Appl Biochem Biotechnol (2013) 169:546–553
DOI 10.1007/s12010-012-9993-0

Hypolipidaemic Effects of Methanol Extract of *Holoptelea integrifolia* (Roxb.) Planchon Bark in Diet-Induced Obese Rats

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Received: 4 August 2012 / Accepted: 28 November 2012 /
Published online: 14 December 2012

Abstract The leaf and bark paste of *Holoptelea integrifolia* is traditionally used for the treatment of obesity in Asian countries. However, no scientific studies have been undertaken to reveal the actual mechanism of action. The present study aimed to investigate the hypolipidaemic effect of *H. integrifolia* and its mechanism in diet-induced obese rat model. After 4 weeks of oral administration, blood samples were collected for the estimation of serum lipids, lecithin: cholesterolacyltransferase (LCAT) apolipoproteins (apo) and liver for HMG-CoA reductase (HMGR) assay. The faecal samples were also collected to estimate the faecal fat content. The *H. integrifolia* treatment markedly lowered body weight, serum lipids and apo B and increase high-density lipoprotein-cholesterol and apo A1 concentrations. In this study, HMGR activity was enormously reduced, which helps to reduce cholesterol biosynthesis and an increase in LCAT activity was also observed. The detailed faecal analysis showed a remarkable increase in faecal lipids, which indicates the ability to inhibit intestinal fat absorption. The methanol fraction of *H. integrifolia* on LC-MS and tandem mass spectrometry analysis shows the presence of a compound, 3-(7-ethoxy-4-methyl-2-oxo-2H-chromen-3-yl)propanoate (C1). The result showed that the significant hypolipidaemic effect of *H. integrifolia* may be linked to its ability to inhibit HMGR activity and block intestinal fat absorption.

Keywords *Holoptelea integrifolia* · Diet-induced obesity · HMG-CoA reductase · LCAT · Apolipoproteins

Introduction

Obesity is the overabundance of body weight for a particular age, sex and height due to the imbalance between energy intake and its expenditure. Obesity remains a major global public health issue because of its increasing prevalence, cutting across all sex, age groups, ethnicity

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or race [1]. *Holoptelea integrifolia* (Roxb.) Planchon (Family: Ulmaceae) is a large, glabrous deciduous tree, commonly known as Indian elm. The species is distributed in India, Sri Lanka and China [2]. It contains several classes of chemical constituents like alkaloids, terpeniods, glycosides, steroids, sterols, saponins, tannins, carbohydrates, proteins and flavonoids [3]. The leaves and stem bark of this plant were used by local people as antiviral, antioxidant, antimicrobial and antiobesity preparations [4, 5] and in the management of cancer [6]. The methanolic extract of *H. integrifolia* increases lipolysis by 70.2 % in prolipolytic activity assay in differentiated adipocytes and shows significant reduction in lipid profile in diet-induced obese rats in which levels of serum cholesterol and LDL were reduced by 40 and 45 %, respectively, whereas the level of HDL was enhanced by 25 % [4]. *H. integrifolia* is a plant included in lekhaneya gana—a pharmacological classification mentioned in 'Charakasamhita' which means 'reduce excess fat' [7]. The purpose of the present study was to ascertain the antiobesity effect and mechanism of action of methanol fraction of *H. integrifolia* using diet-induced obese rats as model.

Materials and Methods

Materials

Mevinolin and sodium iodoacetic acid were obtained from Sigma-Aldrich (St. Louis, USA). The diagnostic kits for total cholesterol (T-c), triglycerides (TG), high-density lipoprotein-cholesterol (HDL-c) and immunoturbidimetic assay kits for apolipoprotein A1 and B were obtained from Agappe Diagnostics, Switzerland GmbH. apolipoprotein (apo) A1 and B standards were purchased from Denka Seiken Co Ltd., Japan. All other chemicals were obtained from Merck (Germany).

Preparation of the Bark Extract of *H. integrifolia*

Fresh barks of *H. integrifolia* were collected from the hill area of Kallanode (Calicut, India) (coordinates, 11°31′49″ N, 75°52′21″ E). The plant was identified and authenticated by the Centre for Medicinal Plants Research (CMPR) Kottakkal, India. Voucher specimen was processed and deposited in the herbarium at CMPR Kottakkal (voucher no, CMPR 3949). Methanol extract was then prepared using a Soxhlet apparatus for 48 h. The solvent was removed using a rotary evaporator at 40 °C (yield 8.3 % (*w*/*w*)). The methanol fraction was loaded to a silica gel column (18×500 mm) and then eluted with MeOH/water solvent in different proportions. The 70 % MeOH soluble fraction was used for treating diet-induced obese rats [3].

Experimental Animals

Sprague–Dawley strain rats, body weight 160±10 g, were used for experiments. The animals were selected, such that the weight difference within and between groups does not exceed ±20 % of the average body weight of the sample population. They were housed in polypropylene cages at controlled temperature (22±3 °C) and humidity (50±10 %) and were kept in 12 h light cycle. Rats were fed with standard diet and water ad libitum and were acclimated 7 days before they were used. The experiment was approved by the institutional animal ethics committee (KULS/IAEC 2011-04).

Experimental Protocol

Rats were randomly divided into two groups: normal control group (n=10) and high-fat (HF) group (n=40). Animals in control group were fed with a basic diet for 4 weeks. All animals in HF group were fed with a high-fat diet for 4 weeks [8, 9]. The ingredients of high-fat diet were shown in Table 1. After 4 weeks, blood samples were taken from rats of both groups for the analysis of T-c, TG, HDL-c and low-density lipoprotein-cholesterol (LDL-c) [8]. The high-fat group rats which met the hyperlipidaemic criteria were used for *H. integrifolia* extract treatment.

The rats in normal control group were continued to be fed with a basic diet. The rats in the HF model group were further randomly divided into four groups with ten rats per group: the model control, *H. integrifolia* 200, *H. integrifolia* 200 and positive control group. The model control group was continued to be fed with a high-fat diet; The rats in *H. integrifolia* 200 and *H. integrifolia* 400 groups were continued to be fed with a high-fat diet and orally given *H. integrifolia* extract at a dosage of 200 and 400 mg/kg body weight (BW), respectively. The rats of positive control group were continued to be fed with a high-fat diet and orally given mevinolin (3.0 mg/kg BW) daily for 4 weeks [8]. The acute toxicity of the *H. integrifolia* extract was performed and the doses were chosen according to the acute toxicological study results divided by security factor 10 [10].

Estimation of Serum Lipids

Serum samples were taken from rats and centrifuged at 3,000 rpm for 15 min at 4 °C for the lipid analysis. In vitro quantitative determination of T-c, TG and HDL-c was performed using rat serum by enzymatic kit procedure (Agappe Diagnostics, Switzerland GmbH) [11, 12]. All of the results were expressed as milligrams per deciliter serum.

HMG-CoA Reductase and Lecithin: Cholesterolacyltransferase Activity Assay

At the end of the experiments, the overnight fasted rats were sacrificed and liver was taken for HMG-CoA reductase assay [13]. The ratio of HMG-CoA to mevalonate was taken as an index of enzyme activity which catalyzes the conversion of HMG to mevalonate. Lecithin cholesterolacyltransferase (LCAT) activity was determined according to the self-substrate method which directly quantifies free cholesterol [14].

Table 1 High-fat diet ingredients

S/N	Ingredients	HF diet (g/100 g diet)
1	Basic diet	82.8
2	Lard	10
3	Cholesterol	2
4	Bile salt	0.5
5	Propylthiouracil	0.2
6	AIN-76 vitamin mix[a]	3.5
7	AIN-76 mineral mix[a]	1

HF rats were fed with high-fat diet

[a] Prepared as per formula recommended by the American Institute on Nutrition ad hoc Committee on Standards for Nutritional Studies (1980) [8, 9]

Estimation of Apolipoprotein A1 and Apolipoprotein B

The in vitro quantitative determination of apo A1 and B was performed using immunoturbidimetric method [15] (Agappe Diagnostics, Switzerland GmbH).

Estimation of Faecal Fat Content

Faeces were collected from the cages in the last week of the 4-week treatment period. It was freeze dried for 48 h and stored. The faeces were ground into fine powder and extracted with a chloroform–methanol (2:1) mixture in accordance to the method described by Folch et al. [16]. Faecal T-c and TG were measured using the enzymatic kits. All the experiments were done in triplicate.

LC-MS Analysis of Methanol Fraction of *H. integrifolia* Extract

The methanol fraction of *H. integrifolia* extract, which showed the HMG-CoA reductase (HMGR) inhibiting activity was used to perform liquid chromatography (LC) mass spectrometry (MS) and MS-MS analysis (Waters ESI QToF System) and structural elucidation of molecules, was performed using Massfragment (MassLynx Mass Spectrometry Software Waters Corporation, Milford, USA). The MS^E mode of acquisition was used to acquire the data with low and high collision energy. The same scan was performed in positive and negative modes. The fragmentation patterns obtained in high collision energy were confirmed using MS-MS mode of acquisition.

Statistical Analysis

All values are presented as mean±standard deviation (SD). Statistical comparisons of the groups were made by ANOVA, and each group was compared with the others by post hoc Fisher's PLSD test (SPSS Inc., IBM, USA). Statistical significance was defined as $P<0.05$.

Results

Effect of *H. integrifolia* on Body Weight and Serum Lipids in Diet-Induced Obese Rats

The estimated lipid levels in normal control, high-fat model control, extract-treated groups and positive control are given in Table 2. The oral administration of methanol fraction of *H. integrifolia* extract was able to reduce the body weight compared to HF diet rats. The T-c, TG and LDL-c levels in *H. integrifolia* extract-treated groups were lower than model control group. In the extract-treated groups, HDL-c level was found to be increased. The mevinolin-treated group had also shown a significant hypocholesterolemic effect compared with the model control.

Effect of *H. integrifolia* on the Hepatic and Faecal Lipids in Diet-Induced Obese Rats

Faecal analysis of *H. integrifolia* extract-treated rats shows a remarkable increment in faecal T-c and TG, compared to model control. On the other hand, the mevinolin-treated group does not show any significant change in faecal fat content compared with the model control rats.

Table 2 Effect of *H. integrifolia* on serum lipids, faecal lipids, LCAT and apolipoproteins in diet-induced obese rats

	Normal control	High-fat model	*H. integrifolia* 200 mg/kg	*H. integrifolia* 400 mg/kg	Positive control
Serum lipids ($mg dL^{-1}$)					
T-c	72.34±7.64	111.8±6.67[a]	92.85±4.79[b]	86.3±2.76[b]	83.7±1.93
TG	78.10±4.14	107.1±6.62[a]	93.41±5.76[b]	84.7±3.32[b]	81.81±1.77
HDL-c	35.1±3.49	22.43±4.08[a]	25.98±1.86[b]	29.90±1.90[b]	31.6±1.65
LDL-c	21.62±8.02	67.96±9.88[a]	48.18±5.32[b]	39.46±2.17[b]	35.75±1.29
Faecal lipids ($mg dL^{-1}$)					
T-c	85.98±3.89	92.75±1.62[a]	95.75±1.72[b]	88.38±2.96[b]	52.31±1.57
TG	15.03±1.21	17.81±1.17[a]	21.0±1.72[b]	16.3±1.38[b]	12.75±1.20
LCAT (μg/ml/h)	30.98±2.53	37.01±2.6[a]	41.86±2.13[b]	47.33±1.30[b]	42.66±1.60
Apolipoproteins					
Apo A1	6.28±0.37	4.26±0.12[a]	4.27±0.22	4.93±0.23[b]	5.31±0.29
Apo B	2.82±0.29	8.11±0.41[a]	6.55±0.58[b]	5.21±0.26[b]	4.5±0.32

The values are mean±SD for ten rats

Normal control rat fed with normal diet, *high-fat model* rat fed with high-fat diet, *H. integrifolia 200 mg/kg* high-fat diet rats treated with 200 mg/kg body weight of *H. integrifolia* extract, *H. integrifolia 400 mg/kg* high-fat diet rats treated with 400 mg/kg body weight of *H. integrifolia* extract, *positive control* high-fat diet rats treated with mevinolin (3.0 mg/kg body weight)

[a] $P<0.05$ compared with control group

[b] $P<0.05$ compared with untreated model group

Effect of *H. integrifolia* on Enzymes HMGR and LCAT in Diet-Induced Obese Rats

Estimation of hepatic HMGR activity showed a significant decrease in extract-treated hyperlipidaemic rats (Fig. 1). In particular, hepatic HMGR was significantly lower by 70.27 % in the *H. integrifolia* 400 mg/kg extract-treated group. In *H. integrifolia*-treated groups, the value in LCAT was significantly activated compared to the model control group.

Effect of *H. integrifolia* on apo a1 and apo B Levels in Diet-Induced Obese Rats

The administration of *H. integrifolia* extract did not significantly alter the apo A1 at low concentration, 200 mg/kg BW. The high concentration 400 mg/kg BW *H. integrifolia* extract was able to increase plasmatic apo A1. On the other hand, the *H. integrifolia* treatment to

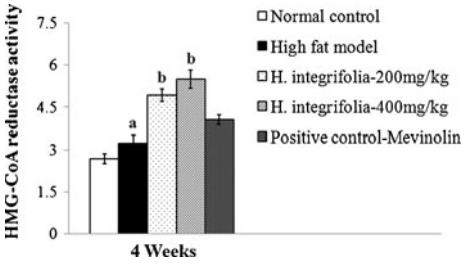

Fig. 1 Graph showing the effect of *H. integrifolia* on HMGR in diet-induced obese rats. The values are mean±SD for ten rats. *a* $P<0.05$ compared with control group. *b* $P<0.05$ compared with untreated model group

high-fat animals resulted in a significant decrease in apo B level in a dose-dependent way, compared to untreated model group.

LC-MS Analysis of Methanol Extract of *H. integrifolia*

The LC-MS and MS-MS analysis of methanol fraction of *H. integrifolia* extract showed the presence of compound 3-(7-ethoxy-4-methyl-2-oxo-2H-chromen-3-yl)propanoate (C1). The MS-MS spectrum, library search and structure of obtained compound (C1) at retention time of 12.7 min are shown in Fig. 2.

Discussion

The elevated level of T-c, TG and LDL-c and the decrease in HDL-c result in threefold increased risk of obesity and cardiovascular diseases. In our study, the oral administration of *H. integrifolia* methanol fraction reduced the body weight significantly and was able to reduce serum T-c, TG, LDL-c and VLDL-c levels compared to high-fat model control rats. Recent studies showed that triglycerides are independently related to obesity and coronary heart disease. The reduction in TG level is a promising result as most of the antihypercholesterolemic drugs were not able to reduce triglyceride levels [17].

LDL-c is the chief cholesterol carrier in the blood, around the network of arteries. The ill effect of LDL-c is not only because each particle is made up mostly of cholesterol, but also of its terminus. The excess LDL-c may permeate the inner arterial wall and result in development of atherosclerotic lesions. The results indicate that both the *H. integrifolia* dose treatment was able to accelerate LDL clearance in blood [18].

HDL-c level in *H. integrifolia*-treated groups was enhanced which can lower a risk of developing heart diseases. Positive effect of HDL-c is largely attributed to its central function in the reverse cholesterol transport, a process where HDL circulates around the

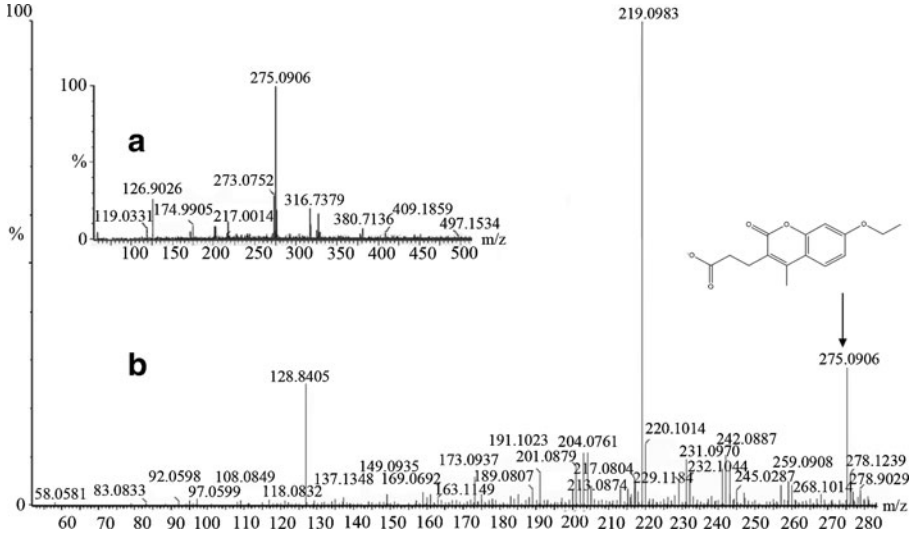

Fig. 2 **a** MS-MS spectrum of *H. integrifolia* extract and **b** MassFragment Analysis of Mass 275.0906 *m/z* and the structure of 3-(7-ethoxy-4-methyl-2-oxo-2H-chromen-3-yl)propanoate (C1)

body, picking up excess cholesterol and bringing it back to the liver for disposal [19]. This effect of *H. integrifolia* may be due to its ability to the increase the LCAT activity, which may contribute to the regulation of blood lipids.

Faecal T-c and TG were markedly increased in extract-treated groups as compared with the HF control group. The results lead to the assumption that the components of *H. integrifolia* extract at this level of concentration may exert an inhibitory effect on intestinal fat absorption [20].

The *H. integrifolia* treatment shows a substantial decrease in HMGR activity, which blocks the cholesterol biosynthesis. HMGR, the rate-limiting enzyme, takes part in a key step in the mevalonate pathway. Moreover, HMG-CoA has an alternative metabolic pathway for its breakdown when HMGR is inhibited, so that there is no build-up of potentially toxic precursors [21–23]. The above factors make HMGR an attractive target to block cholesterol biosynthesis and control obesity. Similar results were observed for HMGR in Korean soybean paste [24].

LCAT is an enzyme that catalyzes the formation of cholesteryl esters on HDL and by that promotes maturation of HDL particles in plasma and facilitates reverse cholesterol transport by maintaining a concentration gradient for the diffusion of cellular unesterified cholesterol to HDL-c. *H. integrifolia* treatment could activate LCAT and thereby increase the HDL-c levels.

Apo A1 constitutes the major component of HDL-c. It is an activator of the enzyme LCAT and removes free cholesterol from extrahepatic tissues. Apo B present in LDL-c is the ligand concerned with the uptake of cholesterol. Measurement of apo A1 and B aids in identifying coronary artery disease. Elevated levels of apo B and low apo A1 levels indicate an increased risk of cardiovascular disease even when T-c and LDL-c levels are normal [15, 25]. The *H. integrifolia* treatment resulted in notable increase in apo A1 and decrease in apo B levels. The decrease in apo B/apo A1 ratio shows the anti-atherogenic potential of *H. integrifolia* extract.

The LC-MS analysis of methanol fraction of *H. integrifolia* showed the presence of compound C1. All statins share an HMG-like moiety, which may be present in an inactive lactone form. In vivo, these prodrugs are enzymatically hydrolyzed to their active hydroxyl acid forms [23].

Similar to statins, the compound C1 also possesses an HMG-like moiety, which is structurally similar to HMG-CoA, substrate of HMGR. Structural formulas of HMG-CoA, compound C1 and compactin are shown in Fig. 3.

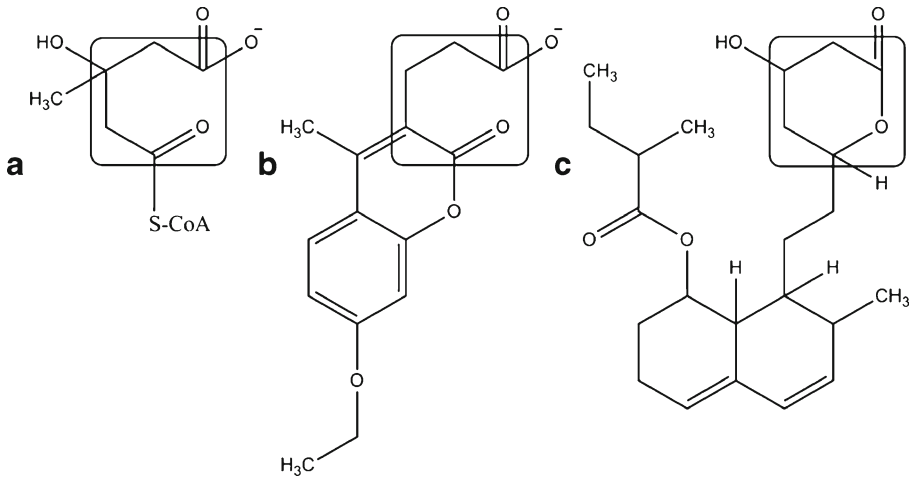

Fig. 3 *a–c* Structural formulas of HMG-CoA, 3-(7-ethoxy-4-methyl-2-oxo-2H-chromen-3-yl)propanoate and compactin. The similar portions are shown in *square boxes*

The above observations strongly support the fact the C1 can bind strongly with active site of HMGR and may block the cholesterol synthesis. The above results were overwhelming and indicate the dual activity of *H. integrifolia* extract. The extract is able to reduce HMGR activity and block intestinal fat absorption which may result in decrease in lipid profile and body weight. Thus, the oral treatment with *H. integrifolia* extract is able to ameliorate the diet-induced obesity.

On the basis of these data, it is concluded that the *H. integrifolia* exhibits cardioprotective potential and lipid-lowering ability. The results constitute a valid scientific groundwork for its medicinal application. The experimental data are in good agreement with the lekhaneya gana property of *H. integrifolia* remarked in Charakasamhita (700 BC).

Acknowledgments One of the authors, Mr. Arun KS, is thankful to (UGC-BSR), New Delhi for providing the fellowship to carry out the research work.

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