Phytochemical and Pharmacological Validation of the Polyherbal Extract on Rodents

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Abstract

Hyperlipedemic is the greatest risk factor for coronary heart diseases. It is characterized by elevated serum total cholesterol, low density lipoprotein, very low density lipoprotein and decreased high density lipoprotein levels. Methanolic extract of *Terminalia arjuna* (bark), *Phyllanthus emblica* (fruits), *Withania somnifera* (leaves), *Convolvulus pluricaulis* (whole plant), *Piper betel* (leaves), *Allium sativum* (bulb), *Piper longum* (dry fruits), *Zingiber officinale* (rhizomes), *Tribulus terrestris* (whole plant) and *Cardamom* (dry fruits) these herbs were tested against Triton X100 induced hyperlipedemic in adult albino rats. The therapeutic dose is calculated as 200mg/kg as per the toxicity guidelines OECD 423. Fenofibrate 65mg/kg is used as a standard drug. The methanolic extracts shows a significant decrease in the levels of serum cholesterol, Triglycerides, LDL, VLDL and significant increase in the level of serum HDL against Triton induced hyperlipedemic rats. The results shows that the polyherbal extract possess potential antihyperlipedemic action suggesting the potential role in coronary artery disease.

KEYWORDS: Polyherbs, Methanolic extract, Hyperlipidemia, antioxidant, Triton X100

INTRODUCTION

Medicinal plants provide important ingredients in modern medicines, nutraceuticals, food supplements, folk medicines, and lead compounds for synthetic drugs[1]. Several studies indicated that medicinal plants contain compounds like peptides, unsaturated long chain fatty acids, aldehydes, alkaloids, essential oils, phenols and water or ethanol soluble compounds, which are significant in therapeutic applications against the pathogens like; bacteria, fungi and viruses[2]. Screening medicinal plants for novel bioactive compounds are the sole remedy since, plant based drugs are biodegradable, safe and have fewer side effects. Thus, the therapeutic potential of the herbs opens up new vista in the future pharmacological research of herbal drug development[3]. The objective of the present investigation was to validate the methanolic extract of *Terminalia arjuna*[4] (bark), *Phyllanthus emblica*[5] (fruits), *Withania somnifera*[6] (leaves), *Convolvulus pluricaulis*[7] (whole plant), *Piper betel*[8] (leaves), *Allium sativum*[9] (bulb), *Piper longum*[10] (dry fruits), *Zingiber officinale*[11] (rhizomes), *Tribulus terrestris*[12] (whole plant) and *Cardamom*[13] phytochemically and preclinically.

Hyperlipidemia is a state of high lipid profile or high cholesterol levels in the plasma, which can lead to severe health complications like congestive cardiac failure, atherosclerosis and pancreatitis[14].Lipid-lowering strategy may have a beneficial role in normalizing vascular function and greatly decreasing the frequency of clinical events associated with atherosclerosis, combined with the ability of antioxidants to alleviate vasomotor disturbances in hypercholesterolemia and to slow the progression of atherosclerosis. Thus the desirable medicament must not be confined just to reduce the lipid levels in plasma but also should be efficient to protect from free radical damage[15].

MATERIALS AND METHODS

The plant materials were collected locally and they identified and authentified by Dr.P.Jayaraman, Director, Plant Anatomy and Research Centre, Chennai. Tyloxapol or Triton WR-1339 (a non-ionic detergent, iso octyl polyoxy ethylene phenol and formaldehyde polymer) was purchased from Hi Media, Hyderabad. Fenofibrate was purchased from Moral Labs, Chennai. Cholesterol and HDL-Cholesterol enzyme kit purchased from Span Diagnostics Ltd., Triglycerides and LDL-Cholesterol kits from Euro Diagnostic System. All other chemicals were of analytical grade and obtained locally.

Extraction

All drugs were shade dried at room temperature and were powdered in a Wiley mill. One kilogram of powdered drug was packed in a Soxhlet apparatus, extracted with petroleum ether and methanol. The percentage yield of both the extract was calculated.[4] The pet ether and methanolic extract was concentrated in a rotary evaporator. They were used for the phytochemical, instrumental, toxicity study and pharmacological validation.

Phytochemical analysis

The pet ether and methanolic extracts of the polyherbal subjected to systematic qualitative phytochemical screening to identify the phytoconstituents.[5]

INSTRUMENTAL ANALYSIS

The methanolic extract was analysed by Paper chromatography, Thin layer chromatography, UV, FT-IR and HPTLC methods. The phytoconstituents present in the extract were identified and analysed. [6, 7]

Paper chromatography

The polyherbal extract was dissolved in their suitable solvent and their spots were kept on the Whatman filter paper of pore size 0.24. Respective stationary phases and detecting agents were chosen to identify the presence of tannins, alkaloids, glycosides, flavonoids and proteins.[8]

Tannins	-	Hexane(8ml):Chloroform(2ml):Methanol(1ml)
Alkaloids	-	Toluene(4.5ml):Chloroform(5):Methanol(0.75)
Glycosides	-	Ethylacetate(8.2ml):Formicacid(0.9ml)Water(0.9ml)
Flavonoids	-	Chloroform(5ml):Methanol(4ml):Water(1ml)
Proteins	-	N-Butanol(8ml):Aceticacid(1ml):Water(1ml)

Thin layer chromatography

The polyherbal extract was dissolved in their suitable solvent and their spots were kept on the Silica gel G coated plates. [9, 10] Respective stationary phases and detecting agents were chosen to identify the presence of tannins, alkaloids, glycosides, flavonoids and proteins.

UV spectroscopy

Stock solution

Accurately weighed 10mg of Extract was dissolved in 10ml of methanol in a 10ml of volumetric flask and volume was made up to the mark with to get a concentration of 1000µg/ml.[11]

Primary stock solution

From primary stock solutions of 1ml was pipetted out in a 10ml of volumetric flask and volume was made up to the mark to get a concentration of 100µg/ml.

Secondary stock solution

From secondary stock solutions 0.2 ml was pipetted out and diluted to 10ml with methanol to get a concentration range of 2µg/ml. Then it was analysed spectrometrically.

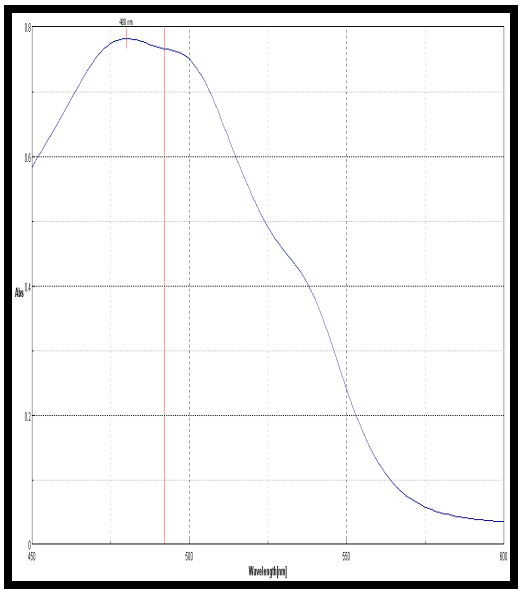
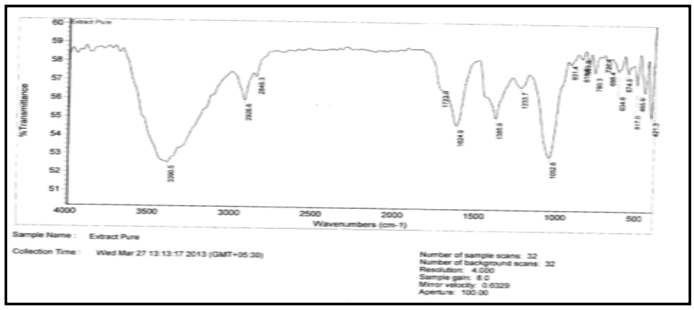


Figure – 1 UV Spectrum of the methanolic polyherbal extract

Fourier transform infra red spectroscopy

The FT-IR analysis of the plant extract in KBr pellets by using FT-IR spectroscopy Shimadzu, IR affinity 1, Japan) at moderate scanning speed between 4000 – 400 cm^{-1}.[12]

Figure – 2 FT-IR spectrum of the polyherbal methanolic extract



High pressure thin layer chromatography

Chromatography was performed on silica gel F254 HPTLC pre-coated plates. Samples were applied on the plates as band of 7mm width using a Camag Linomat V sample applicator at the distance of 14mm from the edge of the plates. The mobile phase was constituted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (V/V/V/V). After development, plates were dried and derivatised in NP-PEG reagent. The finger prints were evaluated at 366 nm in fluorescence mode with WinCats and VideoScan software.[13]

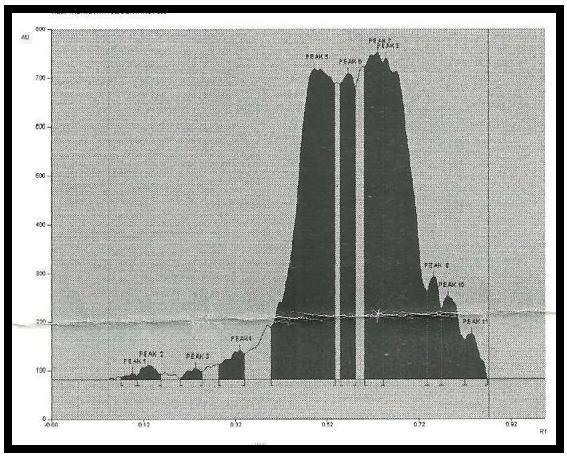


Figure – 3 HPTLC Chromatogram of the polyherbal extract

Animals

Normal healthy adult albino rats of either sex (180 – 250 gm) were housed under standard environmental

conditions at temperature (25±2° C) and light and dark (12:12h). Rats were fed with standard pellet diet (National Institute of Nutrition, Hyderabad) and water *ad libitum*. The experiment was carried out according to the guidelines of the CPCSEA and IAEC, Department of Pharmacology, Teegala Ram Reddy College of Pharmacy, Meerpet, Hyderabad.

Acute toxicity study

Acute oral toxicity study was performed as per OECD – 423 guideline (Acute Toxic Oral Class method), albino rats (n=6) of either sex selected by random sampling were used for acute toxicity study. The animals were kept fasting for over night and provided only with water, after which the extract was administered orally at 5, 50, 300, 2000mg/kg/b.w. by gastric intubations and observed for 14 days. If mortality was observed then the dose administered was assigned as toxic dose.[14] From that $1/10^{th}$ of the dose will be taken as the therapeutic dose *i.e*) $1/10^{th}$ of $LD_{50} = ED_{50}$.

PHARMACOLOGICAL SCREENING

Triton x 100 induced hyperlipidaemia

The animals were fasted over night with the free access of water and Triton X 100 was administered intraperitonealy at the dose of 100mg/kg in the morning. After 72 hours the animals were given the test drug and the standard drug every day morning for upto 7 days.

Group 1-Control received distilled water (1ml/kg)
Group 2-Triton X 100 (100mg/kg)
Group3-Methanolicextract (200mg/kg)
Group 4- Fenofibrate (65mg/kg)

8^{th} day blood collection and analysis of Total cholesterol, Triglycerides, High density lipoproteins, Low density lipoproteins, Very low density lipoproteins and Total protein.

Biochemical estimation

The rats were sacrificed after 48 hours of last dose by cervical decapitation. The blood samples were collected separately by cardiac puncture and allowed to clot, for 30 min at room temperature. The clear serum was separated by centrifugation at 2500 rpm for 10min. The serum Triglycerides, HDL, LDL, VLDL, Total cholesterol, Total protein levels were determined by standard kits by using Auto analyzer and Very low density lipoprotein cholesterol (VLDL-c) was calculated by using Friedwald formula VLDL-c = TG/5.[15]

Statistical analysis

Statistical evaluation the data was done by one-way ANOVA followed by Dunnett's multiple comparison tests using Graph pad prism software version 5.0 and the values were expressed as Mean±SEM.

Results and discussion

The percentage yield for petroleum ether extract 9.11% and methanolic extract 51.3% shows the number and quantity of active phytocontituents soluble in the particular solvent used based upon their polarity nature. The phytochemical analysis showed the presence of a few and traces of phytoconstituents in the pet ether extract and alkaloids, tannins, saponins, flavonoids, phenol, sugar were present in the methanolic extract. The polyherbal methanolic extract absorbed the UV radiation between the wavelengths of 450 – 600nm. It may be the possible phytocompounds of flavonoids, alkaloids, tannins or any other phytocompounds. Since it was a crude extract the exact phytocompounds may not be known

but the phytocompounds which absorbs the radiation in this region was present in the extract. This directly involves in the biological activity of the phyto medicine.

The presence of the functional groups directly or indirectly involved in the pharmacological actions of the selective Indian medicinal plants. The functional groups of the phytoconstituents present in the extract may modify the lipid levels which lead to the selective biological response in the preclinical animal models. Functional groups of the phytochemicals similar to the functional group of the body biochemical act according to the SAR and there by produce the required pharmacological response. The results were shown in Table – 1.

Chromatographic analysis of the extract shows the presence of the active phyto ingredient in the polyherbal extract according to the Rf values eluted in the paper and the TLC plates. Based upon the Rf value each and every active principle can be identified. In all the mobile phases used for Paper and TLC shown some Rf values according to the eluted compound. So it was confirmed that active ingredients were present in the methanolic extract according to the mobile phase used for the specified phyto compound. Those compounds may be responsible for the antihyperlipedemic action. . The results were shown in Table – 2.

In HPTLC, the number of peaks at various Rf values shows the different compounds present in the crude extracts. They were isolated at particular finger print region with their respective peak areas and peak height. The extract contains many peaks confirms many number of active constituents present in it. Those extracts were chosen for the preclinical evaluation. The results were shown in Table – 3.

The extract didn't show any specific toxic symptom during toxicity study at the dose of 5, 50, 300 mg/kg. But at the dose of 2000mg/kg two animals were died. So the lethal dose was fixed as 2000 mg/kg and from this $1/10^{th}$ of the lethal dose, 200 mg/kg therapeutic dose was calculated.

Triton WR 1339 is the non-ionic detergent, induces acute hyperlipidemia consisting of two phases. In phase I, cholesterol levels raise 2-3 times within 24 hours of administration. The increased cholesterol levels decreases nearly to control levels within next 24 hours in phase II. The mechanism of the Triton induced hypercholesterolemia in phase I is thought to be due to increased hepatic synthesis of cholesterol through the ability of Triton to interfere with the uptake of plasma lipids by the tissue. Thus, our study was designed to test antihyperlipedemic activity of polyherbal extract within 24 hours of Triton WR 1339 administration as it is used only to induce acute but not chronic. The results were shown in Table – 4.

There is an inverse relationship between plasma HDL-cholesterol level and coronary heart disease. The levels of serum lipid profile, total cholesterol, triglycerides, LDL, VLDL and HDL in control, test and standard drug treated were presented in the table. Lowering of serum lipid profiles through dietary or drug therapy seems to be associated with a decrease in the risk of vascular disease. The increased fatty acid concentration also increases the beta-oxidation of fatty acids, producing more acetyl Co-A and cholesterol. The increased concentration of free fatty

acid may be due to lipid break down and this may cause increased generation of NADPH dependent microsomal lipid peroxidation. Phospholipids were increased in animals treated with Triton. Phospholipids present in the cell membrane and make up vast majority of the surface lipoprotein forming a lipid layer that acts as an interface with both polar plasma environment and non-polar lipoprotein of lipoprotein core. Administration of the extracts showed decreased the levels of phospholipids. As a conclusion the methanolic polyherbal extract shows significant anti hyperlipedemic action when compared with the standard drug Fenofibrate. Hence the methanolic polyherbal extract can be safely used as an antihyperlipedemic medicine used against hyper lipedemia.

Table – 1

FT- IR analysis of the polyherbal methanolic extract

S.No	Peak value nm^{-1}	Functional group
1	3390.5	NH Amine - Stretching
2	1624	NH Amine - Bending
3	1385	C-N Amine - Stretching
4	2926, 2849	C-H Alkane Aliphatic - Stretching
5	780.3, 727.4, 698.4	Aliphatic C-H Bending

Table – 2

Rf values of the polyherbal methanolic extract in paper chromatography and thin layer chromatography

Test	Rf value		Rf value	
	Petroleum ether extract		Methanolic extract	
Tannins	0.3	0.2	0.9	0.3
Alkaloids	0.2	0.9	0.3	0.4
Glycosides	0.6	0.2	0.9	0.5
Flavonoids	0.5	0.9	0.7	0.8

Table – 3

HPTLC analysis

Peak	Rf	Peak height	Peak area
Peak 1	0.1	12.418	202.054
Peak 2	0.13	27.527	702.205
Peak 3	0.24	22.127	510.771
Peak 4	0.33	58.608	1679.0
Peak 5	0.5	638.801	39306.14
Peak 6	0.57	628.104	14807.59
Peak 7	0.64	670.951	17830.59
Peak 8	0.65	660.557	30697.14
Peak 9	0.76	210.436	4000.716
Peak 10	0.79	170.669	4729.008
Peak 11	0.84	92.574	2068.397

Table – 4

Effect of polyherbal extract on Triton X 100 induced hyperlipidemia

Treatment	TC mg/dl	TG mg/dl	HDL mg/dl	LDL mg/dl	VLDL mg/dl
Control	70.29±2.25	65.59±2.10	22.67±1.54	34.47±3.15	13.12±0.41
Triton X 100	156.7±32.61	139.7±2.30	18.34±1.09	110.2±2.71	29.17±0.56
Methanolic extract 200mg/kg	96.13±2.01*	93.46±2.31*	23.3±1.10*	53.4±2.51*	18.47±0.47*
Fenofibrate 65mg/kg	84.33±1.47*	89.86±1.75*	25.07±1.57*	41.29±2.54*	17.91±0.35*

n=6, Values are **mean ± SEM**. Differences were assessed statistically using one-way ANOVA followed by Dunnett's test**.** *(**p<0.001**)

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