

ALTERATIONS INDUCED BY N-NITROSODIMETHYLAMINE AND ETHANOLIC ROOT EXTRACT OF *OPERCULINA TURPETHUM* IN SERUM LIPID PROFILE OF MALE ALBINO MICE

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Received: 27 February 2012, Revised and Accepted: 9 May 2012

ABSTRACT

The present study investigated the protective effect of ethanolic root extract of *Operculina turpethum* on serum lipid profile in male albino mice intoxicated with N-Nitrosodimethylamine (NDMA). N-nitrosodimethylamine is a potent hepatotoxin and carcinogen and has been reported to be found in the chloraminated drinking water from treatment plants and distribution systems. *Operculina turpethum* Linn is convolvulaceous plant, the roots are bitter, acrid, sweet, thermogenic, purgative, carminative, anthelmintic, expectorant, antipyretic, hepatic stimulant and effective against colic obesity and high lipid levels. Phytochemicals detected in the ethanolic extract are Flavonoids, Polyphenols, Alkaloids, Saponins, Enzymes, and Minerals. Adult male albino mice, treated with NDMA at a concentration of 10 mg/kg body weight and *Operculina turpethum* root extract was given orally in doses of 300 and 400 mg/kg body weight at 5 h after the administration of NDMA. NDMA treated mice showed a significant decrease in the levels of low density lipoprotein (LDL) in the serum of mice received OTE at a concentration 300mg/kg b.wt. Further, an increase in the level of high density lipoprotein (HDL) in serum of mice who received OTE (400 mg/kg b.wt.) after intoxication with NDMA and a significant decrease in the level of cholesterol and triglycerides of mice administrated with OTE at a concentration of 300mg/kg b.wt. was observed. Administration of ethanolic extract at a dose of 300 mg/kg b.wt. significantly attenuated the alterations caused by the intoxication of NDMA when compared with the standard BHA and restored the levels of triglycerides in the serum of mice.

Keywords: Lipid profile, NDMA, *Operculina turpethum*, Cholesterol.

INTRODUCTION

Coronary heart disease (CHD) remains the principal cause of death in both developed and developing countries. Studies have shown that high levels of total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL) and apolipoproteins and low levels of high density lipoproteins cholesterol (HDL) are the risk factors of CHD. High intake of fats and dyslipidemia are risk factors for its development. Reactive oxygen species may contribute to the events of atherogenesis and leading to the progression of atherogenic lesions by promoting oxidation of low density lipoproteins¹. Liver play an important role in protein and lipoprotein catabolism and synthesis. (NDMA) N-Nitrosodimethylamine is toxic to the liver as it causes fibrosis that results in change in the lipid metabolism and hence the lipid content of the blood also leads to the formation of reactive oxygen species. Reactive oxygen species have been implicated as causative agents in many degenerative diseases, and also in the promotion phase in carcinogenesis.²

High blood lipids are associated with hypertension and lipid peroxidation with drug toxicity. However in case of toxicity micronutrients such as vitamins A and C naturally present in most plants serve as antioxidants.³ Naturally occurring antioxidants in leafy vegetables and seeds, such as ascorbic acid, vitamin E, and phenolic compounds, possess the ability to reduce the oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, arthritis, immune deficiency diseases and ageing.⁴

The plant *Operculina turpethum*, which is commonly known as *trivit*, is a large stout perennial twinner with milky juice and fleshy branched roots. *Operculina turpethum* Linn, belongs to the family Convolvulaceae. It is widely grown throughout india and it is occasionally cultivated in gardens as an ornament. It has been used as a folk medicine in many countries to treat constipation, jaundice, rheumatism, chronic gout, piles and tumors, obesity and many other diseases. The bark of the plant contains a glycosidic resin, which has the insoluble glycoside turpethin and two ether soluble glycosides. In addition, it also contains a minor amount of essential oil, glucose and fructose.⁵ N-Nitrosodimethylamine (NDMA) is a member of a family of extremely potent carcinogens, the N-nitrosamines. Humans are exposed to a wide range of N-nitrosocompounds (NOCs) from diet, tobacco smoking, work place and drinking water. NDMA is an emerging drinking water contaminant that is of interest to the

environmental community because of its miscibility with water, as well as its carcinogenicity and toxicity.⁶

In this investigation we intended to look at the possibility of having lipid changes in the blood of mice treated with NDMA and the extract of *Operculina turpethum* roots.

MATERIALS AND METHODS

Drugs and Chemicals

Draggendorff's reagent, Chloroform, Fehling's solution, Glacial acetic acid, H₂SO₄, Ferric chloride. All chemicals used in the study were of analytical reagent grade and were purchased from reliable firms (SRL (India), MERCK, RANBAXY, HIMEDIA). NDMA was purchased from SIGMA. Kits for the estimation of total cholesterol, triglyceride and HDL-cholesterol were purchased from Erba (Transasia Bio-Medicals Ltd. Jaipur, India).

Animal Care and Monitoring

Healthy male Swiss albino mice (*Mus musculus*) (4-6 months old, weighing 20-30 g) were procured from C.C.S. Haryana Agricultural University (Hisar, India). They were housed under standard laboratory conditions of light (12:12 h L: D cycle), temperature (23 ± 2°C) and relative humidity (55 ± 5%). Animals had free access to standard food pellet diet (Hindustan Lever Limited: metal contents in parts per million dry weight: Cu 10.0, Zn 45.0, Mn 55.0, Co 5.0, Fe 75.0) and drinking water *ad libitum* throughout the study. The animals were provided standard mice pellet feed and tap water *ad libitum*.

Plant Material

Operculina turpethum was collected from Pharmacological garden of CCSHAU Hisar, Haryana, India in the month of November 2010. The plant was identified with the help of available literature and authenticated by Botanist of Krishi Vigyan Kendra Rohtak, Haryana, India.

Preparation of Ethanolic Extract

The freshly collected *Operculina turpethum* roots were dried in shade and coarse powder was extracted. Dried powdered material was placed in the Soxhlet thimble with 80% ethanol in 500 ml flat bottom flask. Further refluxed for 18 h. at 80°C for two days.

Collected solvent was cooled and poured in a glass plate. The marc was dried in hot air oven below 50°C for 48 h and kept in desiccator for 2 days⁷. The yield of the extract was 18.5% w/w of powdered plant material for further exploration. Collected dried extract was stored at 5°C in air tight containers.

Preliminary Phytochemical Screening

Test for steroids

Extract (20 mg) was treated with acetic anhydride (2.5 ml) and chloroform (2.5 ml). Then concentrated solution of sulphuric acid was added slowly and green bluish color was observed for steroids⁸.

Test for flavonoids

There are two methods used to test flavonoids. First, 10% of dilute ammonia (5 ml) was added to a portion of an aqueous filtrate of the extract. Concentrated sulphuric acid (1 ml) was added. A yellow colouration that disappears on standing indicates the presence of flavonoids. Second, a few drops of 1% aluminium solution were added to a portion of the filtrate. A yellow colouration indicates the presence of flavonoids⁸.

Test for saponins

To 0.5 g of extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion⁸.

Test for terpenoids

To 0.5 g of the extract was added 2 ml of chloroform. Concentrated sulphuric acid (H₂SO₄) 3ml was carefully added to form a layer. Formation of reddish brown colouration of the interface indicates the presence of terpenoids⁸.

Test for alkaloids

About 0.5 g of extract was diluted in 10 ml of 1% aqueous hydrochloric acid, boiled and filtered. Dilute ammonia 2ml was added to 5 ml of the filtrate. Chloroform 5ml was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) was observed indicating the presence of alkaloids⁸.

Test for cardiac glycosides

To 0.5 g of extract diluted in 5 ml of water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underplayed with 1 ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout the layer⁸.

Test for reducing sugars

The aqueous ethanol extract (0.5 g in 5 ml of water) was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction⁸.

ETHICAL CLEARANCE

The animal experiments were carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The Institutional Animal Ethics Committee approved experimental design performed in this study for the use of Swiss Albino mice as an animal model for the study.

EXPERIMENTAL DESIGN

The male Swiss Albino mice (*mus musculus*) weighing 25-30g were randomly selected from laboratory stocks and will be placed into various groups (8-10).

- Group I - Control
- Group II - NDMA treated (10 mg/kg body weight)
- Group III - NDMA + OTE (300mg/kg body weight)
- Group IV - NDMA+ OTE (400mg/kg body weight)
- Group V - OTE (300 mg/kg body weight)
- Group VI - OTE(400mg/kg body weight)
- Group VII - NDMA + Standard antioxidant(BHA1%)
- Group VIII - BHA (1%)

The doses of the plant extract, NDMA and standard antioxidant were decided on the basis of previously published reports (Riaz Ahmad, Sarfaraz Ahmed, NizamUddin Khan, Absar-ul Hasnaina.,2009)⁹. NDMA was given on three consecutive days of each week for three successive weeks along with the plant extract simultaneously. The animals were sacrificed after 21 days of the start of experiment.

BODY WEIGHT : The body weight of the animals was calculated before and after the experiment and is expressed in grams. The change in the body weight was calculated according to the following formula .

$$\text{Change in the body weight \%} = \frac{\text{Change in the body weight}}{\text{Initial body weight}} \times 100$$

Collection of Blood and Serum

Twenty-first day after the start of the experiment, the animals were procured and the blood was collected by puncturing the retro-orbital plexus from the eye. Serum samples were separated from the clot by centrifugation at 3000 g for 10 min using centrifuge. Serum samples were separated into sterile plain tubes and stored in the refrigerator for analyses¹⁰.

Biochemical Estimation

Serum total cholesterol (TC), triglyceride (TG) and HDL, LDL were estimated by using diagnostic kits Erba (Transasia Bio-Medicals Ltd. Jaipur, India). VLDL was calculated as per Friedewald's equation.¹¹ Results were expressed in mg/dl.

$$\text{VLDL-cholesterol} = \frac{\text{Serum triglyceride} - \text{Cholesterol}}{5}$$

Inhibition of Lipid Peroxidation

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using liver homogenate as lipid-rich media, as described by Ruberto et al¹². Briefly, 0.5 mL of liver homogenate was added to 0.1 mL of the extract. The volume was then made upto 1.0 mL with distilled water. Thereafter, 0.05 ml of FeSO₄ was added and the mixture was incubated at 37°C for 30 min. Then, 1.5 mL of acetic acid was added, followed by 1.5 mL of TBA in SDS. The resulting mixture was vortex mixed and heated at 95°C for 1 hour. After cooling, 5 mL of butanol was added and the mixture was centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm and the percentage inhibition was calculated with the formula

$$\% \text{ inhibition of lipid peroxidation} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Compliance with Good Laboratory Practice (GLP)

The studies were carried out according to Good Laboratory Practice (GLP) regulations of Organization for Economic Cooperation and Development - OECD (UNDP/World Bank/WHO, 2001).

Statistical Analysis

Statistical analysis of data was determined with the use of Standard student's 't'-test method and hypothesis testing method included one-way analysis of variance (ANOVA) p < 0.05, p<0.01 were regarded as significant. The group data are expressed as mean ± SD for six animals in each group.

RESULTS

The results of the phytochemical screening of the ethanolic root extract of *Operculina turpethum* showed the presence of various secondary metabolites of which proanthocyanidins, phenols, flavonoids, alkaloid, saponin, and cardiac glycosides were the most prominent and the result of phytochemical test has been summarized in (Table1). *Operculina turpethum* is tested negative for terpenoids¹³. The presence of alkaloid and saponins in the plant also indicates that the plant extracts could be used for the antifungal activity. Flavonoids and alkaloids are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers¹⁴. These finding suggest that this plant is a potential source of natural antioxidants that could serve great importance as therapeutic agents, anti-inflammatory, anti-analgesic and anti-hyperlipidemic, which is implicated in the pathogenesis of numerous disorders, e.g. cardiovascular, atherosclerosis, rheumatoid arthritis etc.

Table 1: Analysis of Phytochemicals of Ethanolic Extract of *Operculina Turpethum* (Ot) Roots.

Phytochemicals	Ethanolic Extract <i>Operculina turpethum</i>
Alkaloids	++
Saponins	+++
Phytosterols	++
Phenols	+++
Flavonoids	+++
Terpenoids	-
Cardiac Glycosides	+++
Proanthocyanidin	+++

+++ Maximum Presence of the compound, ++ Moderate, + Least Presence; - Absence of the compound.

The changes in the body weight of animals were calculated at the end of the experiment and are presented in Table 2.

Table 2: Analysis of the body weight of mice in different groups before and after treatment

Groups	Initial Weight (gm)	Final weight (gm)	%Change
Gp I Control	25.5±0.55	25.2±0.37	1.17
Gp II NDMA	26.0±0.82	21.3±0.47 ^a	18.07
Gp III NDMA +OTE (300 mg/kg b.wt.)	25.7±1.25	24.2±1.2 ^a	5.83
Gp IV NDMA+OTE (400 mg/kg b.wt.)	25.8±0.37	23.8±0.69	7.74
Gp V OTE (300mg/kg b.wt.)	25.0±0.58	24.7±0.94 ^a	5.22
Gp VI OTE (400 mg/kg b.wt.)	25.6±0.75	24.5±0.55 ^a	4.55
Gp VII NDMA +BHA	25.8±1.07	24.2±1.34 ^a	6.31
Gp VIII BHA	25.6±1.03	27±0.63 ^a	5.18

Values are expressed as Mean ± SD, (n=6), ^aP<0.05, values compared to initial weight. (Student t-test)

It is evident from the results that there is not much change in the weight of animals in control group with time. The initial average body weight of the mice was (25.5±0.55) Control, (26.0±0.82) NDMA, (25.7±1.25) NDMA +OTE 300 mg/kg body weight, (25.8±0.37) NDMA + OTE 400 mg/kg, (25.0±0.58) OTE 300 mg/kg body weight, (25.6±0.75) OTE 400 mg/kg body weight, (25.8±1.07) NDMA +BHA, (25.6±1.03) BHA. The weight of the animals after the completion of the experiment were significantly reduced (25.2±0.37) Control, (21.3±0.47) NDMA, (24.2±1.2) NDMA +OTE 300 mg/kg body weight, (23.8±0.69) NDMA +OTE 400 mg/kg body weight, (24.7±0.94) OTE 300mg/kg body weight, (24.5±0.55) OTE 400 mg/kg body weight, (24.2±1.34) NDMA + BHA and increased as (27±0.63) BHA respectively. Decrease in the body weight was observed throughout the study duration in almost all the groups except group VIII. This deduction in the body weight can be

attributed to the fact that the compound NDMA caused a significant toxicity in mice. There is a significant increase in the body weight in group VIII which can be attributed to the antioxidant effect of BHA. The harmful effect caused by the NDMA is significantly recovered by the administration of the OTE at the concentration of 300 mg/kg b.wt and at the concentration of 400 mg/kg b.wt. respectively.

Using liver homogenate as a medium of peroxidation, percentage lipid peroxidation inhibition by ethanolic root extract of *Operculina turpethum* and standard ascorbic acid¹⁵ were calculated. The percentage inhibition of OTE (*Operculina turpethum* extract) was found to be significantly ($p<0.01$) comparable to that with the standard ascorbic acid. This reveals that the plant extract is capable of inhibiting lipid peroxidation and the maximum inhibition was calculated at the concentration of 5000µg/ml in liver homogenate which is quite promising to have a positive effect.

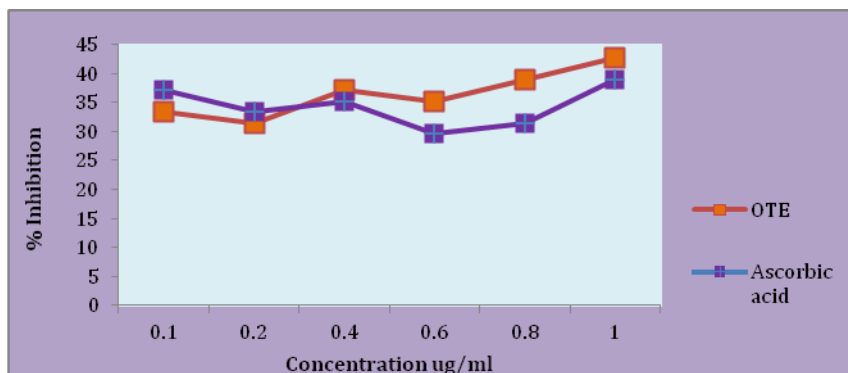


Figure 1: analysis of the %inhibition of lipid peroxidation by ascorbic acid and *operculina turpethum* root extract in liver.

Table 3: Effect of *Operculina Turpethum* Root Extract on Serum Lipid Profile of Mice after 21 Day Treatment.

Groups	Total Cholesterol mg/dl	HDL mg/dl	LDL mg/dl	Triglycerides mg/dl	VLDL mg/dl	Total Lipids mg/100ml
Group I	104.4±9.1 ^π	38.2±9.1 ^π	58.8±10.9 ^π	171.8±90.2 ^π	17.32±11.3 ^π	432±79.5 ^π
Group II	122.6±30.8 [*]	39.8±5.0 ^{ns}	65±7.2 [*]	232.2±90.8 [*]	21.92±14.8 [*]	492±125.6 [*]
Group III	92.7±11.1 ^π	35.8±5.5 ^μ	51.3±8.2 ^π	111.3±64.6 ^π	11.13±5.4 ^π	356.7±55.7 ^π
Group IV	127.8±14.5 ^μ	47.8±6.4 ^μ	74±9.4 ^μ	177.8±65.8 ^{ns}	12.4±10.1 ^μ	508±76.6 ^μ
Group V	114.0±26.5 [*]	39±6.4 ^{ns}	56.8±10.2 [*]	93.7±46.4 [*]	9.8±2.2 [*]	425±90.9 [*]
Group VI	156.7±52.3 [*]	41.8±6.7 [#]	84.3±29.6 [#]	99.5±72.6 [*]	11.4±5.3 [#]	580±214.4 [#]
Group VII	89.58±15.2 ^π	40.8±8.1 ^π	72±11.4 ^π	220.5±122.2 ^π	21.3±18.3 ^{ns}	545±87.8 ^π
Group VIII	129.3±10.0 [#]	29.3±4.3 [#]	64.8±5.7 [#]	150.7±77.6 [#]	12.9±7.9 [#]	501.7±33.1 [#]

Values are expressed as Mean ± SD, # p<0.05, *p<0.01 when compared to control group and ^μp<0.05, ^πp<0.01 when compared to treated group (ANOVA); n = 6.(ns; non significant)

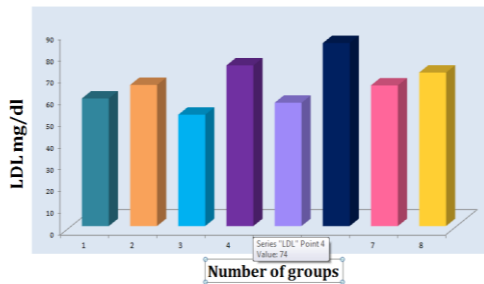


Figure 2: Variation in the Low Density Lipoprotein Concentration in Different Groups of Mice after Treatment.

The serum lipid profile results of this present study revealed that oral administration of *Operculina turpethum* root extract to the animals for 21 days at two different doses showed a significant decrease in total cholesterol, LDL and triglyceride concentration, and a significant increase in HDL concentration when compared with the untreated group whereas certain non significant changes have also been observed in the results. Very low density and low density lipoprotein was markedly reduced in the treated group in comparison with the untreated, as shown in Table 3 when compared with the control group values.

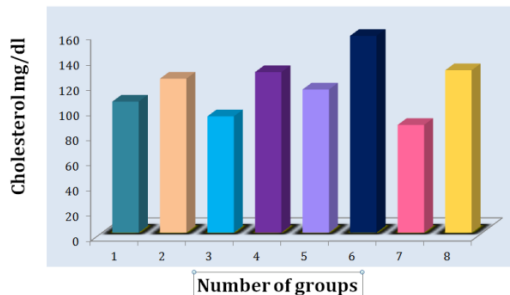


Figure 3: variation in the cholesterol concentration in groups of mice after treatment.

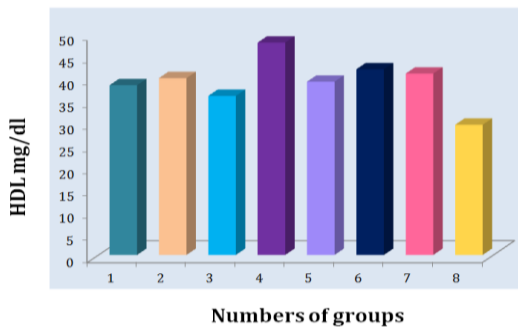


Figure 4: variation in the high density lipoprotein concentration in different groups of mice after treatment.

The high density lipoprotein levels are increased in the mice given with the extract at the concentration of 400 mg/kg body weight.

DISCUSSION

Atherogenicity with subsequent cardiovascular manifestations is one of the major causes of death and morbidity in the world. These abnormalities affect the body weight in one or the other way¹⁶. In the present study there was a drastical change in the body weight of animals of group II which is a sensitive indicator that the compound NDMA has caused toxicity leading to hepatic fibrosis and reduction in the organ weight. On the other hand the loss in the weight is significantly recovered in animals treated with the plant extract. Gross pathological observation of the organs showed no gross abnormalities in the morphologies, features, consistencies and appearances of the liver, kidney, heart, spleen, lungs and testes of the male mice treated for 21 days with the extract and only the changes in the weight were observed.

Lipid peroxidation is an accumulated effect of reactive oxygen species (ROS), which leads to deterioration of biological systems. It may be initiated by reactive free radicals, which subtract an allylic hydrogen atom from a methylene group of polyunsaturated fatty acid side chains¹⁷. The main target available for reactive oxygen species for attack is polyunsaturated fatty acids (PUFA), which is the precursor for lipid peroxide formation. The damage has been shown to cause different diseases like cancer, cardiovascular diseases and diabetes whereas phytochemical constituents of plants have been reported as scavengers of free radicals and inhibitors of lipid peroxidation. HDL are susceptible to oxidation, which affects their cardioprotective properties¹⁸. Vitamin C inhibits lipid peroxidation in HDL and preserves the antioxidant activity associated with this lipoprotein fraction. Hence we used the ascorbic acid as a standard to compare the inhibitory effect of *Operculina turpethum* plant extract. The abilities of the fractions of *Operculina turpethum* to inhibit the process of lipid peroxidation were tested using the method of Ruberto *et al*.

Hyperlipidaemia and other abnormal blood lipid profile are largely of genetic origin or due to unwholesome nutritional habits. Lipids and other substances accumulate on arterial wall, forming plaque, which occlude the vascular lumen and obstruct the blood flow to vital organs such as the heart, brain, liver, or kidney. Obstruction of blood supply to the heart, brain, liver or kidney cause coronary heart diseases, stroke or kidney failure¹⁹. The cholesterol in blood serum was significantly high in mice groups given with the dose of 400 mg/kg b.wt. when compared to control group whereas at the dose of 300 mg/kg b.wt. its significantly reduced. In our study, high cholesterol level by NDMA intoxication is may also be due to decreased activity of cytochrome P450 enzymes and due to hepatic dysfunction in mice. *Operculina turpethum* extract worked best at the concentration of 300 mg/kg body weight and can depress the hepatic activity of lipogenic, cholesterogenic enzymes such as malic enzymes, fatty acid synthase, glucose-6-phosphate dehydrogenase causing the reduction in the cholesterol level.

The rise in cholesterol and triglycerides in serum lipid profile may also be attributed to increased lipolysis, mediated by increased norepinephrine release which act through interference with the

intracellular functions of Ca^{+2} in the cytoplasm. All these events may lead to increased production of ROS, inducing oxidative stress resulting in metabolic dysfunction²⁰. The observed LDL effect in the study may be attributed to the effect of saponins. Saponins are capable of precipitating cholesterol from micelles and interfere with enterohepatic circulation of bile acids, making it unavailable for intestinal absorption²¹. Saponins are also known antinutritional factors which reduce the uptake of certain nutrition especially cholesterol at the gut through intraluminal physiochemical interactions. Hence, saponins have been reported to have hypocholesterolic effect. Presence of saponins has been reported in the pant extract and this saponin may explain the antilipidemic effect of the extract at a particular concentration observed in this study. The importance of this LDL-cholesterol lowering effect is that the extract may aid in the prevention or reduction of cardiovascular diseases. The significant reduction in the level of log (TG/HDL-cholesterol) promises a decrease risk of vascular disease, since high atherogenic index of log TG/HDL-cholesterol has been positively correlated with cardiovascular disease²².

The high density lipoprotein levels are increased in the mice given with the extract at the concentration of 400 mg/kg body weight. The administration of ethanolic extract for a period of 21 days resulted in a significant increase in HDL, when compared with untreated animals. It is well documented that while low level of HDL is indicative of high risk for coronary artery disease, an increase in HDL level is considered beneficial. Epidemiological studies have also shown that high HDL levels could potentially contribute to anti-atherogenesis, including inhibition of LDL oxidation to protect the endothelial cells from the cytotoxic effects of oxidized LDL²³. In conclusion, various functional components, such as flavonoids, saponins, phenols and glycosides in the plant, could play important roles in altering body fat and regulating lipid metabolism. However, further study is needed to clarify the hypolipidemic activity and its profound and subtle mechanisms at the cellular level whereas the studies on the isolation, identification and characterization of the active principles are in progress.

ACKNOWLEDGMENT

The authors are thankful to the authorities of Banasthali University for providing support to the study.

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