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Evalaution of Antihyperlipidemic Activity of *Boerhavia Diffusa* Plant Leaves Extarct in Mice

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A B S T R A C T
Major complications of hyperlipidemia are atherosclerotic heart disease, heart attack and heart stroke, but atherosclerosis is primary cause of death. Developing countries are reliant on medicinal plants as their main source of treatment for diseases. As *Boerhavia Diffusa* has the native habitat the production is more so it is locally available cost effective with no side effects. As *Boerhavia Diffusa* is cost effective and beneficiary in metabolism of cholesterol[22], so it has been taken in to consideration in order "To evaluate Anti-hyperlipidemic activity of Methanolic Extract and Phenolic Extracts of *Boerhavia Diffusa* in triton X -100 induced hyperlipidemic rats".

Keywords: *Boerhavia Diffusa*, Anti-hyperlipidemic activity, Methanolic Extract

A R T I C L E I N F O

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Contents

1. Introduction

Lipid is the scientific term for fats in the blood. At Normal levels, lipids perform important functions in your body, but can cause health problems if they are present in excess. The term hyperlipidemia means high lipid levels. Hyperlipidemia includes several conditions, but it usually means that you have high cholesterol and high triglyceride levels.[1] Lipid and lipoprotein abnormalities are common in the general population, and are regarded as a modifiable risk factor for cardiovascular disease due to their influence on atherosclerosis. In addition, some forms may predispose to acute pancreatitis.

Hyperlipoproteinemia Type I

It is a form of hyperlipoproteinemia associated with deficiencies of lipoprotein lipase.

Hyperlipoproteinemia Type II

Hyperlipoproteinemia type II, by far the most common form, is further classified into type IIa and type IIb, depending mainly on whether there is elevation in the triglyceride level in addition to LDL cholesterol.

Type IIa

This may be sporadic (due to dietary factors), polygenic, or truly familial as a result of a mutation either in the LDL receptor gene on chromosome 19 (0.2% of the population) or the ApoB gene (0.2%). The familial form is characterized by tendon xanthoma, xanthelasma and premature cardiovascular disease. The incidence of this disease is about 1 in 500 for heterozygotes, and 1 in 1,000,000 for homozygotes.

Type IIb

The high VLDL levels are due to overproduction of substrates, including triglycerides, acetyl CoA, and an increase in B-100 synthesis. They may also be caused by the decreased clearance of LDL. Prevalence in the population is 10%.

- Familial combined hyperlipoproteinemia (FCH)
- Secondary combined hyperlipoproteinemia (usually in the context of metabolic syndrome, for which it is a diagnostic criterion)

Hyperlipoproteinemia Type III

This form is due to high chylomicrons and IDL (intermediate density lipoprotein). Also known as "broad beta disease" or "dysbetalipoproteinemia", the most common cause for this form is the presence of ApoE E2/E2 genotype. It is due to cholesterol-rich VLDL (β-VLDL). Prevalence is 0.02% of the population.

Hyperlipoproteinemia Type IV

This form is due to high triglycerides. It is also known as "hypertriglyceridemia" (or "pure hypertriglyceridemia"). According to the NCEP-ATPIII definition of high triglycerides (>200 mg/dl), prevalence is about 16% of adult population.

Hyperlipoproteinemia Type V

This type is very similar to type I, but with high VLDL in addition to chylomicrons. It is also associated with glucose intolerance and hyperuricemia. Non-classified forms are extremely rare:

- Hypo-alpha lipoproteinemia
- Hypo-beta lipoproteinemia (prevalence 0.01-0.1%)

2. Materials and Methods

Table 1: Materials and Instruments

Materials and Instruments	Supplier /Manufacturer
Atorvastatin	Dr.Reddys Lab, Hyderabad.
Normal saline	Claris life sciences. Ltd. Ahmedabad, India.
Chloroform	Molychem, Mumbai.
Diethyl ether	Finar Ltd, Ahmedabad, India.
Triton X-100	Unisource Chemical Pvt, Ltd
Shimadzu Electronic Balance	Toshvin Analytical Pvt.Ltd, Mumbai, India
U.V spectrophotometer	Shimadzu UV-1800.
Ultra homogenizer	Biologics, inc, USA.
Centrifuge RM-12C	Remi Electro Technik Ltd, Mumbai India.
Rotary Evaporator	Heidolph Rotary Flask Evaporator,India.
Total cholesterol kits	Excel Diagnostics Pvt, Ltd, India
Triglycerides Kits	Excel Diagnostics Pvt, Ltd, India
HDL-Cholesterol Kits	Excel Diagnostics Pvt Ltd , India

Collection and Authentification of Plant Material

The Aerial Parts of *Boerhavia diffusa* for the study were procured and authenticated

Extraction of Plant Material

The plant is grinded in to a coarse powder with the help of suitable grinder.

Cold Extraction (Methanol Extraction)[38]

In this work the cold extraction process was done with the help of methanol. About 200gms of powdered material was taken in a clean, flat bottomed glass container and soaked in 750 ml of methanol. The container with its contents were sealed and kept for period of 7 days accompanied by continuous shaking with the shaker. The whole mixture then went under a coarse filtration by a piece of a clean, white cotton wool.

Evaporation of Solvent

The filtrates (methanol extract) obtained were evaporated using Rotary evaporator in a porcelain dish. They rendered a gummy concentrate of greenish black. The extract was kept in vaccum dissecator for 7 days.

Preliminary Phytochemical Screening

Preliminary phytochemical screening of the *Boerhavia diffusa* extract was carried out for the analysis of Alkaloids, Carbohydrates, Tannins, Saponins, Steroids, Phenols, Flavonoids as per the standard methods [40].

Animals

Healthy Adult Male mice of 5 weeks old with Average weight in the range of 30-40gms were selected. Animals are housed 4 per cage in temperature controlled (27 ^{0}C ±3 ^{0}c) room with light/dark cycle in a ratio of 12:12 hrs is to be maintained. The Animals are allowed to acclimatize to the environment for seven days and are supplied with a standard diet and water *ad libitum*. The guidelines of committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Govt of India were followed and prior permission was sought from the Institutional Animal Ethics Committee (IAEC) for conducting the study.

Acute toxicity studies: The Acute Toxicity Studies was performed using female mice as per OECD Guideline No.423 (Short term toxicity). Male mice were selected of weight around 50 ±10 gm for main test. Single animals are dosed in sequence usually at 48 h intervals. A Dose Progression Factor of 3.2 is used. Using the default dose progression factor, doses would be selected from the sequence (1.75, 5.5, 17.5, 55, 175, 550, 1750, and 5000). However, the time intervals between dosing are determined by the onset, duration, and severity of toxic signs. Treatment of an animal at the next dose should be delayed until one is confident of survival of the previously dosed animal. If the animal survives, the second animal receives a higher dose. If the first animal dies or appears moribund, the second animal receives a lower dose. The toxicological effects were observed in terms of mortality expressed as LD50. The number of animals dying or surviving during a period was noted.

Method of Induction: The systemic administration of the surfactant Triton X-100 to mice results in a biphasic elevation of plasma cholesterol and triglycerides. Hyperlipidemia was induced in Wister albino mice by single intraperitoneal injection of freshly prepared solution of Triton-X-100 (100mg/kg) in physiological saline solution after overnight fasting for 1h.[13]

Experimental Animal Protocol

Experimental mice, straved for 18 hr, were provided water *ad libitum*. The mice were divided in to six groups containing four animals in each group.

Group – I: Normal Control. (Normal saline 10ml/kg orally for 7 days)

Group – II: Hyperlipedemic control, (Triton x 100.)

Group – III: Hyperlipedemic mice treated with MEBD at dose of 500mg/kg. for 7days

Group – IV: Hyperlipedemic mice treated with PEBD at dose of 400mg/kg for 7days.

Group – V: Hyperlipedemic mice treated with PEBD at dose of 500mg/kg for 7days.

Group–VI: Hyperlipedemic mice treated with Atorvostatin at 10 mg/kg for 7days.

All the groups recives single i.p. injection of Trition X-100 at dose of 100mg/kg, simultaneously with Group- II, Group – III, Group – IV, Group – V, Group – VI, expect Group – I (Normal control). After 72 hours of Triton X-100 injection. The Group – VI receives Atorvostatin at dose of 10 mg/kg, was prepared by suspending bulk Atorvastatin in aqueous 0.5% methyl cellulose[42] for 7 days. The Group– III, receive MEBD, at daily dose of 500mg/kg orally for 7 days and Group – IV, Group – V receives PEBD at daily dose of 400mg/kg and 500mg/kg orally for 7 days

Blood Sample Collection and Analysis

The mice are anesthetized by ether and then Blood samples were collected on 0^{th} and 8^{th} day[13] from retro-orbital plexus of rat using micro capillary technique from mice of all the groups[43], and centrifuged at 3000 rpm for 15 min so as to get serum. The serum is analyzed for total cholesterol, triglycerides and HDL levels using biochemical kits (diagnostic kit.)[44]. VLDL, and LDL- Cholesterol were calculated by the below formula

Serum LDL- Cholesterol concentration was calculated According to the equation of Fried and wald[45].

LDL-Cholesterol=Total Cholesterol – (HDL-Cholesterol +TG/5)

VLDL-C = TG/5

Bio Chemical Assays for lipids

Estimation Procedures: Plasma Lipid Profile Estimation

Total cholesterol LDL cholesterol, HDL Cholesterol, VLDL cholesterol, Triglycerides levels were measured using commercial kits.

Estimation of Triglycerides. (GPO/PAP Method)[46]

Principle: Triglycerides are hydrolyzed by lipase to glycerol and free fatty Acids. Glycerol is phosphorylated by ATP in the presence of glycerolkinase (GK) to glycerol – 3 –phosphate (G-3-P). Which is oxidized by the enzyme Glycerol–3–phosphoxidase (G-P-O) producing hydrogen peroxide. Hydrogen peroxide so formed reacts with 4-Amino-Hydrogen peroxidave (POP), to produce a brown. colour complex. The intensity of the colour developed is proportional to the triglyceride concentrate

Procedure

Wave length: 546 (Green Filter)

Temperature: 37 C

Reaction type: End point with standard.

Pipettle in to clean dry tube labelled Blank (B), Standard (S) and Test (T) and then add following:

	Blank	Standard	Test
Enzyme reagent	1.0 ml	1.0 ml	1.0 ml
Standard	-	0.01ml	-
Serum / Plasma	-	-	0.01 ml

Mix well and incubate for 10 minute at 37^0 C. Read absorbance of standard and test against blank.

Calculations

Triglyceride concentration in mg% = Absorbance of test /Absorbance of Standard X 200

Estimation of cholesterol (Total cholesterol).

CHOD/POD Method[47].

Clinical Significance

Heart disease is often the result of cholesterol deposits on the arteries. While not the only factor for heart disease, serum cholesterol levels are often checked to determine the risk of heart disease on patient.

Principle

Enzymatic determinations of total cholesterol according to the following reactions:-

Cholesterol Ester $+H_2O$ $\xrightarrow{\text{Cholesterol Esterase}}$ Cholesterol + Fatty Acids.

Cholesterol $+O_2$ $\xrightarrow{\text{Cholesterol Oxidase}}$ 4– Cholesterol –3-one $+ H_2O$

$2H_2O_2$+Phenol $\xrightarrow{\text{4-Aminoantipyrineperoxidase}}$ Quininoneimine dye+ $4H_2O$

Procedure

Wave Length: 500 nm (green filter)

Temperature: 37^0C.

Reaction type: End point with standard.

	Blank	**Std**	**Test**
Enzyme Reagent	1 ml	1ml	1 ml
Deionized Water	0.01 ml	-	-
Standard	-	0.01 ml	-
Serum / Plasma	-	-	0.01 ml

Pipette in a clean dry test tube labelled as Blank (B), Standard (S), Test (T).

Mix and read the optical density (OD) at 500nm against blank after 5min incubation (37^0c). The final colour is stable for atleast 1 hour.

Calculations

Cholesterol concentration in mg% = Absorbances of Test/ Absorbances of Standard×200 (Standard).

Estimation of HDL cholesterol.

Procedure: It includes two steps.

Step: 1-precipitation

Serum	0.2 ml
HDL precipitating reagent	0.3 ml

Step: 2 – Colour development

Take 3 clean glass tubes labelled as blank (B), standard (S), and test (T). Mix well and stand at room temperature for 10 min, centrifuge at 3000 rpm for 10 min.

	Blank	**Standard**	**Test**
Enzyme reagent	1 ml	1ml	1 ml
Cholesterol (Standard)	-	0.01 ml	-
Supernatant serum Step-1	-	-	0.1 ml
Distilled water	0.1 ml	0.1 ml	-

Incubation for 5 min at 37^0c and read the optical density at 500nm against blank.

Calculations

HDL cholesterol = Absorbance of test /Absorbance of standard × 50 (Standard concentration).

LDL Calculation

It is calculated using formula: LDL = TC-HDL-TG/5.0 (mg/dl). VLDL is calculated using formula:

VLDL = Triglycerides (mg/dl) / 5,

According to these guidelines, the normal range of lipid profile

Total cholesterol	**< 200 mg/dl**
Triglycerides	< 200 mg/dl
HDL	> 40 mg/dl
LDL	< 150 mg/dl
VLDL	5-30 mg/dl

LDL/HDL and TC/HDL ≤ 5 mg/dl are the favorable risk factor.

Statistical Analysis: Results are expressed as Mean ± S.D .all the results were compared with control subject one-way analysis of variance (ANOVA), followed by the dunnet t-test using Graph Pad Prism Software 6 version. P Values < 0.05 were as considered statistically significant.

3. Results and Discussion

%Yield of Methanolic Extract from Aerial Parts of *Boerhavia diffusa* was found to be **25.7**

% Yield value of Phenolic Extract from Aerial Parts of *Boerhavia diffusa* was found to be **12.6**

Preliminary Phytochemical Screening

Investigation revealed the presence of Alkaloid, Tannin, Saponin, Phenol in Methanolic Extract of *Boerhavia diffusa* while only Phenol were present in Phenolic Extract of *Boerhavia diffusa*.

Table 2: Preliminary Phytochemical Screening

Phytochemical	**Results**
Steroid	+
Alkaloid	+
Tannin	+
Carbohydrate	-
Phenol	+
Flavonoid	+
Saponin	_

(+) Present: **(-)** Absent

Acute toxicity studies

As per (OECD) draft guidelines 423 adopted, Female albino mice were administered with *Boerhavia diffusa* and doses was be selected in the sequence (1.75- 5000) using the default dose progression factor, for the purpose of toxicity study. Animals are observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours and daily thereafter, for a total of 14 days,. In all the cases, no death was observed within 14 days. Additional observations like behavioral changes in skin, fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous systems and somato motor activity and behavior pattern were also found to be normal. Attention was also given to observation of tremors and convulsions, salivation, diarrhoea, lethargy, sleep and coma. Overall results suggested the LD_{50} value as 5000 mg/kg. Hence therapeutic dose was calculated (i.e. 400mg/kg and 500 mg/kg) of the lethal dose for the purpose of antihyperlipidemic investigations. Phytochemical Investigation revealed the presence of Alkaloid, Tannin, Saponin, Phenol in Methanolic Extract of *Boerhavia diffusa* while only Phenol were present in Phenolic Extract of *Boerhavia diffusa* %Yield value of Methanolic Extract from Aerial Parts of *Boerhavia diffusa* was found to be **25.7** % Yield value of Phenolic Extract from Aerial Parts of *Boerhavia diffusa* was found to be **12.6 %.** Administration of Triton-X-100 (100mg/kg) to all the fasted mice caused an elevation of TC, TG, VLDL and LDL and reduction in HDL levels. After 72 hrs of induction of Triton X-100 results in hyperlipidemia which is compared with normal control group .which results in significantly increased serum lipid levels in hyperlipidemic group. The change in lipid levels in group number III to VI, were comparable with group of Hyperlipidemic control (i.e Triton X-100 ,Group- II) . The Standard group (i.e Atorvostatin group) significantly lowers the serum lipid level ($P<0.001$). The results of the study clearly indicate that MEBD Extract and PEBD Extract at a dose of 500 mg/kg & 400 mg/kg significantly lowered serum lipid levels ($P<0.01$). PEBD Extract at a dose of 500 mg/kg significantly lowered serum lipid levels, ($P<0.001$) i.e. antihyperlipidemic activity which was found to be more effective in higher dose of PEBD as compared to MEBD and lower dose of PEBD when administered orally in triton induced hyperlipidemic models. MEBD Extract having very low hypolipidemic activity. PEBD Extracts showed a dose dependant decrease in the levels of cholesterol, Triglyceride, LDL-C and VLDL-C level. Among three groups (i.e. group number III-V), Group number- V reduced the elevated lipid levels more significantly than the other Groups.($P<0.001$). Flavonoids have exhibited a variety of pharmacological activities, including the antiatherogenesis and antioxidant effect. Thus the present result strongly suggests that the hypolipidemic activity of this medicinal plant could be attributed to the presence of Tannis, Phenols and flavonoids in the Extracts.

Table 1: 3 Lipids Levels Obtained on 8th Day (After Treatment)

S.N	GROUPS	TC	TG	HDL	LDL	VLDL
I	Normal Control	64.03 ± 1.45	82.66 ± 2.46	38.91 ± 2.33	8.45 ± 3.43	16.53 ± 0.49
II	Hyperlipidemic Control	192.47 ± 5.05	168.9±5.28	21.86±2.74	136.82±7.00	33.79±1.05
III	MEBD 500mg/kg	124.19 ± 9.5*	127.7 ± 10.5*	29.1 ± 2.9***	86.8 ± 6.6*	25.5 ± 2.05***
IV	PEBD400mg/kg.	120.4 ± 9.4*	109.3 ± 6.6*	30.0 ± 3.3**	70.1 ± 10.5***	22.5 ± 2.3***
V	PEBD 500mg/kg.	132.7 ±9.25*	102.5 ±9.2*	33.1 ± 3.1**	56.1 ± 5.9*	28.1 ± 1.4***
VI	Std. Atorvostatin10mg/kg	92.29 ± 5.63*	102.26 ± 7.68*	39.18 ± 3.14**	32.91 ± 7.61*	20.44 ± 1.53**

All the data are expressed as MEAN ± S.D (n=4),*P = < 0.001, **P = < 0.01, ***P = < 0.05. vs GROUP .II
TC: Total Cholesterol; TG: Triglycerides; HDL-C: High Density Lipoprotein cholesterol ;
LDL-C: Low Density Lipoprotein- cholesterol; VLDL-C: Very Low Density Lipoprotein;
MEBD: Methanolic Extract of Boerhavia diffusa; PEBD: Phenolic Extract of Boerhavia diffusa

4. Conclusion

The results concluded that PEBD (500 mg/kg) have definite antihyperlipidemic activity in Triton X-100 induced hyperlipidemic model and which is equipotent activity when compared with Atorvastatin treated groups. Further studies on this extract may lead to identify the possible mechanism of action and isolation of active principle from the same.

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