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Screening of polyherbal formulation for its potential anti-hyperlipidemic and antioxidant activity

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Abstract
Polyherbal formulation was prepared based on the ideal tree concept. Phytochemical screening was performed according to the standard methods. Acute toxicity was performed according to OECD guidelines, using 2000 mg/ kg BW as a limit dose. Anti-hyperlipidemic activity of the aqueous extract of polyherbal formulation (50 mg/kg, p.o.) was determined in Triton X-100 induced (100 mg/kg) hyperlipidemic albino wistar rats and compared against simvastatin (10 mg/kg, p.o,) as a standard .On 21st day serum lipid levels were estimated by using respective diagnostic kits. Results are expressed as mean ± SD and subjected to One Way Analysis of Variance (ANOVA) followed by Dunnett's test and values $P<0.01$ were considered to be significant. Antioxidant activity was determined by using hydrogen peroxide scavenging activity method. HPTLC analysis of the polyherbal formulation was performed using quercetin as a marker compound. Cell viability studies were performed to know the cell viability and for the estimation of IC_{50} values, by MTT assay on Vero monkey normal kidney epithelial cell lines. Polyherbal formulation at the dose of 50 mg/kg, lowered TC, TG, VLDL-C, LDL-C levels with simultaneous increase in high density lipoproteins cholesterol (HDL-C) levels ($P<0.01$). HPTLC analysis showed the presence of quercetin, which was also confirmed by showing significant antioxidant activity as compared to ascorbic acid. Phytochemical analysis also revealed the presence of flavanoids. Cell viability studies showed the IC_{50} value of >200, indicating the safety profile of the polyherbal formulation. This is the first study which investigates the anti-hyperlipidemic effect at a low dose level of 50 mg/kg BW.

Keywords: Polyherbal formulation, anti-hyperlipidemic activity, Triton X-100, OECD guidelines.

1. Introduction
Hyperlipidemia is a disorder of lipoprotein metabolism, including lipoprotein overproduction or deficiency and manifested by elevation of the serum total cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride concentrations, and a decrease in the high-density lipoprotein (HDL) cholesterol concentration [1]. Hyperlipidemia is the major risk factor contributing to the prevalence and progression of cardiovascular complications like atherosclerosis causing angina, peripheral arterial disease, strokes and heart attacks. Allopathic anti-hyperlipidemic drugs available in the market are having side effects like hepatotoxicity, hyperuricemia and myositis [2]. A vast number of plants have been reported in ayurvedic literature used for the treatment of hyperlipidemia having no side effects and well tolerated.
All the plants used in the formulation were reported anti-hyperlipidemic activity, but their synergistic combination was not yet tested. In view of the above concept, the present study was designed to investigate the anti-hyperlipidemic effect of the aqueous extract of polyherbal formulation in Triton X-100 induced hyperlipidemic rat model.

2. Experimental
2.1 Identification and authentication
Identification and authentication of the drugs were done by Dr K. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara University, Tirupati, A.P. India.

2.2 Plant material
Anti-lipidemic drugs used were: *Trachyspermum ammi* (30 parts, Fruits), *Boswellia serrata* (10 parts, Oleo-gum resin), *Juglans regia* (10 parts, Kernals), *Curcuma longa* (10 parts, Rhizomes), *Acacia arabica* (10 parts, Gum), *Glycine max* (30 parts, Seeds) (Table 1).

Table 1: Composition of polyherbal formulation

Biological Name	Part Used	Strength (%)
Trachyspermum ammi	Fruits	30
Juglans regia	Kernels	10
Curcuma longa	Rhizomes	10
Boswellia serrata	Oleo-gum resin	10
Acacia arabica	Gum	10
Glycine max	Seeds	30

2.3 Development of polyherbal (PHF) formulation
All the procured and authenticated individual drugs were cleaned thoroughly to remove any extraneous matter by hand sorting and dried for about twenty days. The individual drugs were then crushed using a mixer grinder and passed through mesh no. 100. The individual drugs were then weighed as per the quantity required and mixed geometrically using a blender. The mixed formulations were weighed and stored properly in airtight containers for experimental purposes [3].

2.4 Preliminary Phytochemical analysis
Preliminary phytochemical analysis for the identification of primary and secondary metabolites was done using standard methods [4].

2.5 Pharmacological study
The approval of the Institutional Animal Ethics Committee (IAEC) (1156/AC/07/CPCSEA) of DIT-University, Dehradun, was taken before starting of experiments. Albino Wistar rats of either sex (170-210 g) were used for the assessment of acute oral toxicity and anti-hyperlipidaemic activity [3].

2.6 Determination of acute oral toxicity
In an acute toxicity study the limit test dose of 2000 mg/kg was used as suggested by organization for Economic Co-Operation and Development (OECD) guidelines 420 [3].

2.7 Preparation of solutions
Polyherbal formulation (Test) and standard drug simvastatin were dissolved in distilled water. Both the drugs were administered orally for experimental purpose. For induction of hyperlipidaemia Triton X-100 was administered i.p. and prepared as a solution in physiological saline. Test drugs (50 mg/kg BW) and Triton X-100 (100 mg/kg) [5, 6] were freshly prepared each time before use.

2.8 Assessment of Anti-Hyperlipidemic Activity (Triton X-100 Induced) [5-7]
a. Animals: For the antihyperlipidaemic activity of polyherbal formulation, albino wistar rats of either sex were used. The animals were divided as follows:
Group I: Normal control (Saline only)
Group II: Hyperlipidemic control (100 mg/kg)
Group III: Standard drug treated, Simvastatin (10 mg/kg)
Group IV Polyherbal formulation (50 mg/kg)
All the groups except group I (normal control) simultaneously received a single i.p. injection of Triton X-100 to induce hyperlipidaemia.

b. Procedure
Animals were divided into four groups of six rats each and kept for overnight fasting before initiation of experiment. All the groups of animals received drugs and Triton as per the protocol. At the end of the study (on 21st day), blood samples were collected by tail vein method from anesthetized (slight exposure to chloroform) rats.

The serum was separated by centrifugation at 3000 rpm for 15 minutes in micro centrifuge (star 21, India).The various biochemical parameters (TC, TG, HDL-cholesterol) were estimated using analyzer (Model no. Rapid star 21, SEAC, India) using respective diagnostic kits (ERBA MANNHEIM diagnostics).

Total Cholesterol was estimated according to CHOD-PAP method, based on the Modified Roeschlaus method [8], Triglycerides: GPO–Trinder method, End point [9], HDL cholesterol: Phosphotungstic acid method, End point [10], Atherogenic index [11]. Accordingly, LDL-Cholesterol and VLDL-Cholesterol were calculated by using Friedwald's formula [12].

c. Statistical analysis
Results are expressed as mean ± SD and subjected to One Way Analysis of Variance (ANOVA) followed by Dunnett's test and values $P<0.01$ were considered to be significant.

2.9 Antioxidant activity
Antioxidant activity of polyherbal formulation to scavenge hydrogen peroxide was performed according to the method of Ruch *et al.* [13]. A solution of hydrogen peroxide (40 mm) is prepared in phosphate buffer (50 mMpH 7.4). The Concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (10-100 µg/ml) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as:

$$\text{Scavenged } H_2O_2\ \% = [(A0-A1)/A0 \times 100],$$

Where; A0 is the absorbance of control and A1 is the absorbance of test

2.10 HPTLC analysis [14, 15]
2.10.1 Preparation of standard solution
A stock solution of standard quercetin (1000 µg/mL) was prepared by transferring 10 mg of quercetin, accurately weighed, into a 10 mL volumetric flask, dissolving in 5 mL methanol. It was then sonicated for 10 minutes and the final volume of the solutions was made up to 10 mL with the methanol to get stock solutions containing 1000 µg/mL.

2.10.2 Preparation of sample solution
Polyherbal formulation was extracted in methanol, dried and concentrated under vacuum. 10 mg of the dried methanolic extract was dissolved in 10 ml of methanol. (Concentration-1000 µg/ml).

2.10.3 HPTLC development
The sample was spotted using Camag microlitre syringe (2 µl) on a precoated silica gel plates F 254 (10 cm X 10 cm, E. Merck). The plates were developed in a solvent system in glass chamber, previously saturated with the solvent for 30 min. TLC plates were air dried and scanning was performed on a Camag TLC Scanner at 366 nm

2.10.4 Quercetin estimation
Silica gel F 254 plates were used as a stationary phase. Ethyl acetate: Toluene (3: 7) was used as a mobile phase.

Limit of detection was 1000 µg/mL. Quercetin (1000 µg/mL) was used as a standard. Scanning wavelength was 366 nm, migration distance was 90 mm, and selected wavelength for overlaid spectra was 272 nm.

2.11 Cell viability studies

Cell viability studies were performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT assay) [16-21] on Vero monkey normal kidney epithelial cell lines. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan by the action of mitochondrial enzyme succinate dehydrogenase in viable cells. The cells are then solubilised with an organic solvent (eg. isopropanol) and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.
The time period of treatment was 48 hours. The safety profile of Polyherbal formulation was determined at 540 nm at varying concentrations of polyherbal formulations i.e. 25, 50, 75 and 100 µg/ml concentrations. 0.5% solution of DMSO was taken as controls. Mean cell viability and IC_{50} values were calculated Results were compared against doxorubicin as standard. The IC_{50} were calculated by best fit equations using the Curve Expert statistical program.

3. Results

3.1 Phytochemical screening

Phytochemical screening showed the presence of flavanoids in the polyherbal formulation (Table 2).

Table 2: Phytochemical Screening

Carbohydrates	Alkaloids	Glycosides	Tannins	Proteins	Saponins	Flavonoids
+ve	- ve	+ve	- ve	+ve	- ve	+ve

+ve = present, - ve = absent

3.2 Acute toxicity studies

No signs and toxic symptoms were observed during the acute toxicity study using the aqueous extract after oral administration of dose up to 2000 mg/kg indicating that the formulation was non-toxic and safe.

3.3 Antihyperlipidemic activity

Serum lipids in control and experimental groups of rats were estimated .Total cholesterol was found as 149±30 as compared to standard control 143±7, Triglycerides were found to be 102±6.8 as compared to standard control 97±4.6, LDL cholesterol was found to be 93±27.9 as compared to standard control 86±6.6, HDL cholesterol was found to be 36±2.8 as compared to standard control 32±3.3 ,VLDL cholesterol was found to be 21±1.5 as compared to standard control 19±1 and Artherogenic index was found to be 94±27.9 as compared to standard control 87±6.6 (Table 3, Figure 1).

Table 3: Serum lipids in control and experimental groups of rats.

Groups	TC	TG	LDL	HDL	VLDL	AI
Control	137±3.6	77±5.8	89±4.6	32±3.3	15±1	89±4.8
Induced	276±5.5***	159±7.3***	221±5***	24±2.1***	32±1.5***	222±5.3***
Standard	143±7^{a3}	97±4.6^{***,a3}	86±6.6^{a3}	32±3.3*	19±1^{***,a3}	87±6.6^{a3}
PHF	149±30^{a3}	102±6.8^{***,a3}	93±27.9^{a3}	36±2.8^{a3}	21±1.5^{***,a3}	94±27.9^{a3}

Values are given as mean± SD, n=6 six rats in each group, one way ANOVA followed by dunnett's multiple comparison test.
*, *** *P*<0.05 & 0.001 as compared to control respectively; a3 *P*<0.001 as compared to hyperlipidemic control; b1 *P*<0.05 as compared to standard;

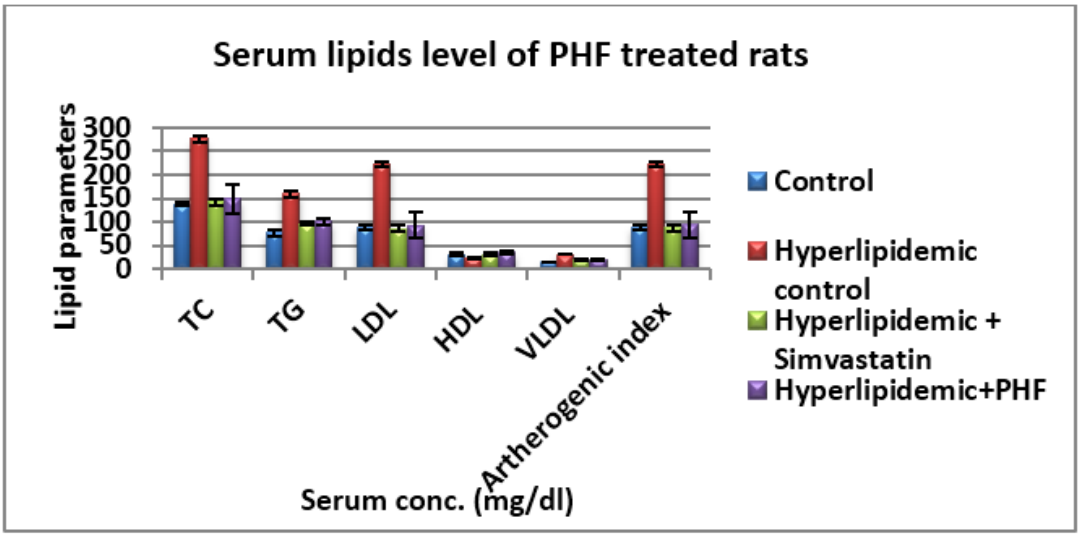

Fig 1: Serum lipid levels of PHF treated rats

3.4 Antioxidant activity

Polyherbal formulation showed % inhibition of 1.82, 4.21, 8.11, 14.36 and 20.97 at 10 µg/mL, 25 µg/mL, 50 µg/mL, 75 µg/mL and 100 µg/mL of concentrations, respectively, when compared with standard ascorbic acid (Table 4, Figure 2).

Table 4: Comparative % inhibition of H_2O_2

Conc. (µg/mL)	Standard	PHF
10	2.57	1.82
25	7.49	4.21
50	12.54	8.11
75	15.96	14.36
100	24.96	20.97

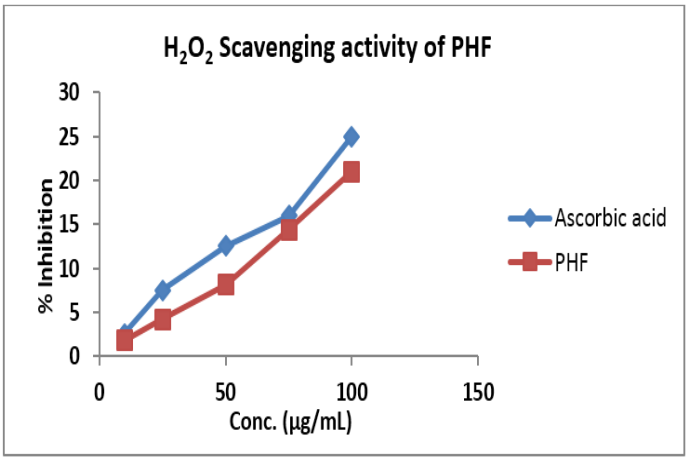

Fig 2: H_2O_2 scavenging activity of PHF

3.5 HPTLC analysis

HPTLC analysis shows the presence of quercetin in the polyherbal formulation as indicated by the similar R_f value of 0.90 as that of standard quercetin (Table 5, Figure 3, 4, 5).

Table 5: Screening data for R_f values

Tracks	Samples	Start R_f	Maximum R_f	End R_f
Track-5	PHF	0.72	0.82	0.90
Track-6	Quercetin	0.73	0.82	0.90

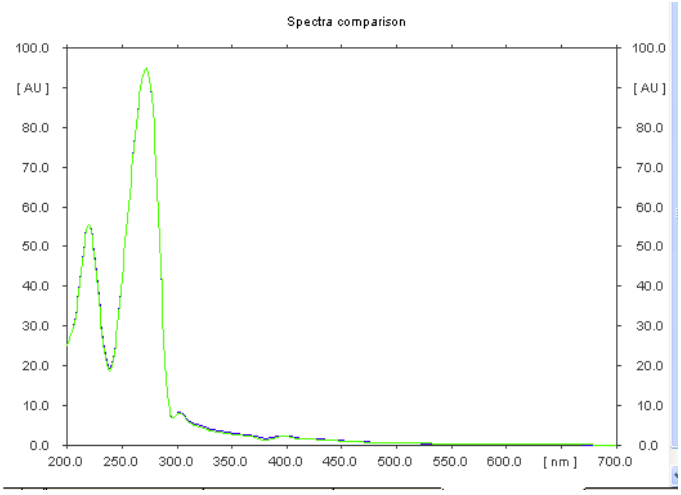

Fig 3: Spectral comparison of PHF with quercetin

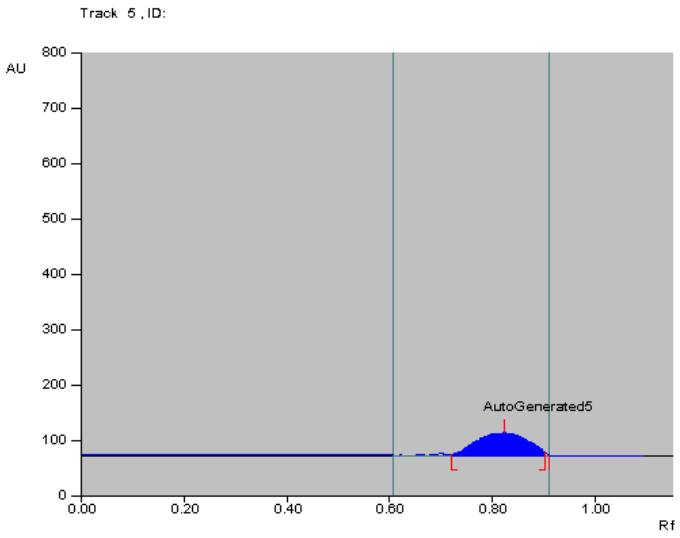

Fig 4: HPTLC Chromatogram of PHF

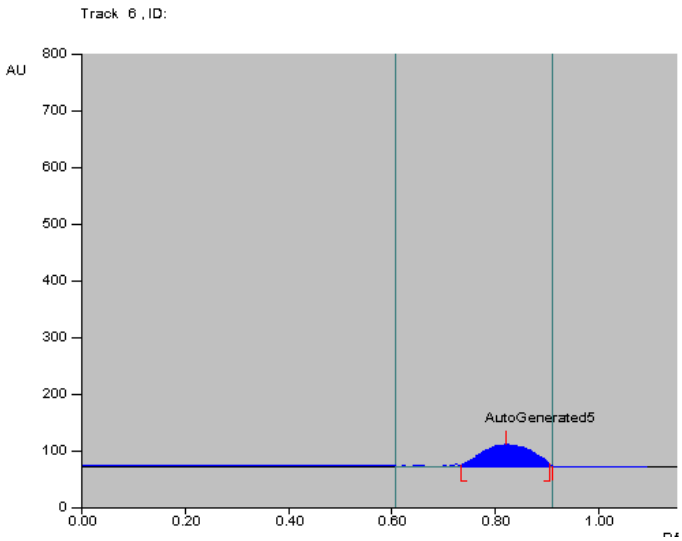

Fig 5: HPTLC Chromatogram of standard Quercetin

3.6 Cell viability studies

Polyherbal formulation showed mean cell viability of 93.9%, 88.3%, 81.1% and 74.6% at 25, 50, 75 and 100 µg/ml of concentrations. Doxorubicin (anti-cancer drug) showed mean cell viability of 98.1%, 73.5%, 49.5% and 29.6% at 0.05, 0.5, 5 and 50 µg/ml of concentrations. IC_{50} value for Doxorubicin was found to be 5.1. Cell viability studies showed IC_{50} values >200 indicating that the polyherbal formulation were safe and does not shown any signs of toxicity on the vero monkey normal kidney epithelial cell lines (Table 6, Figure 6).

Table 6: Mean cell viability and IC_{50} values

S.no	Samples	Conc.(µg/ml)	Mean cell viability	IC_{50}
1	PHF	25	93.9	>200
		50	88.3	
		75	81.1	
		100	74.6	
2	Doxorubicin	0.05	98.1	5.1
		0.5	73.5	
		5	49.5	
		50	29.6	
3	DMSO (0.5%)	0.5%	99.82	-
4	Medium	-	100	-

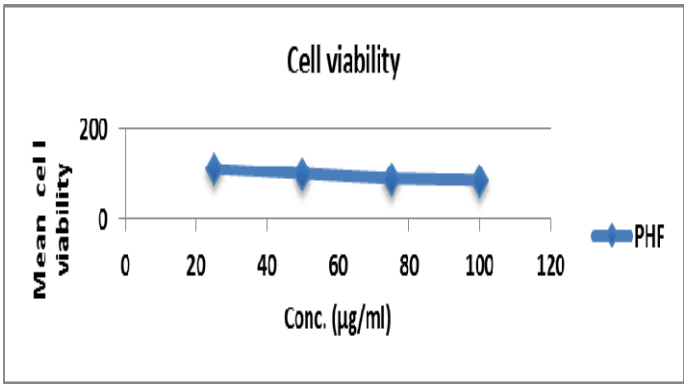

Fig 6: Cell viability studies

4. Discussion

The Antihyperlipidemic activity of polyherbal formulation was may be due to the presence of quercetin, which is a polyphenolic compound with a known antioxidant and anti-hyperlipidemic activity. Quercetin is a well known scavenger of free radicals, which boosts the level of HDL and decreases the level of LDL. Overlaid spectra of polyherbal formulation with standard quercetin also confirm the presence of quercetin.

5. Conclusions

Elevated circulating low-density lipoprotein-cholesterol (LDL-C) is a risk factor for cardiovascular disease [22]. The oxidation of LDL is believed to play an important role in the pathogenesis of atherosclerosis [23].

Quercetin's antioxidant action protects against LDL cholesterol oxidation. This may be beneficial because oxidation causes LDL cholesterol to stick to artery walls [24].

In view of the importance of medicinal plants due to their safety profile and well tolerated, the study of antihyperlipidemic activity of polyherbal formulation was carried out by acting on multiple targets at the same time; the polyherbal formulation enhances the therapeutic efficacy by improving the bioavailability thereby reducing the doses required for individual components in the formulation. Synergism also attenuates undesirable side effects [25].

6. Competing interests

The author(s) declare that they have no competing interests

7. Acknowledgements

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