LDL-C) and triglycerides (TG) are recognized as major risk factors for coronary heart diseases. Other complications related to hyperlipidemia are atherosclerosis, hypertension and obesity (NCEP, 2006). In many cases, hyperlipidemia is also caused due to over-ingestion of alcohol, abnormal diet and in disease condition; hence more attention has been paid for its treatment and prevention along with the use of strict dietary management (Nomura et al., 1996). Hyperlipidemia can be reduced by two possible ways, viz., by blocking endogenous synthesis or by decreasing absorption. Both these factors can be evaluated in normal animals without artificial diets using Triton WR 1339 (Moss and Dajani, 1971).

Currently available hypolipidemic drugs includes statins and fibrates. Statins help in correcting the altered serum lipid levels by inhibiting the synthesis of cholesterol and the fibrates acts by improving the clearance of TG rich lipoprotein (Mahley and Bersot, 2006). However, consumption of these synthetic drugs have been associated with side effects such as increased uric acid levels in blood, diarrhea, motion sickness, myositis, gastric disturbance, dry skin and abnormal liver function (Kumar et al., 2008). Hence, there is an increase in demand for newer plant based products with an ability to reduce or regulate serum TC and TG concentrations. Since plant based products are less damaging than synthetic drugs they have better tolerance even when used in a long term basis (Kaliora et al., 2006).

Crude methanolic extracts of all the five plants were used in the present study. When using crude extract, a factor that can affect the outcome in terms of the biological activity is the synergism between the different active constituents that may be present in the extract. Synergism can lead to better activity as well as decrease in potential toxicity of some individual constituents. Synergism can be due to the individual action of different constituents present in the extract at multiple target sites/parameters (Kicklighter et al., 2003).

Triton-induced hyperlipidemia animal models were used for assessing the antihyperlipidemic activity of the extract, which is a easy and rapid method for the assessment of the test drugs. This method can be considered as a functional
tool for preliminary screening of antihyperlipidemic drugs (Sannoumaru and Shimizu, 1996).

In a constant research to discover new leads from medicinal plants for the treatment of obesity linked with hyperlipidemia, various commonly available medicinal plants were screened for antihyperlipidemic efficacy. Screening of plants was carried out in two stages. In stage I five medicinal plants were screened for their antihyperlipidemic effect using the Triton WR-1339 induced hyperlipidemic mice model. Mice were used for screening of plants as they are economical for use. Total cholesterol was estimated to check the antihyperlipidemic efficacy of the extracts. Normal mice have traditionally not been ideal models for research of lipoprotein disorders since they typically have very low levels of TC and LDL-C but high levels of HDL-C. This is in contrast to humans in whom the reverse is true because unlike humans mice do not possess plasma cholesteryl ester transfer protein (CETP) and therefore, about 70% of the plasma TC is found in HDL particles (Karimi, 2012). Mice also have an ability to maintain their cholesterol profiles even when exposed to high cholesterol diets (Maxwell et al., 2003). Hence the further studies were continued on rat models. The second stage of screening include evaluation of the selected plant extracts for their dose dependent activity in lowering serum lipid levels in Triton WR 1339 induced hyperlipidemic rats. The study was subsequently shifted to rat model as they are sturdier than mice and they can provide more blood volume to analyze various other blood related parameters. The experiments for screening of the plant extracts were divided into two stages.

**STAGE I: Screening of Five Medicinal Plants for the Antihyperlipidemic Activity in Triton WR 1339 Induced Hyperlipidemic Mice Model.**

**2.2 MATERIALS AND METHODS**

**2.2.1 Collection of Plants Material and Extraction**

Leaves of *Hibiscus rosa sinensis*, fruits of *Trichosanthes anguina*, *Amorphophallus campanulatus*, and *Luffa cylindrica* and seeds of *Foeneculum vulgare* were procured locally. The respective parts of these plants were air dried followed by drying in hot air oven at 80 °C for 1 h. The dried material was cooled and ground in a blender to obtain coarse powders that were stored separately in
an air-tight container so as to avoid any contamination till further studies were carried out. Fifty gram of each plant powder was extracted with 300 ml of methanol in soxhlet apparatus. The extract was filtered and dried at 40 °C to obtain a semisolid material. The dried methanolic extracts of plants were stored at 4-8 °C till further pharmacological studies were carried out.

2.2.2 Chemicals
Triton WR-1339 was bought from Sigma Aldrich, USA, while atorvastatin was obtained from Cipla Ltd., Bangalore. Diagnostic kits for total cholesterol (TC) was purchased from Span Diagnostics India Ltd., Surat. All other chemicals used in this study were of analytical grade (AR) and were obtained locally.

2.2.3 Animals
Swiss albino mice of either sex, weighing 25-30 g were procured from Bharat Serums and Vaccines Ltd., Thane, Mumbai. The animals were housed in standard environmental conditions and fed with food and water *ad libitum*. The mice were acclimatized to the laboratory conditions for 10 days prior to initiation of the experiment. The experimental protocol was approved by the Institutional Animal Ethics Committee (CPCSEA/IAEC/SPTM/P-36/2011) and all experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. The animals were divided into eight groups, of six mice each.

Group 1: Normal control
Group 2: Triton control
Group 3: Simvastatin (80 mg/kg body weight)
Group 4: *H. rosa sinensis* (250 mg/kg body weight)
Group 5: *T. anguina* (250 mg/kg body weight)
Group 6: *A. campanulatus* (250 mg/kg body weight)
Group 7: *L. cylindrica* (250 mg/kg body weight)
Group 8: *F. vulgare* (250 mg/kg body weight)
2.2.4 Induction of Hyperlipidemia
The mice were fasted overnight. Triton WR-1339 dissolved in (0.9%) saline was injected intraperitoneally (300 mg/kg body weight) in Group 2-8, to induce hyperlipidemia. Mice Group 1 were injected with saline.

2.2.5 Pharmacological Evaluation
The plant extracts and the standard drug simvastatin were dispersed in 0.5% carboxymethylcellulose (CMC) as drug vehicle. Eight hour following Triton injection, Groups 4-8 were orally administered with different extracts at a median dose of 250 mg/kg body weight, by gastric intubation. Simultaneously, the positive control group (Group 3) was treated with simvastatin at a dose of 80 mg/kg body weight whereas, the vehicle 0.5% CMC, alone was administered to the normal and Triton control group by gastric intubation. At the end of the study, i.e. 24 h after extract administration, blood was withdrawn by retro-orbital sinus puncture and serum was separated by centrifugation at 5000 rpm for 10 min and stored at -20 °C. The amount of serum TC was estimated using diagnostic kits.

2.2.6 Statistical Analysis
The results have been expressed as mean±SD. Statistical analysis was carried out using GraphPad Prism®5 software. One way analysis of variance (ANOVA) was performed followed by Dunnett’s multiple comparison tests. The plant extracts and drug treated group were compared with the triton control group and P<0.05 was considered significant.

2.3 RESULTS
Figure 2.1 shows the effect of five plant extracts and simvastatin on the TC levels. The injection of Triton WR-1339 caused a significant elevation (P<0.0001) of serum TC levels in all the groups in comparison with normal control group. Administration of *H. rosa sinensis*, *T. anguina*, *A. campanulatus* and *F. vulgare* extracts at a dose of 250 mg/kg body weight showed a significant decrease in serum TC levels with maximum percentage reduction shown by *H. rosa sinensis* and *T. anguina* by approx. 46% and 47%, respectively. *L. cylindrical* showed 14.4% decrease in serum TC. However, the decrease was not statistically significant.
Figure 2.1: Effect of plant extracts and simvastatin on serum total cholesterol levels. The values are expressed as mean ± S.D. Treatment groups were compared with HFD control (*P<0.05, **P<0.01, ***P<0.0001) and normal control with HFD control (#P<0.0001)

STAGE II: Determination of Dose Dependent Activity of Selected Plant Extracts Using Triton WR 1339 Induced Hyperlipidemic Rat Model.

2.4 MATERIALS AND METHODS
2.4.1 Collection of Plant Material and Extraction
Leaves of H. rosa sinensis and fruits of T. anguina were collected in bulk and the plant material were identified and authenticated at The Blatter’s Herbarium, St. Xavier's College, Mumbai, India (H. rosa sinensis voucher specimen no. PDS 55, T. anguina Voucher specimen 17178 E. Blatter). The extraction of methanolic extract of H. rosa sinensis (MEHR) and T. anguina (META) were done as per the method described earlier in the section 2.2.1
2.4.2 Chemicals

Diagnostic kits for TC, triglycerides (TG) and high density lipoprotein cholesterol (HDL-C) were purchased from Span Diagnostics India Ltd., Surat. All other chemicals used in this study were of analytical grade (AR) and were obtained locally.

2.4.3 Animals

Adult albino wistar rats of either sex, weighing 150-200 g were taken for this current study. All the details such as procurement, housing and CPCESA no. have been described earlier in the section 2.2.3.

2.4.4 Experimental Design for Dose Dependent Activity of MEHR and META

The animals were divided into six groups 1-6, each consisting of six rats.

Group 1 : Normal control
Group 2 : Triton control
Group 3 : Atorvastatin control
Group 4 : MEHR/META 125 mg/kg body weight
Group 5 : MEHR/META 250 mg/kg body weight
Group 6 : MEHR/META 500 mg/kg body weight

The induction of hyperlipidemia in rats was carried out as mentioned earlier in the section 2.2.4. Extracts and the standard drug were prepared in 0.5% CMC. Twenty four hours after the administration of Triton WR-1339, animals from Groups 4-6 were treated with the extracts at doses of 125, 250, and 500 mg/kg body weight respectively, by gastric intubation using gavage needle. Simultaneously, the standard drug atorvastatin at a dose of 30 mg/kg body weight was orally administered to Group 3, which served as positive control. Whereas, 0.5% CMC alone was administered to Group 1 and 2. Twenty four hours following administration with atorvastatin and the plant extracts, blood was withdrawn by retro-orbital sinus puncture and serum was separated by centrifugation at 5000 rpm for 10 minutes and stored at -20 °C. The amount of
serum TC, TG and HDL was estimated using diagnostic kits. LDL-C and very low density lipoprotein cholesterol (VLDL-C) were calculated using the Friedewald formula.

2.4.5 Statistical Analysis
The results have been expressed as mean ± SD. Statistical analysis was carried out using GraphPad Prism® 5 software. ANOVA was performed followed by Dunnett’s multiple comparison tests. MEHR, META and drug treated groups were compared with triton control group and \( P<0.05 \) was considered significant.

2.5 RESULTS
2.5.1 Effect of MEHR on Serum Lipid Profile
The effect of MEHR on lipid profile of Triton WR 1339 induced hyperlipidemic rats is shown in figure 2.2. Injection of Triton WR 1339 caused hyperlipidemia as shown by elevated serum TC, TG, LDL-C, VLDL-C levels \((P<0.0001)\) and decrease in HDL-C levels in rats of Group 2 \((P<0.0001)\). Administration of MEHR at a dose of 250 mg/kg body weight resulted in the significant decrease of serum TC and TG levels but did not show any significant effect in the LDL-C and VLDL-C levels. However, serum TC, TG, LDL and VLDL levels were significantly reduced \((P<0.001)\) and HDL levels were significantly increased at a dose of 500 mg/kg body weight when compared to the untreated triton control group. The MEHR showed a decrease of 54.6% in TC, 77.5% in TG, 77% LDL, 41.9% VLDL and increase of 107.9% in HDL levels. Treatment with atorvastatin in triton induced animal caused a significant decrease of TC, TG, LDL, VLDL levels by 44.2%, 57.7%, 38.7%, 26.4% respectively and increase in HDL levels by 49.8% in comparison with hyperlipidemic animals.
Figure 2.2: Effect of MEHR on serum TC(A), TG(B), VLDL(C), LDL(D) and HDL(E) levels in Triton WR 1339 induced hyperlipidemic rats. Treatment groups were compared with HFD control (*P<0.05, **P<0.01, ***P<0.0001) and normal control with HFD control (#P<0.0001).
2.5.2 Effect of META on Serum Lipid Profile

The effect of three different doses of META and atorvastatin on serum lipid profile in experimental rats is shown in Figure 2.3. The injection of Triton WR 1339 caused a significant elevation of serum TC, TG, LDL-C and VLDL-C levels \( (P<0.05) \) when compared with normal control group. Treatment with META at a dose of 125 and 250 mg/kg body weight did not have any significant effect on the lipid and lipoprotein levels. Treatment with META at the dose of 500 mg/kg b.w. resulted in significant decrease in levels of TC by 54\%, TG by 75\% and VLDL-C by 66\%. Also, significant increase in serum HDL-C levels were observed in comparison with triton group by 117\%. 
**Figure 2.3**: Effect of META on serum TC(A), HDL(B), LDL(C), TG(D) and VLDL(E) levels in Triton WR 1339 induced hyperlipidemic rats. Treatment groups were compared with HFD control (*P<0.05, **P<0.01, ***P<0.0001) and normal control with HFD control (#P<0.0001)
2.6 DISCUSSION

In the current study, Triton WR-1339 non-ionic surfactant was used to induce hyperlipidemic state in mice and rats (Kellner et al., 1951). It was previously reported that administration of Triton WR 1339 to adult rats leads to induction of hyperlipidemia (Schultz and Parkinson, 1972). Triton WR 1339 leads to blockage of triglyceride-rich lipoprotein clearance, that in turn is responsible for acute hyperlipidemia in albino wistar rats (Yusuf et al., 2011). The significant elevation in serum TC and TG levels is due to increased VLDL secretion by the liver accompanied by a noticeable decline in VLDL and LDL catabolism (Otway and Robinson, 1967).

In the first stage of the study, screening for antihyperlipidemic potential of five selected plants was carried out in Triton WR 1339 induced hyperlipidemic mice model. The effect of methanolic extracts of *H. rosa sinensis*, *T. anguina*, *A. campanulatus*, *L. cylindrica* and *F. vulgare* at a dose of 250 mg/kg body weight on serum TC levels was analyzed. From the results it was observed that triton injection in mice displayed hyperlipidemia as shown by their increased levels of serum TC when compared with normal control group. Among the five plant extracts MEHR (24%) and META (16%) showed maximum percentage reduction in total cholesterol and hence, were selected for the further evaluation.

In the second stage of screening, evaluation of the selected plant extracts viz., MEHR and META for their dose dependent activity in lowering serum lipid levels was carried out. Three doses (125 mg/kg b.w., 250 mg/kg b.w. and 500 mg/kg b.w.) of both plant extracts were tested. Triton injection resulted in induction of hyperlipidemia in rats as reflected by the increased serum lipoprotein levels. Both the plant extracts showed maximum efficacy at the dose of 500 mg/kg b.w. Hence this dose was selected for further studies.

The present findings also indicate that the antihyperlipidemic effect exerted by MEHR and META could probably be associated with reduced absorption of cholesterol in intestine thereby resulting in increase of lipids in fecal excretion or could be due to interference with the cholesterol biosynthesis (Purohit and Vyas, 2006). It is well demonstrated that decrease of HDL-cholesterol levels is a major cause for coronary heart disease (Malloy, 1994). Current findings indicate increase in the HDL levels after the treatment with MEHR and META in
hyperlipidemic rats could play a role in prevention of coronary heart disease. Vijayaraj et al (2013) have demonstrated similar results with flower extract of *Cassia auriculata* while Sivaelango et al. (2012) elucidated the ability of ethanolic seed extract of *Spermacoce hispida* to reduce lipid levels in Triton induced hyperlipidemic rats. Sikarwar and Patil (2012) have also reported antihyperlipidemic effect of root extract of *Salacia chinensis* using triton induced hyperlipidemic rat model.

The presence of alkaloids, phytosterols, flavonoid, saponins and β-sitosterol (will be discussed in chapter 3) in MEHR and META might have resulted in the lipid lowering activity. As number of bioactive compounds (Ikeda and Sugano, 1983) such as stigmasterols, orientin, bergapten, tannins, and steroids (sitosterol and sitosterol-o-β-D-glucoside) have been reported in the improvement of lipid levels (Duester, 2001). Flavonoids are reported to have variety of pharmacological activities and may decrease the risk of cardiovascular disease (Engler and Engler, 2004). In addition phytosterols, specifically, β-sitosterol helps in inhibiting the cholesterol levels in the intestine lumen by competing with dietary and biliary cholesterol for its incorporation into mixed miscelles (Brufau et al., 2008). Reports suggest that the intake of plant materials rich in β-sitosterol caused reduction in total serum cholesterol levels in the experimental animals (Mayes and Botham, 2003; Moghadasian and Frohlich, 1999). Saponins were also reported to increase the lipoprotein lipase activity which in turn leads to the removal of free fatty acid from circulation thereby decreasing the total cholesterol levels (Guimaraes et al., 2000). Additionally, Girija and Lakshman (2011) have demonstrated the presence of steroids, flavonoids and triterpinoids in the methanolic extracts of three plants of *Amaranthus* that caused the reduction of lipid levels.
2.7 BIBLIOGRPAHY


