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RESEARCH ARTICLE

Effect of *Phyllanthus maderaspatensis* L. crude methanolic extract on diet induced hypercholesterolemia in Wistar albino rats (*Mus norvegicus albinus*)

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Abstract: Present experiment was aimed to assess the diet induced hypocholesterolemic activity of crude methanolic extracts of *Phyllanthus maderaspatensis* L. on Wistar rats. The crude whole plant methanolic extract was administered orally at the dosage of 2000 mg/kg body weight per day, to diet induced hypercholesterolemic Wistar albino rats for 14 days to assess the acute toxicity and hypercholesterolemic activity. Lower concentrations were used since no toxicity found during the acute toxicity experiment. Dosages of Methanolic extracts of 400mg, 800 mg and 1200 mg/ kg body weight per day were orally administered to rats for 42 days whilst total cholesterol, triglyceride, were studied. Hardly any acute toxicity signs and liver toxicity were observed with the extract of 2000 mg/kg. The same concentration indicated a reduction of total cholesterol and triglyceride during 14 days. The groups treated with three lower doses of extract exhibited dose dependent negative response with total cholesterol and triglyceride. Results indicated capability of the crude methanolic extract of *P. maderaspatensis* L. in total cholesterol and triglyceride in diet induced hypercholestrolemic Wistar rats.

Keywords: Cholesterol, *Phyllanthus maderaspatensis* L, Wistar albino rats.

INTRODUCTION

The condition with elevated serum cholesterol levels in blood is referred to as hypercholesterolemia (Rajasekaran *et al.*, 2013). Currently, a larger number of research have been concentrated on the possible risk of hypercholesterolemia and associated cardiovascular diseases (Beaglehole *et al*., 2001; Rajasekaran *et al.*, 2013). Cholesterol or triglyceride-rich lipoproteins play an important role in cardiovascular disease (Nordestgaard and Varbo, 2014). Hypercholesterolemia is often associated with dietary induction and/or genetic manipulations in experimental studies (Griffin and Lichtenstein, 2013).

At present, the available medications used for reduction of blood cholesterol levels are associated with unwanted side effects. Medicinal plants have been widely used for many centuries as remedies for human diseases since they contain constituents of therapeutic value (Yadav *et al.*, 2014). Therefore, there is a growing interest in search on hypoholesterolemic secondary metabolites from herbal medicines (Maruthappan and Sakthi, 2010). The plant family Euphorbiaceae is a taxonomically complex and consists of various medicinal properties such as hepatoprotective activity (Asha *et al.*, 2007), lipid lowering activity (Maruthappan and Sakthi, 2010), anti-diabetic activity (Chauhan *et al.*, 2010) and other activities (Xin Mao *et al.*, 2016). The plants of the genus *Phyllanthus* of family Euphorbiaceae (more recently Phyllanthaceae) have high reputation in traditional medicine in China, India, Brazil, and the Southeast Asian countries (Xin Mao *et al.*, 2016). Plants of this genus have been used in treatments since number of species contains medicinally important bioactive compounds.

It is reported that *Phyllanthus* species such as *P. emblica, P. reticulates, P. debilis* (Zafrul-Azam *et al.*, 2012) and *P. maderaspatensis* (Rani and Raju, 2014) are distributed in different ecological zones of Sri Lanka (Holm *et al.*, 1979).

Figure 1: *Phyllanthus maderaspatensis* L.

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In English *P. maderaspatensis* L. is commonly called as "Madras leaf flower". It is a native herbaceous plant in Sri Lanka, India and Africa (Holm *et al.*, 1979: Chaudhary and Akram, 1987: Hutchinson *et al.*, 1958: Moody, 1989).

Research has revealed that *P. maderaspatensis* L. possess anti-bacterial (Annamalai *et al*., 2012: Nirmal *et al*., 2009) anti-microbial (Nirmal *et al*., 2009: Komuraiahl *et al*., 2009), anti-cataleptic (Nirmal *et al*., 2009) and other medicinal properties such as astringent, deobstruent, stomachic, diuretic, febrifugal and antiseptic (Komuraiahl *et al*., 2009). Also, the leaves of *P. maderaspatensis* L. have expectorant and diaphoretic properties useful in strangury and sweats (Schmelzer and Gurib-Fakir, 2008).

In India, *P. maderaspatensis* L. is widely used as an effective hepato-protective agent in the indigenous medicine (Unani) and the powder from dried plant material mixed with milk is used to treat jaundice (Schmelzer and Gurib-Fakir, 2008). The ethanolic extract of *P. maderaspatensis* L. is a popular South Indian dietary supplement (Bommu *et al*., 2008).

Even though dried plant material of *P. maderaspatensis* L. is used in treating biliary diseases (pith dosha - Sinhala) in Sri Lankan folk medicine, the plant is least concern for its other medical value including hyphochlestrolemic activity (Holm *et al*., 1979).

Although *P. reticulates* (Santoshkumar *et al*., 2012) and *P. emblica* (Samaranayaka, 2000) reported to have exhibited anti-hypercholesterolemic activity, there were hardly any scientific reports are available on the anti-hypercholesterolemic activity including dose dependence of *P. maderaspatensis* L. Further, there was lack of studies in acute toxicity and dose dependency of methanolic extract of *P. maderaspatensis* L. in anti-hypercholesterolemic activity. The present study is the first investigation on the methanolic extract of *P. madaraspatentis* L. plant on the diet induced hypercholectrolemic rats. Hence, the study was aimed to assess the hypocholesterolemic activity of crude methanolic extracts of *P. maderaspatensis* L. with dose dependant response on Wistar rats.

MATERIALS AND METHODS

Collection and preparation of plant material

Fresh whole plants of *P. maderaspatensis* L. were collected during the month of January 2017 from Puttalam district, Sri Lanka. These specimens were identified by comparing herbarium specimens deposited in the Botanical Garden, Peradeniya, Sri Lanka and referring related literature. The voucher specimens were prepared in triplicate and were deposited in the herbarium in the Department of Botany, The Open University of Sri Lanka (OUSL/Herbarium/A 0002). The collected samples were shade dried to a constant weight and were powdered mechanically and stored in air-tight containers at room temperature for future use.

Plant extraction

About 500g of air dried mechanically ground powder was subjected to Soxhlet extraction with 80% methanol at 64°C for 6-8hr. The methanol soluble residue was concentrated under vacuum at room temperature using rotary evaporator which yielded a dark greenish semisolid material. Evaporated samples were further dried in vacuum oven to a constant weight. The yield was 31.99% and the extracts were stored in an air tight container between 2-8°C for further studies.

Experimental animal

Healthy adult male Wistar albino rats (*Mus norvegicus albinus*) of 6 months and weighing between 180-200g were purchased from Medical Research Institute, Borella, Sri Lanka. They were kept under standardized animal house conditions (Photoperiod: approximately 12h natural light per day, temperature: 28-30°C, humidity: 55%-60%) with water and standard diet *ad libitum.* Standard diet was prepared according to the menu that obtained from Medical Research Institute, Borella, Sri Lanka (Ingredients of normal rat diet: Maize meal- 5kg, fish meal-1kg, DL Methinin-6g, brown rice-1.25kg, rice polish-0.215kg, soyameal-1kg, wheat flour-1.8kg, milk powder-0.75kg, brown sugar- 0.0202kg, soya oil-250g, grass powder-0.275kg, rice bran- 0.25kg, mineral mix-50g, bone meal-187.5g, NaCl- 25g, Premix vit. E-2.5g, vitamin B tablets-80g). Animal experiments were conducted in accordance with the internationally accepted principles for animal use and care and rules of the Faculty of Natural Sciences, The Open University of Sri Lanka. The experimental animals were acclimatized for 7 days before the commencement of the study. Ethical clearance for the study was obtained from the Ethical review committee of the Institute of Biology, Sri Lanka.

Induction of Cholesterol

Hypercholesterolemia was induced in rats by feeding a mixture of egg yolk (20 ml), butter (50 g) and cow ghee (20 ml). Equal amounts of the mixture (~2 ml) were given to all the rats, except the negative control group, once a day orally throughout the experiment. The rats with blood cholesterol levels greater than 120 mg/dl were considered as hypercholesterolemic (Samaranayaka, 2000) and were chosen for the study.

Experimental Design

First, the acute toxicity of the crude methanol extract of *P. maderaspatensis* L. was evaluated using Wistar albino rats and at the same time whether the extract has any effect in lowering cholesterol level in blood was investigated. As there was no toxic effect and anti-hypercholesterolemic activity was noted, then, the dose-dependent response of the extract on anti-hypercholestrolemic activity was estimated selecting three doses, below the tested acute toxicity concentration.

The crude methanolic extract of *P. maderaspatensis* L. 2000 mg/kg/day was chosen for acute toxicity according to the standard guidelines (OECD/OCDE No: 423) at the end of 14th day. Acute toxicity signs were observed (Ogbonnia, 2003) and analgesic activity (Hot plate test and Tail flick test) was evaluated (Fernando *et al*., 2009). Rats fed with a diet supplemented with cholesterol for 12 days to induce hypercholesterolemia served as the experimental model.

The acute toxicity test was carried out for 14 days consecutively and body weights and the biochemical parameters of rats were measured at the beginning of the experiment and on the 7th and 14th day of the treatment subsequent dose dependant experiment was performed with three concentrations (400, 800, 1200 mg/kg body weight) of crude methanol extract of *P. maderaspatensis* L. was orally administered to groups of rats for 42 days consecutively (Table 1).

Eighteen rats (Wanniarachchi *et al*., 2009) were weighed, numbered, and randomly divided into 3 groups (NCG1, CME-*PM*-2000, PCG1) of 6 animals each and each rat was fed with a dosage of 2000 mg/kg body weight for the purpose of evaluation of acute toxicity, anti-hypercholectrolemic activity and 6 rats in five group (NCG2, CME-*PM*-400, CME-*PM*-800, CME-*PM*-1200, PCG2) included in the determinations of dose dependant response (Table 1).

The treated and untreated animals were deprived of food for overnight and anesthetized with anesthetizing ether and subsequently, 1 ml of blood was collected using 1 ml 27-gauge insulin syringe with disposable needle from the lateral tail vein of rats with the aid of a rat strainer (Parasuraman *et al*., 2010).

In the dose dependent experiments, treatments were carried out for 6 weeks, total cholesterol and triglyceride were measured at the beginning of the experiment and on 14th, 28th and 42nd day of the experiment. At the 42nd day of treatment, liver toxicity was evaluated using Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) tests (Al-Musa and AL-Hashem, 2014).

Measurement of serum biochemical parameters were carried out in the Zoology and Botany Research Laboratories at the Open University of Sri Lanka after centrifuging (micro centrifuge Sigma 1-14, Sigma Laborzentrifugen GmbH, Germany) samples at 3500 rpm for 30 minutes. The serum total cholesterol (TC) and serum triglyceride (TG) were measured using Biolabo, Mazy, France diagnostic kits (Seneviratne *et al*., 2011) and ALT and AST were determined using Humann diagnostic standard kits (Al-Musa and AL-Hashem, 2014).

The intensity of the colour developed measured using UV Spectrophotometer (Spectro UV-VIS Double Beam, UVD 3500, Labomed, InC) against standard.

Phytochemical analysis

Cardiac Glycosides, total flavonoids, total tannins (Kumar *et al*., 2015), phenolic compounds (Akinseye *et al*., 2007), total saponins (Egbuna and Ifemeje, 2016) and steroids (Geetha and Geetha, 2014) were tested to analyse the phytochemicals.

Statistical Analysis

The descriptive statistics such as means and standard deviations were calculated for each treatment. The statistically significant variances of measured parameters of treatments between groups were evaluated using one-way ANOVA test at the 5% probability level. Tukey's Honest Significant Difference (HSD) was used for the purpose of multiple comparison of treatments. The results were expressed as means and standard deviation.

RESULTS

In the rats treated with acute dose 2000mg/ kg, no acute toxic signs such as salivation, sleep, diarrhoea, and tremors, lethargy or mortality were observed up to 14 days. Further, the same dose failed to induce any significant ($p > 0.05$) analgesic activity in both tail flick and hot plate tests.

The results of the lipid profile analysis of treated and controlled rats in anti-hypercholesterolemic activity test are summarized in Table 2. The significant decrease in total cholesterol of CME-*PM*-2000 treated group ($p < 0.05$) was observed when compared with the control group.

The results obtained from the study on the dose dependent response are summarized in Table 3. The higher lipid profiles were observed in all rats fed with the high fat diet at day 0. Then, there were general dose-dependent decreases in the TC and TG level compared with the positive control. Further, there were fluctuation during the treatment time period.

Table 1: Details of evaluating experiment on acute toxicity, anti-hypercholetrolemic activity and dose dependant study.

Experiment	Group	Type of diet	Dosage (mg/kg body weight)
Experiment 1: Toxicity and anti-hypercholestrolemic activity			
	NCG1	Normal saline + Normal diet	Nill
	CME-*PM*-2000	High cholesterol diet (2ml/kg/day)+ Normal saline + Normal diet	2000
	PCG1	High cholesterol diet (2ml/kg/day) + Normal saline + Normal diet	Nil
Experiment 2: Dose dependant study			
	NCG2	Normal saline + Normal diet	Nil
	CME-*PM*-400	High cholesterol diet + Normal saline + Normal diet	400
	CME-*PM*-800	High cholesterol diet + Normal saline + Normal diet	800
	CME-*PM*-1200	High cholesterol diet + Normal saline + Normal diet	1200
	PCG2	High cholesterol diet + Normal saline + Normal diet	Nil

Table 2: Effects of *P. maderaspatensis* L. on serum TC and TG in rats (n=6) at the end of 14 days of study.

	Treatment		
Biochemical Parameter (mg/dl)	**NCG1**	**PCG1**	**CME-*PM*-2000**
Total Cholesterol	81.98±7.03 [a]	129.35.±3.44 [b]	75.28±3.59 [a]
Triglyceride	97.98±7.17 [a]	202.99±22.19 [b]	89.16±14.66 [a]

TC= Total cholesterol; TG= Triglyceride; NCG1= Negative control 1; PCG1= Positive control 1; CME-PM-2000= Methanolic Extract of P. madarapatensis L. dosage of 2000 mg/body weight.

Means followed by the same letter within each raw are not significantly different at $p \leq 0.05$, according to the least significant difference test.

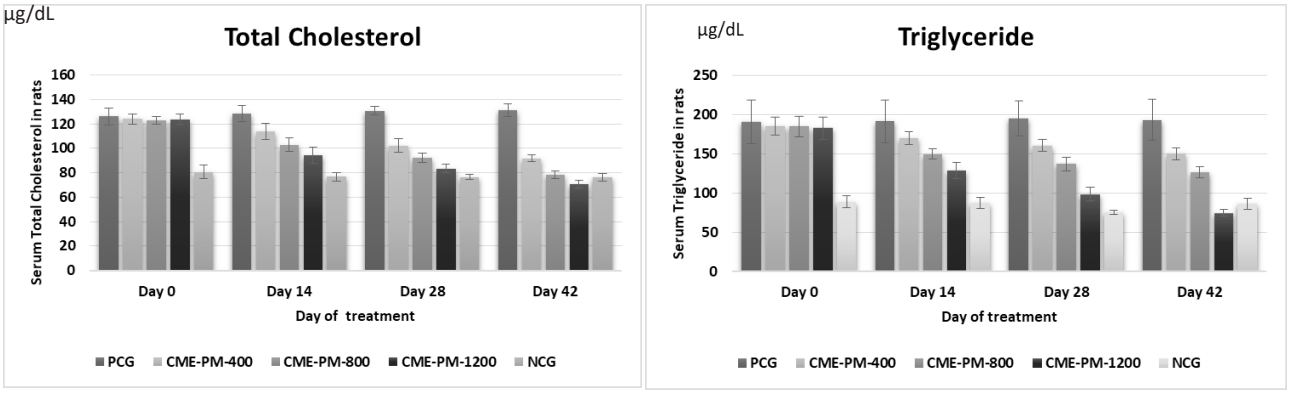

Figure 2: Dose dependent response of serum lipid parameters of rats with number of days after treatment.

PCG= Positive control group; CME-*PM*-400= Methanolic Extract of *P. madarapatensis* L. dosage of 400 mg/body weight; CME-*PM*-800= Methanolic Extract of *P. madarapatensis* L. dosage of 800 mg/body weight; CME-*PM*-1600= Methanolic Extract of *P. madarapatensis* L. dosage of 1600 mg/body weight; NCG= Negative control group

Table 3: Dose dependent response of crude methanolic extract of *P. maderaspatensis* L. on serum lipids at the 0th, 14th, 28th and 42nd day of the study.

Biochemical parameter (µg/dL)	**Day**	**NCG2**	**PCG2**	**CME-*PM*-400**	**CME-*PM*-800**	**CME-*PM*-1200**
Total Cholesterol	day 0	80.61± 5.54[a]	126.02±6.72[b]	123.89±4.07[b]	122.60±3.03[b]	123.85± 4.14[b]
	Day 14	76.64±3.39[a]	128.51±6.67[b]	114.04±6.62[b]	102.98±5.42[b]	94.35±6.53[a]
	Day 28	76.48±2.10[a]	130.55±3.49[b]	102.26±5.52[b]	92.31±3.84[a]	83.48±3.75[a]
	Day 42	76.18±3.38[a]	131.01±5.29[b]	91.73±2.66[b]	78.51±3.20[a]	70.74±3.22[a]
Triglyceride	Day 0	88.57±7.19[a]	190.58±27.83 [b]	185.11±11.63 [b]	184.52±13.02 [b]	182.60±14.16 [b]
	Day 14	87.03±6.57[a]	190.95±27.01 [b]	169.83±8.03 [b]	149.21±6.72 [b]	127.94±10.31 [b]
	Day 28	75.01±3.03[a]	194.56±22.72 [b]	160.31±8.02 [b]	136.64±8.74 [b]	98.41±8.53 [b]
	Day 42	85.74±6.76[a]	192.98±25.92[b]	149.60±8.05[b]	126.27±7.43[b]	74.26±4.32[a]

Means followed by the same letter within each raw are not significantly different at $P \leq 0.05$, according to the least significant difference test.

Increase in CME-*PM* doses showed decreasing trends with TC and TG (Figure2). By the 28th day of the experiment, the group treated with CME-*PM*-1200 treatment and by 42nd day with both CME-*PM*-800 and CME-*PM*-1200 treatments showed insignificant difference ($p \geq 0.05$) in total cholesterol with reference to the negative control.

On 42nd day of treatment, triglyceride level of the group treated with 1200mg/kg and HDL-C all three doses were insignificant ($p \geq 0.05$) with the negative control. However, LDL-C level did not reached to the level of negative control even by 42nd day.

The effects of *P. maderaspatensis* L. on the serum levels of ALT and AST in all groups of rats are shown in Table 03. There were no significant differences among groups at $p<0.05$.

Table 4: Assessment of activities of AST and ALT in the serum of the control and all experimental groups (Mean ± Standard Deviation).

	NCG	PCG	CME-*PM* - 400	CME-*PM* - 800	CME-*PM* -1200
AST (U/L)	46.53±2.79	46.07±2.14	47.38±2.56	47.27±1.87	48.45±2.21
ALT (U/L)	32.68±2.16	35.67±3.91	34.29±2.25	32.40±1.26	32.74±2.25

Phytochemical analysis

The phytochemical screening of methanol extracts of *P. maderaspatensis* L. revealed that tannins, saponins, steroids, terpenoids, flavonoids and phenol are present and Glycosides are absent.

DISCUSSION

Numerous animal and human studies have confirmed the hypercholesterolemic properties of saturated fatty acids and cholesterol, which increase total cholesterol and alter lipoprotein patterns in which the mechanisms remain under study (Olson, 1998). In this study, feeding rats with high cholesterol diet (HCD) for 7 days induced hypercholesterolemia similar to previous studies (Olson, 1998: Kanungo *et al*., 2007). It also indicated that high cholesterol diet significantly increased the serum TC, TG, LDL-C levels ($p \leq 0.05$) and decreased HDL-C level when compared with rats on normal diet (Patel *et al*., 2009).

During the experiment, cholesterol diet was co-administered with extracts. The elevated serum TC and TG levels showed a considerable decline due to the treatment during the experiment. The TC- and, TG- lowering activity of CME-*PM*-1200 was more prominent compared to CME-*PM*-800 and CME-*PM*-400. These results indicate the dose dependent response of *P. maderaspatensis* L. extract which interfere the elevations and declines seen in various components of lipid profile under experimentally induced hypercholesterolemia.

Present study indicated that CME-*PM* decrease the TC level in high cholesterol induced rats, supports to reduction of development of atherosclerosis. Flavonoids have also been reported to increase HDL-C levels and decrease LDL and VLDL levels in hypercholesteremic rats (Schmelzer and Gurib-Fakir, 2008). The presence of flavonoids and polyphenols in CME-*PM* extracts could be considered as attributive compounds in increasing TC in extracts treated rats.

Since acute toxicity signs were not observed in rats treated with CME-*PM*-2000 extracts, it could be concluded that the dosage use in the study is well-tolerated by the test animals. However, ALT and AST levels though differ in magnitude, statistically there were significant difference between normal diet fed group (NCG2) and *P. maderaspatensis* L. extract treated animals groups. Confirmation of the ALT and AST findings require anatomical study of the structural changes / damages in hepatic tissues.

Limitations of the study

The number of animals includes in the treatment groups low and for an in-depth study it is required to increase the number of animals per replicate. The result of a study with limited number of animals per replicate may preclude the generalization of the findings**.** The study included TC and TG, however, other parameters such as LDL, HDL fractions need to be quantify to have complete picture of the effect of crude methanolic extract of *P. madaraspatensis* on the serum cholesterol levels in treated rats.

CONCLUSIONS

Results of present study revealed that the crude methanolic extract of *P. maderaspatensis* L. decrease serum TC and TG in rats. The most effective dose of 1200 mg/Kg body weight and these findings provide some biochemical basis for the use of extract of *P. maderaspatensis* L. as anti-hypercholesterolemic agent and thus could be useful in the treatment of hypercholesterolemic conditions. Further, studies are required to carry out to realise the possible mechanism/s of action.

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