

Cholesterol esterase inhibitory activity of bioactives from leaves of *Mangifera indica* L

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Submitted: 11-11-2014

Revised: 10-12-2014

Published: 21-10-2015

ABSTRACT

Background: In the earlier studies, methanolic extract of *Mangifera indica* L leaf was exhibited hypocholesterol activity. However, the bioactive compounds responsible for the same are not reported so far. **Objective:** To isolate the bioactive compounds with hypocholesterol activity from the leaf extract using cholesterol esterase inhibition assay which can be used for the standardization of extract. **Materials and Methods:** The leaf methanolic extract of *M. indica* (Sindoor variety) was partitioned with ethyl acetate and chromatographed on silica gel to yield twelve fractions and the activity was monitored by using cholesterol esterase inhibition assay. Active fractions were re-chromatographed to yield individual compounds. **Results and Discussion:** A major compound mangiferin present in the extract was screened along with other varieties of mango leaves for cholesterol esterase inhibition assay. However, the result indicates that compounds other than mangiferin may be active in the extract. In vitro pancreatic cholesterol esterase inhibition assay was used for bioactivity guided fractionation (BAGF) to yield bioactive compound for standardization of extract. Bioactivity guided fractionation afford the active fraction containing 3 β -taraxerol with an IC₅₀ value of 0.86 μ g/ml. **Conclusion:** This study demonstrates that *M. indica* methanol extract of leaf have significant hypocholesterol activity which is standardized with 3 β -taraxerol, a standardized extract for hypocholesterol activity resulted in development of dietary supplement from leaves of *Mangifera indica*.

Key words: Bioactivity, Hypocholesterol, *Mangifera indica*, 3 β -taraxerol

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Website:

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DOI: 10.4103/0974-8490.159578

Quick Response Code:



INTRODUCTION

Hypercholesterolemia, is a prerequisite for atherogenesis which leads to myocardial ischemia and other cardiac complications. People with elevated low-density-lipoprotein (LDL) cholesterol are prone to the development of coronary heart disease through multiple stages of the process. Lowering of serum LDL cholesterol is the primary target of anti-hyperlipidemic therapy. A large number of clinical trials on cholesterol-lowering therapy resulted in using 3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors (statins [STs]).^[1] Bile acid sequestrants acted on by interrupting enterohepatic recycling of bile acids, they have shown adverse effects like constipation and bloating hemorrhoidal bleeding. Fibric acid derivatives are another class of drugs reduce cholesterol by increased lipolysis of triglyceride via lipoprotein lipase. Ezetimibe

reduces cholesterol by blocking the uptake of cholesterol into jejunal enterocytes.^[2] STs are drugs of first choice for the patients with hypercholesterolemia, especially in those at high cardiovascular risk, however some of these patients are intolerant to STs.^[3] Dietary ingredients include vitamins, minerals, amino acids, and herbs or botanicals, as well as other substances that can be used to supplement the diet. Several plant based nutraceuticals have been suggested to improve plasma lipid profile.^[4] Red yeast rice, sugar cane-derived policosanols, artichoke leaf extracts are currently used as dietary supplements for their potential LDL-cholesterol-lowering effects.^[5] Extensive literature survey supports *Mangifera indica* L. as one of the ingredients which could be utilized for controlling cholesterol level.

Mangifera indica commonly known as mango is a large avenue tree which seen throughout India. It belongs to the family Anacardiaceae. *M. indica* is one of the most popular of all tropical trees. There are more than 1000 varieties of mango trees all over the world. Most parts of the tree (fruit, seeds, pulp, stem bark, roots and leaves) have medicinal properties.^[6] It is native to tropical Asia

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and has been cultivated in the Indian subcontinent for over 4000 years and is now found naturalized in most tropical countries.^[7] Phytochemical studies on various parts of *M. indica* L. revealed that it contains phenolic acids, phenolic esters, flavonoids, xanthanoid-mangiferin etc. *In vitro* studies revealed that these compounds exhibit many biological activities. Mangiferin, a major compound of *M. indica* have studied for many pharmacological activities like anti-diabetic, rheumatid arthritis, anti-inflammatory, hypolipidemic, antioxidant activities.^[8-12] Flavonoid rich fraction of kernels of *M. indica*, leaf and bark extract have shown anti-atherogenic activity and excretion of cholesterol through faeces.^[13,14] The ethanolic extract of immature leaf have shown favorable hypolipidemic and hepatoprotective activities.^[15] Thus, this study was focused to isolate bioactive compounds through bioactivity guided fractionation for isolation of bioactives from the extract using cholesterol esterase inhibition assay along with their purification and structural elucidation. The active fraction can be used to develop a dietary supplement of *M. indica* for hypocholesterol activity. An effort is also made to develop a high performance liquid chromatography (HPLC) method to determine the amount of bioactive compounds in different varieties of *M. indica* screened for activity.

Experimental details

Plant materials

Fresh leaves of different varieties of *M. indica* like Badami, Totapuri, Sindoor, Sannabeejada Kayi and Valaja were collected from Krishnagiri District, Tamil Nadu, India and identified by Dr. Santhan, Taxonomist, Natural Remedies Pvt. Ltd, Bangalore. Voucher specimens for each variety have been deposited in Agronomy Department of Natural Remedies Pvt. Ltd.

General

The hydrogen nuclear magnetic resonance (¹H NMR) and carbon NMR (¹³C NMR) spectra were recorded using a Bruker AMX-400 (400 MHz) instrument. Deuterated chloroform, dimethyl sulphoxide are used for NMR analysis. HPLC analysis is carried by using Shimadzu LC 2010AHT which uses ultraviolet (UV) and photodiode array detector with LC solution software. The mass spectra were taken on a LC-MSD-Trip-XCT-plus instrument. Fractions were monitored by thin layer chromatography and the spots were visualized under UV (254 nm) light and further developed by spraying with anisaldehyde sulphuric acid and heated at 105°C. TLC was carried out on precoated silica gel 60F₂₅₄ (0.25 mm thick, Merck, Darmstadt, Germany) and column chromatography was performed on silica gel (120 mesh, Swambe chemicals). Standards-gallic acid, mangiferin (phytochemicals scale up Lab, Natural Remedies, Pvt., Ltd.).

Bioassay guided fractionation

The dried leaves of *M. indica* of different varieties were used for extraction by reflux method. Sindoor variety (5 kg), and others 200 g each of Badami, Totapuri, Sannabeejada Kayi and Valaja were extracted three times with methanol. The methanol extracts of all variety were filtered, combined and concentrated at 60°C under vacuum using a rotary evaporator. Final powder form of methanol extract was used for bioassay studies. Sindoor variety extract was used for bioactivity guided fractionation studies due to easy and commercial availability of leaf. The crude methanol extract of Sindoor leaf was subjected to liquid/liquid partition using water and ethyl acetate. The ethyl acetate layer (250 g) subjected to column chromatography (mesh size #60–120).

Compound 1

As shown in the Figure 1, the ethyl acetate fraction from methanol extract was subjected to silica column chromatography by eluting with increasing polarity of solvents like petroleum ether, ethyl acetate and methanol (A: B and B: C-1:0-0:1 and 1:0-0:1) as a gradient to afford eleven fractions.

The 15% ethyl acetate in pet ether fraction (FRN IV) enriched with compound 1 was chromatographed on silica gel (300–400 mesh) using flash chromatography (Combi flash) using petroleum ether and chloroform as mobile phase with increasing polarity. The combination of petroleum ether and chloroform would give different polarity combination than the ethyl acetate combination for better separation. (TLC-precoated silica gel 60 F₂₅₄ plates [Merck]; developing solvent petroleum ether: ethyl acetate [8:2] and the spots are not detected by 254 nm. Hence, it is sprayed with anisaldehyde sulphuric acid reagent followed by heating at 105°C for 5 min). Fractions collected from 20% chloroform to 30% chloroform in petroleum ether was enriched with compound 1, followed by repeated crystallisation by dissolving in hexane to get pure 3 β-taraxerol [Figure 2] characterized based on spectroscopic data [Figures S1-4] matched with literature values.^[16]

Compound 2

The 100% methanol fraction (fraction no XII) from silica column was rechromatographed on silica gel, eluted with increasing polarity using petroleum ether and ethyl acetate followed by increasing percentage of methanol. Each individual collected fraction was concentrated in rotary evaporator to distil the solvents. The 20% methanol in ethyl acetate fraction obtained from above was applied on diaion HP-20 resin column for further purification of compound 2, which was eluted with decreasing polarity using water and acetone. The 20% acetone in water

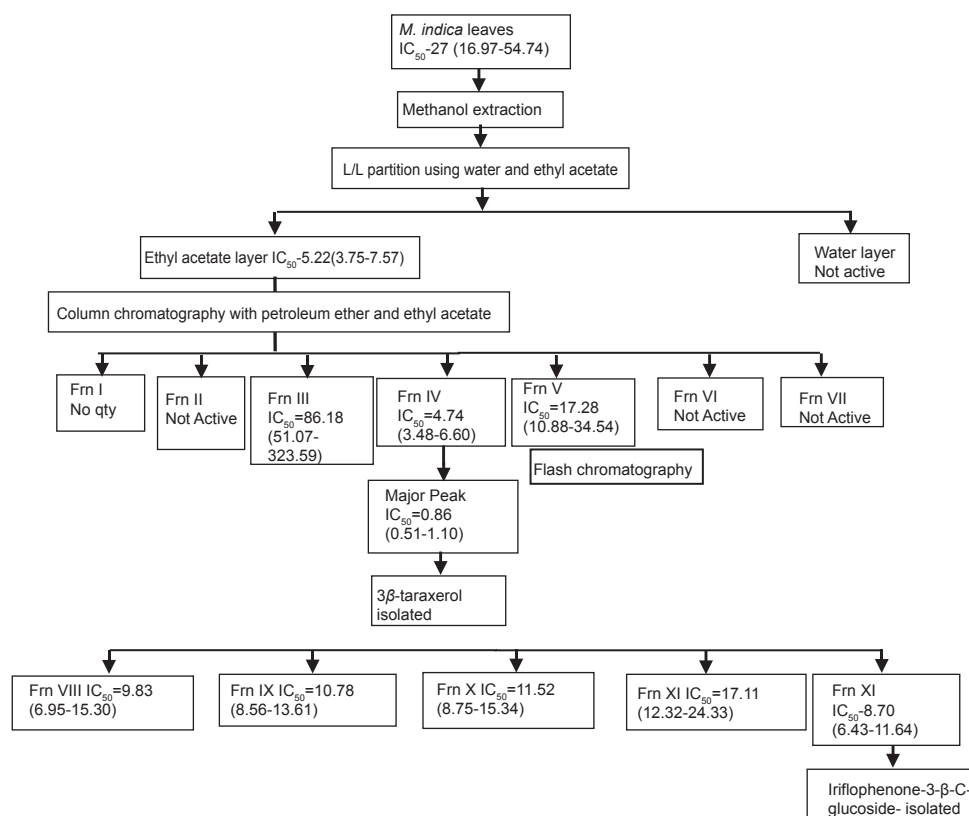


Figure 1: Scheme for bioactivity guided fractionation of *Mangifera indica* leaf methanol extract in cholesterol esterase inhibition assay

fraction was applied on sephadex LH-20 for further purification of compound eluted using water as solvent. Different fractions were collected and combined based on TLC observation. Final purification was carried out by using the prep HPLC (Kromasil C18 preparative HPLC column [250 mm × 21.2 mm, 5 μm]) was used. The preparative HPLC method was developed using isocratic method containing 15% methanol in water to yield compound 2 (360 mg) which was identified as Iriflophenone-3-β-C-glucoside [Figure 3] based on the spectroscopic data [Figures S5-7].^[17]

3 β-taraxerol (compound 1)

Infrared: 3600 (strong broad, OH), 2900 (CH stretching), 1540 (C = C).

Mass spectrum: m/z 408 (M-H₂O).

Hydrogen nuclear magnetic resonance and carbon nuclear magnetic resonance

Hydrogen nuclear magnetic resonance spectrum (CDCl₃-200 MHz)

δ 0.80 (3H, s, 24-Me), 0.82 (3H, s, 28-Me), 0.90 (3H, s, 25, 29-Me), 0.92 (3H, s, 27, 30-Me), 0.95 (3H, s, 23-Me), 0.97 (3H, s, 27-Me), 1.02 (1H, m, C5H), 1.09 (3H, s, 26-Me), 4.14 (1H, d, C3H, J = 7.0 Hz), 5.56 (1H, dd, C15H, J = 8.0, 8.0 Hz).

Carbon nuclear magnetic resonance spectrum (CDCl₃-200 MHz)

δ 15.62 (C-24, 25), 17.68 (C-11), 18.98 (C-6), 21.49 (C-30), 26.08 (C-27), 27.34 (C-2), 28.18 (C-23), 28.98 (C-20), 30.10 (C-26, 28), 33.28 (C-22), 33.53 (C-29), 33.88 (C-21), 35.31 (C-7), 35.97 (C-12), 36.88 (C-16), 37.80 (C-10), 37.91 (C-13), 38.04 (C-1), 37.91 (C-17), 38.82 (C-8), 38.95 (C-4), 41.51 (C-19), 48.95 (C-9), 49.47 (C-18), 55.72 (C-5), 79.25 (C-3), 117.06 (C-15), 158.27 (C-14).

Iriflophenone-3-β-C-glucoside (compound 2)

Infrared: 3312 (strong broad, OH), 2900 (CH stretching), 1606 (C = C).

Mass spectrum: m/z 407 (M-H).

Nuclear magnetic resonance details

¹H-NMR (dimethyl sulfoxide [DMSO]) δ: 7.57 (2H, dd, J = 6.8, 1.6 Hz, H-2', H-6'), 6.79 (2H, dd, J = 7.2 Hz, H-3', H-5), 5.95 (1H, s, H-6), 4.60 (1H, d, J = 9.6, glc H-1), 3.19–3.63 (sugar protons); ¹³C-NMR (DMSO) δ: 194.616 (C-7), 161.36 (C-4'), 158.99 (C-4), 157.36 (C-3), 156.70 (C-2), 131.49 (C-2', C-6'), 130.71 (C-1'), 114.60 (C-3', C-5'), 106.95 (C-1), 103.63 (C-5), 94.79 (C-6), 81.04 (C-5''), 78.28 (C-3''), 74.62 (C-1''), 71.82 (C-2''), 69.6 (C-4''), 60.4 (C-6'').

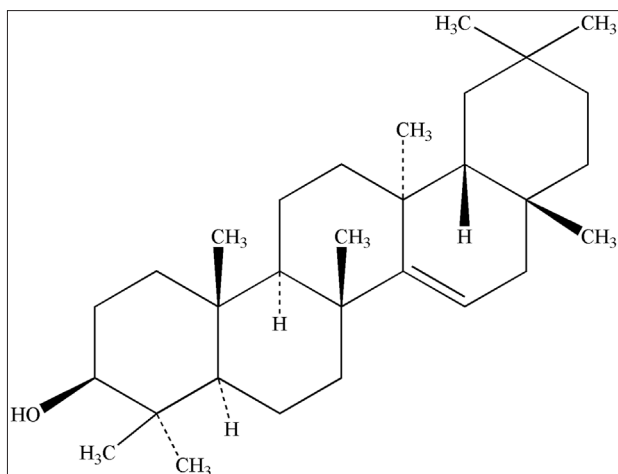


Figure 2: 3 β -taraxerol

High performance liquid chromatography quantification of 3 β -taraxerol

The HPLC instrument employed for the analysis of the amount bioactives consists of Shimadzu SIL-10A auto injector, sample cooler, two CTO-10A pumps, CTO-10A column oven, SPD-M10AVP diode array detector and SCL 10 AVP central unit (Shimadzu Ltd, Kyoto, Japan). A Kromasil C₈ column (250 mm $\times$ 4.6 mm, particle size 5 μ) was used. The isocratic mobile phase consists of a water (solvent A) and methanol (solvent B) in proportion of 5:95. Flow rate of the effluent was 1.5 ml/min, with run time of 30 min, the detector wavelength of 205 nm and the volume of injection was 20 μ L. Sample concentration used in the range of 5–10 mg/ml solutions.

Cholesterol esterase inhibition assay

Hypercholesterolemia is the source for major health issues like coronary heart disease and atherosclerosis. One of the major therapeutic strategies to effectively control these diseases by regulating plasma cholesterol level which is produced by the biosynthesis and dietary intake. Inhibition of human cholesterol esterase is very important target in the treatment of hypercholesterolemia, which is involved in the regulation of plasma cholesterol level.^[18]

The pancreatic cholesterol esterase inhibition assay was performed in triplicates according to Pietsch and Gutschow.^[19] Various concentrations of each compound/fractions were incubated with mixtures containing 100 μ l of 5.16 mM taurocholic acid, 90 μ l of 0.2 mM p-nitrophenylbutyrate in 100 mM sodium phosphate buffer diluted with 100 mM NaCl, pH 7.0. The reaction was initiated by adding 5 μ l of porcine pancreatic cholesterol esterase (1 μ g/mL). After the incubation of 5 min at 25°C, absorbance of the mixtures was measured at 405 nm. Simvastatin was used as a positive control for this study.

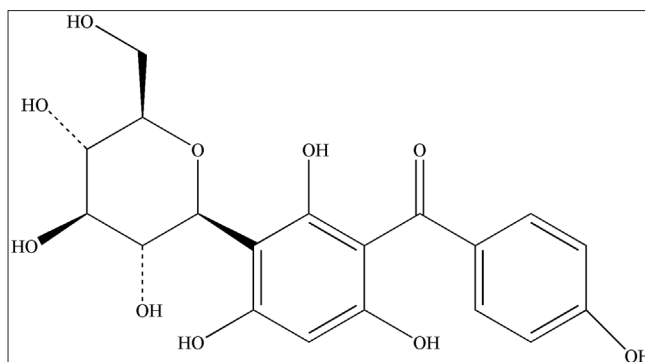


Figure 3: Iridiflophenone-3- β -C-glucoside

RESULTS AND DISCUSSION

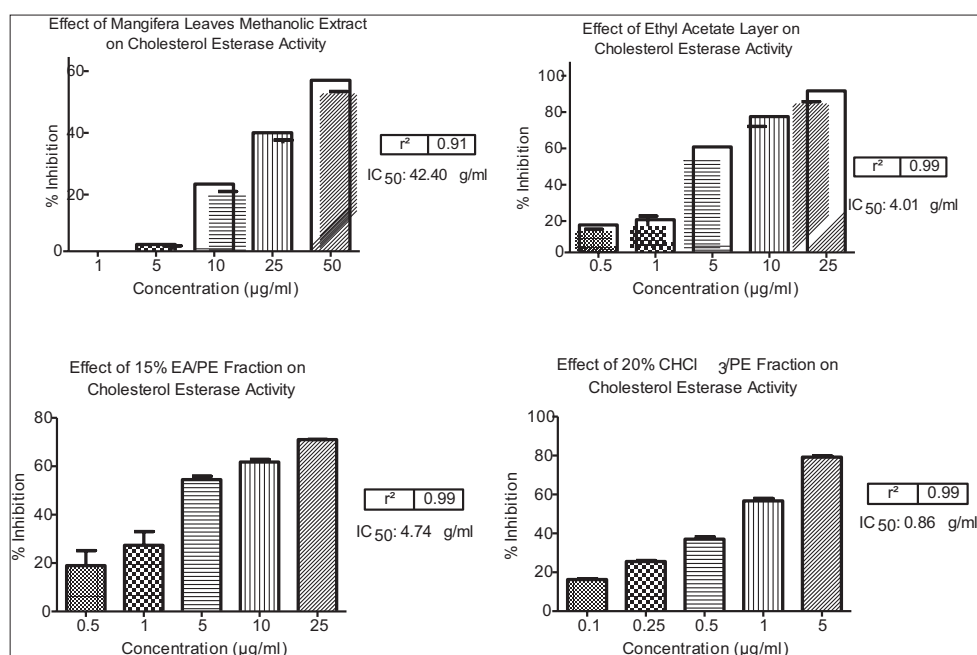
Cholesterol and free fatty acids are liberated within the intestinal lumen after hydrolysis of long chain fatty acid esters of cholesterol by pancreatic cholesterol esterase (EC 3.1.1.13), also known as a bile salt-dependent lipase. The process of hydrolysis of cholesterol ester into cholesterol by cholesterol esterase in the intestinal lumen is required for absorption because cholesterol ester is not directly absorbed by the intestinal epithelial cells. WAY-121,898, a synthetic novel inhibitor of cholesterol esterase was shown the lipid lowering effect upon oral and parenteral administrations.^[20] The efficient decrease in the absorption of dietary cholesterol esters in animal^[21,22] was supported that the hydrolysis of dietary cholesterol ester into cholesterol by cholesterol esterase in the intestinal lumen is an essential process for absorption. Therefore, inhibition of cholesterol esterase enzyme activity could inhibit absorption of dietary cholesterol esters.

The leaf methanol extract of *M. indica* is known to have hypocholesterol activity.^[21] Scientific literature search has indicated that cholesterol lowering constituents are not identified so far. Polyphenols like gallic acid, catechin and epicatechin from the grape seed are exhibiting hypocholesterol activity.^[23] Mangiferin has shown reduced cholesterol, antihyperlipidemic and antiatherogenic properties in diabetic rats.^[24-26] These evidences motivated us to test these compounds for the cholesterol lowering activity. However, the preliminary result revealed that the mangiferin, a polyphenol is not active in cholesterol esterase inhibition assay. The effects of mangiferin on targets other than cholesterol esterase in *in vivo* may results in nonresponding to cholesterol esterase inhibition assay. Hence, bio-active guided studies study is conducted to identify hypocholesterol responsible constituents in the extract. The methanol extract was partitioned between ethyl acetate and water to afford polar (water) and nonpolar (ethyl acetate) fractions. The nonpolar fraction from the methanol extract has shown highest

Table 1: Details of yield, hypocholesterolemic activity and amount of bioactive compounds in different varieties of mango leaves

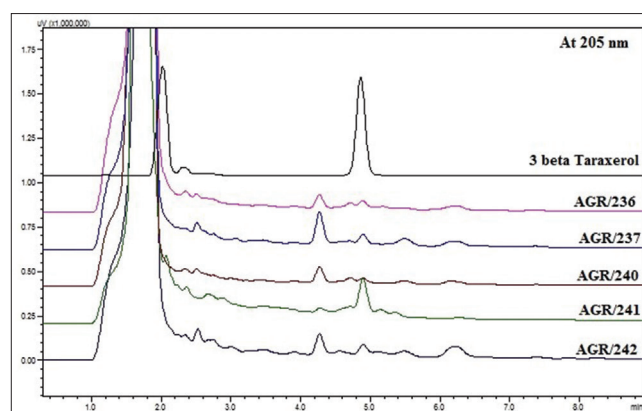
Varieties of MeOH ext of <i>Mangifera indica</i>	Code	Percentage yield (w/w)	IC ₅₀ (µg/ml)	Mangiferin %	Gallic acid %	3 β-taraxerol %
Badami	AGR/236	13.39	18.54	7.33	1.37	0.51
Totapuri	AGR/237	14.8	11.57	5.81	0.32	0.95
Sindoor	AGR/240	16.6	21.32	5.87	0.72	0.49
Sannabeejjada kayi	AGR/241	5.5	11.88	1.49	0.07	0.42
Valaja	AGR/242	11.2	11.22	6.11	0.34	0.82
Mangiferin (standard)	-	-	Not active	-	-	-
20% chloroform/PE frn	-	-	0.86	-	-	-
Iriflophenone-3-β-C-glucoside	-	-	Not active	-	-	-

PE=Petrolium ether

**Figure 4:** Determination of IC₅₀ values for extract and other samples

activity than the polar fraction [Figure 1]. The scheme of isolation of marker compounds indicates that the 3 β-taraxerol (compound 1) enriched fraction has shown IC₅₀ value of 0.86 µg/ml [Figure 4]. However, 3 β-taraxerol isolated from the fraction was not soluble in buffer assay; hence, it was not able to carry out IC₅₀ value of the same.

Methanol extract of different mango are tested in cholesterol esterase inhibition assay to understand the variability of the activity and constituents [Table 1]. Valaja and totapuri variety have shown best activity when compared to other mangoes. Maximum yield of 16% from sindoor variety but the activity is very less. The sterols appeared to have same R_f values in TLC/HPTLC. Hence, there is requirement of developing HPLC based analysis for 3 β-taraxerol. It was found to be in the range of 0.4–0.9% w/w in different variety mango leaves [Figure 5]. The developed method is useful to quantify the 3 β-taraxerol without any interference

**Figure 5:** High performance liquid chromatography chromatograms of different varieties of mango leaves

of the other compounds. However, the method should be validated for quality control purpose. The fraction XII have shown IC₅₀ value of 8.70 µg/ml. the iriflophenone-3-β-C-glucoside (compound 2) isolated

from this fraction XII didn't show the cholesterol esterase inhibition activity.

3 β -taraxerol is a sterol, having basic structure containing oleanan-3-ol lacking the methyl group at position 14, with α -methyl substituent at position 13 and a double bond between positions 14 and 15. Phytosterols are naturally found in various oils from plants. These compete with cholesterol in the mixed micelles, needed for cholesterol absorption by the small intestine. As a result, cholesterol absorption, either from food or from bile salts is lowered by about 50%, leading to a lowering of about 10% of blood cholesterol level, even though an increase in hepatic cholesterol synthesis. This reduction is achieved when phytosterols are given as monotherapy, and also in a combination therapy along with ST.^[27] Phytosterols and dietary fibre act as low density lipoprotein cholesterol lowering agents.^[28] Dietary supplements can help to get an adequate dietary intake of essential nutrients; others may help in reducing the risk of disease. The standardized leaf methanol extract of *M. indica* for anti-hypercholesterol could be used as dietary supplement to prevent hypercholesterolemia and the developed HPLC method may be used as a chromatographic fingerprint for this plant extracts or its formulations.

CONCLUSION

The present study demonstrated the hypocholesterol activity of methanolic extract of *M. indica* leaves in cholesterol esterase inhibition assay along with the isolation of bio-actives from their fractions. HPLC analysis of 3 β -taraxerol is developed to quantify in the methanol extract. 3 β -taraxerol is found to be in the range of 0.4–0.9% w/w in the mango leaves. The developed method is useful to quantify the 3 β -taraxerol without any interference of the other compounds (specific). However, the method should be validated for quality control purpose. Iriflophenone-3-C- β -glucoside was isolated from fraction XII have shown IC₅₀ value of 8.70 μ g/ml. However, the compound isolated from this fraction didn't show the cholesterol esterase inhibition activity. But, iriflophenone-3-C- β -glucoside is an alpha glucosidase inhibitor.^[29] This compound is also need to be tested in other responding *in vitro* assays for better understanding of mechanism of the action of hypocholesterol activity of the fraction generated during fractionation. The leaf methanol extract of *M. indica* is standardized with 3 β -taraxerol for hypocholesterol activity using cholesterol esterase inhibition assay.

ACKNOWLEDGMENTS

The authors thank the National Medicinal Plants Board, (New Delhi) for financial support.

SUPPLEMENT INFORMATION

Carbon nuclear magnetic resonance of 3 β -taraxerol

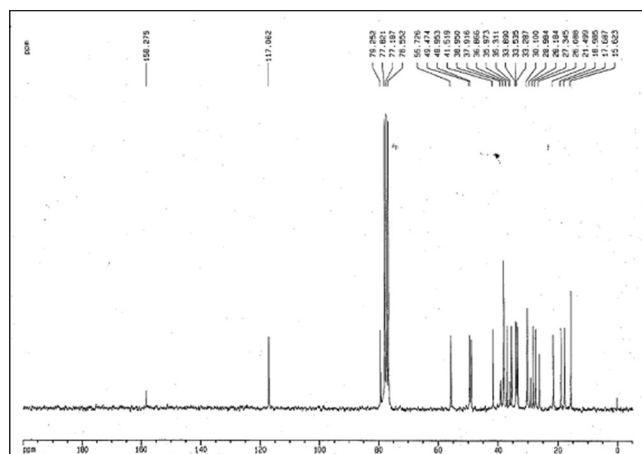


Figure S1: Carbon Nuclear magnetic resonance spectrum in CDC₁₃

Fourier transform infrared spectroscopy of 3 β -taraxerol

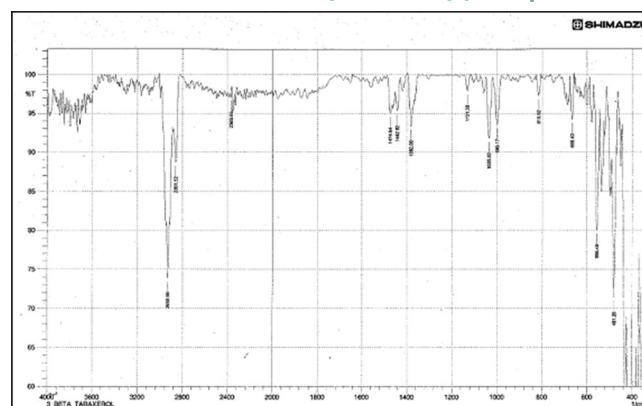


Figure S2: IR spectra in KBr

Hydrogen nuclear magnetic resonance spectrum of 3 β -taraxerol

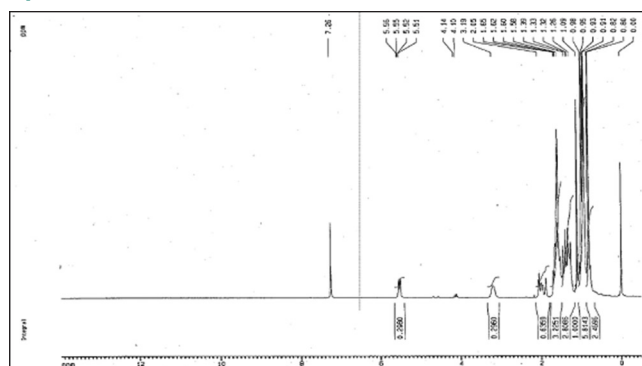
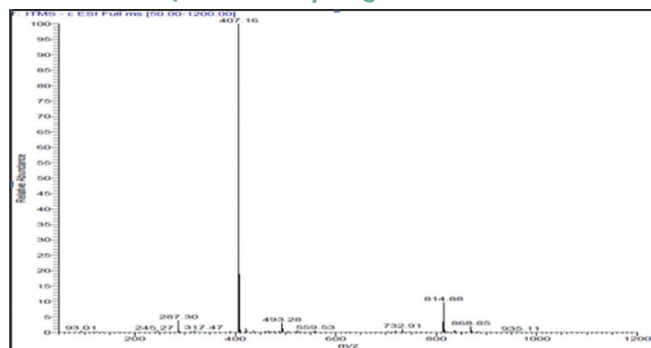


Figure S3: Hydrogen nuclear magnetic resonance spectrum in CDC₁₃

Mass of Iriflophenone-3-β-C-glucoside



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Cite this article as: Gururaja GM, Mundkinajeddu D, Dethe SM, Sangli GK, Abhilash K, Agarwal A. Cholesterol esterase inhibitory activity of bioactives from leaves of *Mangifera indica* L. *Phcog Res* 2015;7:355-62.

Source of Support: The authors thank the National Medicinal Plants Board, (New Delhi) for financial support., **Conflict of Interest:** None declared.