ANTIATHEROSCLECRATIC, HYPOLIPIDEMIC & ANTIOXIDATIVE OF ETHANOLIC EXTRACT OF PRIVA CORDIFOLIA INDUCED ALBINO RATS

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Abstract

Today, there is broad interest in drugs emanated from plants. This interest primarily originates from the belief that phytomedicine is safe and dependable, compared with that of costly synthetic drugs that have adverse effects. To determine the potential and promote the use of herbal medicine, it is essential to intensify the study of medicinal plants that find place in folklore. Therefore, such plants should be investigated to better understand their properties, safety and efficacy. The present study was conducted to develop newer lead for better and safer therapeutic agents traditional medicinal use, namely, Priva cordifolia. Preliminary phytochemical analysis, The phytochemical studies concluded that various extracts of *Priva cordifolia* are rich in Glycosides, Phenols, Flavonoids. In-vivo atherosclerotic activity, the extracts were tested in high fat diet induced atherosclerosis in Albino rat model. Total cholesterol (TC), low density lipids (LDL), high density lipids (HDL), triglycerides (TG), free fatty acids (FFA) and ratios of cholesterol to lipids were also calculated. The values were tabulated and there was a significant reduction in all the lipids except HDL in extract and standard drug treated groups. Extracts showed dose dependant increase in HDL which is an indicator of good cardiovascular health of rats. The extracts at 400mg/kg showed a similar activity as standard. EEPC at 400mg/kg where TC was reduced to 80, LDL to 43 and TG to 132.EEPC exhibited an inhibition of 71% and that of ascorbic acid was 79% at 500µg/ml and the IC_{50} of the extract was 222.79µg/ml, while that of ascorbic acid was 202.72µg/ml.

Keywords: *Priva cordifolia,* Phytomedicine, Anti-Atherosclerotic activity, C-reactive protein, DPPH radical scavenging activity.

INTRODUCTION

Cardiovascular diseases are major health problem, secretarial for 31% of all deaths worldwide (Figure 1). WHO noted that cardiovascular disease (CVD) has no geographic, socioeconomic, or sex boundaries and estimated that, far from being confined to the most developed countries. CVD are also leading cause of death in developing countries as well. [1] Almost 23.6 million people will die from CVD (mainly from heart disease and stroke) till 2030. It is projected to remain the single leading cause of death. Indians constitutes around 1/6th of the humanity and have a much higher rate of cardiovascular disease than other ethnic groups in the worlds. [2]

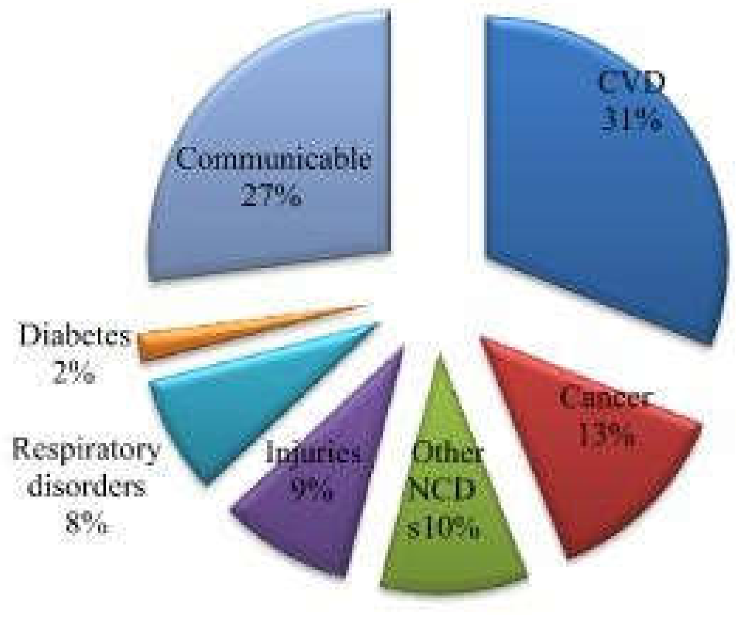

Figure 1. Worldwide role of various disorders responsible for mortality

Cardiovascular disease belongs to the class of disorders which involve the heart or blood vessels (*e.g.* arteries). CVD is usually associated with one or more characteristics, acknowledged as risk factors that describe "any attribute, characteristic or exposure of an individual that increases the likelihood of developing a disease or injury.[3]

Material & method

The Verbenaceae family is made up of approximately 100 genera and 2600 species occurring in temperate, subtropical and tropical regions of both hemispheres. Its representatives have varied habit, from large trees (*Tectoma grandis* L.) until herbs (*Verbena officinalis* L.). According to Bentham and Hooker, the systematic position of the family Verbenaceae (1876) belongs to the cohort Lamiales, series – Bicarpellatae and subclass - Gamonetalae of class Dicots.[4]

Method

Fresh leaves of Priva cordifolia were collected from Bhopal. The collectedleaves were shade dried, crushed and coarsely powdered and then passed to sieve in Sieve no. 40.

Extraction procedure

The dried and powdered leaves of Priva cordifolia were extracted by soxhlet apparatus. About 1 kg of the coarsely powdered drug was extracted successively with petroleum ether (60-80°C), followed by ethyl acetate and ethanol.[5] The marc was air dried before extracting with next solvent, below 50°C. Upon repeated siphonizations the extract obtained was subjected for distillation followed by evaporation by rotary evaporator. Finally, marc was macerated with chloroform water to obtain an aqueous extract.[6] The extracts obtained were weighed and yield in percentage were calculated. The yield value was determined by using the formula.

$$\text{Yield Value} = \frac{\text{Weight of the extract obtained}}{\text{Total amount of crude drug taken}} \times 100$$

All the concentrated extracts were stored in desiccators for further use. All the solvents used for the current study are of analytical grade.

PHARMACOLOGICAL STUDIES

Animal procurement:

Albino rats of 6–8 weeks of age were used. Experiment was conducted in 30 male rats. The animalswere housed in institutional animal house facility in polypropylene cages at 25 ± 2º C and fed with standard pellet diet and water ad libitum. Daily intake of food was quantified. The proposal was duly approved by Institutional Animal Ethical Committee as per CPCSEA guidelines.

In-vivo Anti-Atherosclerotic activity of Ethanol extract of Priva cordifolia (EEPC)

The animals were divided into five groups of 6 rats each. All the rats were weighed and body weights were noted down carefully in each group. Group I rats served as control and were fed normal pellet diet. Group II-V were fed on high fat diet (HFD) for 120 days to develop atherosclerosis. Group II was without any drug. Group III was administered with oral dose of ethanol extract of Priva cordifolia at 200mg/kg body weight (EEPC 200mg/kg). Group IV was administered with oral dose of ethanol extract of Priva cordifolia at 400mg/kg body weight (EEPC 400mg/kg). Group V was administered with oral dose of Standard drug, Simvastatin.[7] After 120 days, the rats were fasted for 12 h before collection of blood samples. They were weighed and body weights were noted down carefully. Each rat was anesthetized with diethyl ether and blood was withdrawn from the retro orbital plexus, transferred to a centrifuge tube and allowed to clot. These separated components were used for further analysis. All the samples were stored in refrigerator at 4ºC and used for determination of various parameters like C-Reactive Protein, Blood analysis for cholesterol, TG (Triglycerides), HDL (High density lipids), VLDL (Very Low Density Lipids) and LDL (Low Density Lipids) level, Atherogenic index.

Table 1: Composition of High fat diet to induce atherosclerosis

Ingredient	**%w/w**
Cholesterol	5.0
Salt mixture	4.0
Casein	20.0
Cellulose	6.0
Choline chloride	0.2
Sugar	25.0
Butter	40.0

Estimation of C - reactive protein

AKTiv sensor cassettes, 1-ethyl-3-[3-dimethylamino propyl] carbodimide hydrochloride (EDC), Dulbecco's modified phosphate buffered saline (PBS), N- hydroxysuccinimide (NHS) (Akubio Ltd., UK), Bovine Serum Albumin (BSA), Tris Sodium Chloride, Tween-20, Sheep IgG were purchased from Sigma-Aldrich (Poole, UK). CRP ELISA was purchased from Kalon Biological (UK).

ELISA:

The extracted sample from rats was tested for CRP level using a validated commercial ELISA kit from Kalon Biological (Kalon Biological, U.K.). The assay was conducted according to the manufacturer's user instructions, with serum diluted toas low as 1 in 5 to as much as 1 in 10,000 in the supplied sample diluent.[8] Standard Resonant Acoustic Profiling (RAP) Assay for CRPSerum Sample Preparation: Serum with human CRP was supplied by Kalon Biological (UK). Rat blood was prepared as follows. Rat blood collected in EDTA tubes was kept refrigerated andused within 24 hours of collection. The blood was centrifuged in 1.5 mL microcentrifuge tubes at 3000 × g for five minutes at 4°C, the upper layer was then aspirated. The whole blood volume was reconstituted by addition of the serum appropriately diluted in normal serum to give blood spiked with human CRP at 1.5 μg/mL (average CRP) and 15 μg/mL (High).[9]

Procedure:

All assays were performed at room temperature under continuous flow at 25 μL/min with a running buffer of Tris-Buffered Saline (TBS) and 0.005% Tween-20. CRP standards were prepared in a sample buffer comprising TBS containing 0.005% Tween-20 and 100 μg/mL BSA from a concentrated stock solution (94.8 μg/mL CRP). CRP rat serum was also appropriately diluted in the same sample buffer from a 1/50 to 1/6000 dilution. CRP samples were prepared in sample buffer (TBS, 0.1% BSA, 0.005% Tween-20). These were injected for 5 minutes, and the initial rate of association was monitored.[10]

Data Analysis Methods

RAP data was analysed as initial rates of signal generation (Hz/s) upon injection of CRP or antibody onto a test channel containing immobilised anti-CRP. The data was displayed and analyzed using RAPid Workbench v1.0.25 (Akubio Ltd., Cambridge, U.K.).

Blood analysis: Lipid profile

The plant extracts were investigated for maintenance of cholesterol, TG (Triglycerides), HDL (High density lipids), VLDL (Very Low Density Lipids) and LDL(Low Density Lipids) level and were assayed using Span diagnostic kits. The measurements of the triglycerides, HDL Cholesterol and total cholesterol were performed with the kits manufactured by Transasia Biomedicals Ltd (In technical collaboration with ERBA diagnostics Mannheim, Germany) according to the specifications of the manufacturers, in ERBA Chem 5 semi autoanalyser. The triglycerides were measured enzymatically by use of Glycerol kinase and glycerol phosphate oxidase method, the total cholesterol by Cholesterol-oxidase and peroxidase method and the HDL cholesterol was measured after using a precipitating agent (phosphotungstate and magnesium). The LDL-C measurement with the homogeneous method was performed with the kits manufactured by Transasia Biomedicals Ltd. The method is based on the selective protection of LDL-C with the addition of reagent R1 (Good's buffer, pH 6.8, 25 mmol/l cholesterol oxidase 5000 U/L, N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (H- DAOS) 0.64 mmol/l, catalase 10000 U/L and Ascorbate oxidase 5000 U/L) . The cholesterol of the other lipoproteins is processed by cholesterol oxidase, and the hydrogen peroxide formed is broken down by catalase. After 5 minutes, with theaddition of reagent 2 (Good's buffer, pH 7.1, 25 mmol/l, 4- aminoantipyrine 3.4 mmol/l, peroxidase 20,000U/L), LDL-C is released for enzymatic processing and yields a blue colour complex with H-DAOS and 4-aminoantipyrine, which is measured at 600 nm. The concentration in sample was compared with the absorbance of the LDL calibrator. The tests were done on ERBA Chem 5 semi autoanalyser. These measurements were carried out for blood samples and organ samples as well and the results were tabulated in the results section.[11]

Atherogenic index:

The atherogenic index was calculated using the following formula

$$\text{Atherogenic Index} = Log\left[\frac{TG}{HDLc}\right]$$

In vitro Antioxidant Activity

DPPH radical scavenging activity

The DPPH radical is a stable free radical and is mostly used to calculate the radical scavenging activity of antioxidant component. This method is depended on thereduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the production of the non radical form DPPH-H. The free radical scavenging activity of EEPC was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the reported method. In short, 0.1mm solution of DPPH inmethanol was prepared, and 1mL of the prepared solution was added to 3 ml of the solution of extract in methanol at different concentration (125, 250, 500 & 1000 µg/mL).[12] The absorbance was measured using a UV-spectrophotometer at 517 nm. Ascorbic acid was used as the reference standard. A lower absorbance value of reaction mixture suggests higher freeradical scavenging activity. The capability to scavenging the DPPH radical was calculated using the following formula.

DPPH scavenging effect (% inhibition) = {(A0 – A1)|A0} × 100

Where, A0 is the absorbance of the control and A1 is the absorbance in presence of the extract and reference. All the tests were performed in triplicates and the results were averaged.

PHARMACOGNOSTICAL STUDIES

Preliminary Phytochemical Screening

Table No.2 : Preliminary Phytochemical Screening of various extracts of *Privacordifolia*

S.NO	TEST	Pet. Ether extract of PC	Ethyl acetate extract of PC	Ethanol extract of PC	Aqueous extract of PC
1	Alkaloids	-	-	-	-
2	Glycosides	-	-	-	-
3	Carbohydrates	-	-	+	+
4	Steroids	+	+	+	-
5	Triterpenoids	+	+	+	-
6	Fixed oils & fats	-	-	-	-
7	Tannins	-	-	-	-
8	Proteins & amino acids	-	+	+	+
9	Gums & mucilages	-	-	-	-
10	Flavonoids	-	-	+	+
11	Saponins	-	-	-	-
12	Phenols & Phenolic compounds	-	-	+	+

'+' indicates Presence; '-' indicates Absence; PC – *Priva cordifolia*

In-vivo atherosclerotic activity, the extracts were tested in high fat diet induced atherosclerosis in Albino rat model. Total cholesterol (TC), low density lipids (LDL), high density lipids (HDL), triglycerides (TG), free fatty acids (FFA) andratios of cholesterol to lipids were also calculated.[13] The values were tabulated and there was a significant reduction in all the lipids except HDL in extract and standard drug treated groups. Extracts showed dose dependant increase in HDL which is an indicator of good cardiovascular health of rats. The extracts at 400mg/kg showed a similar activity as standard. EEPC at 400mg/kg where TC was reduced to 80, LDL to 43 and TG to 132 given.

Table no.3: Effect of Plant extracts on the lipid profile of High fat diet fed rats.

Grou p s	TC (mg/L)	LDL (mg/L)	HDL (mg/L)	TG (mg/L)	FFA (mg/L)	TC/HD L ratio	LDL/HD L ratio
I	58.61±1.32	36.74±1.78	42.12±2.08	109.81±4.14	69.22±3.61	1.59±0.09	0.85±0.14
II	265.21±5.67	118.23±2.85	28.91±0.99	209.11±6.82	90.68±3.71	9.46±1.42	4.21±1.45
III	137.88±2.74	62.06±2.14	38.69±1.65	163.07±4.70	72.16±3.27	3.06±1.05	1.63±0.57
IV	80.42±2.09	43.74±3.05	43.03±1.07	132.68±7.90	70.56±5.70	2.66±0.67	1.01±0.62
V	65.87±2.77	39.09±1.56	42.87±2.82	116.82±6.71	70.11±4.65	1.54±0.19	0.92±0.85

The values are expressed as mean ± SD. significantly different a-*P*< 0.01 compared with the standard drug (group VII), and ∗-*P*< 0.001 compared with high fat diet fed rats (group II), I-Normal control-Normal pellet diet; II-High fat diet fed group; III- EEPC = Ethanol Extract of *Priva cordifolia*at 200mg/kg; IV-EEPC = Ethanol Extract of *Priva cordifolia* at 400mg/kg; V-Standard drug, Simvastatin.

In vitro Antioxidant Activity

i) DPPH Radical Scavenging Activity

The reactivity of EEPC was analyzed with DPPH, a stable free radical. As DPPH picks up one electron in the presence of a free radical scavenger, the absorptiondecreases and the resulting discoloration is stoichiometrically related to the number ofelectrons gained . The DPPH radical scavenging (%) activity is shown in the Fig 2. EEPC exhibited an inhibition of 71% and that of ascorbic acid was 79% at 500µg/ml and the IC50 of the extract was 222.79µg/ml, while that of ascorbic acid was 202.72µg/ml.

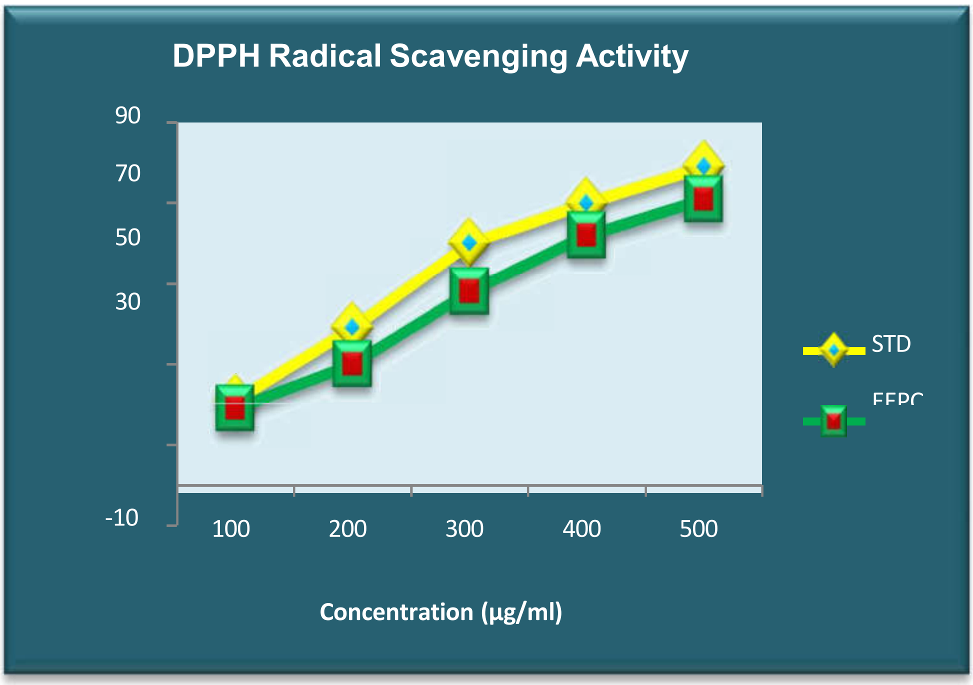

Fig 2. DPPH radical scavenging activity of EEPC

SUMMARY

In concise, the present work was an attempt to explore phytoconstituents of *Priva cordifolia* for protective effects in various preclinical models of atherosclerosis. These plants were selected based on reported traditionaluse and utility of the phytoconstituents present in related species in various disorders. However, no scientific data is reported on these plants, thus this provides us an opportunity for pharmacognostical standardization of the plants and stimulus for further scientific exploration of these plants.

The objective of this work includes pharmacognostic and pharmacological investigation of ***Priva cordifolia.***

Ethanol extract of *Priva cordifolia* revealed the presence of glycosides, flavonoids, phenols and triterpenoids as major phytoconstituents. High fat diet induced atherosclerosis is commonly used animal model to screen anti- atherosclerotic potential of the drug. The present study demonstrates the beneficial effect of EEPC in high fat diet induced atherosclerosis model. Atherogenic index of extracts was found to be very low compared to high fat diet fed rats. But interestingly, normal diet fed rats showed a little atherogenicity index which might be due to lack of exercise and sedentary life style of rats in cages. But extracts could get the atherogenicity index to almost similar to normal diet fed rats which can be again attributed to the involvement of chemical constituents in fatty acid or cholesterol synthesis. C-reactive protein (CRP) leads plasma LDL plasma concentration as a better cardiovascular risk marker and a better predictor of choice for atherosclerosis . As inflammation process is part of pathophysiology of atherosclerosis, it is believed that CRP has a role in the same and is estimated to evaluate the atherogenicity.

The major cause of Atherosclerosis is blood with high concentrations of cholesterol (TC), LDL (Low density lipids) and Triglycerides (TG) and very low concentration of HDL (High density lipids) in serum. The extract treated groups showed significant changes in HDL level when compared to the control, which indicates that the levels of HDL were brought back to more than normal after treatment with EEPC in rats fed with high fat diet. This indicates that the regular consumption of extract will help prevent in the atherosclerosis by improving the HLD. Several researches support the hypothesis that the oxidation of LDL will playa significant role in early stages of atherosclerosis, while thromboembolism is one of the brutal consequences of this disease. Results of the present study infer that the high level of LDL was significantly reduced in rats treated with EEPC. Flavonoids and polyphenols are reported to increase HDL concentration and decrease LDL, TG and cholesterol levels in high fat diet fed rats. The presence of flavonoids and polyphenols in EEPC might be the reasons for increasing HDL and decreasing other lipid levels in rats. The exogenous or endogenous molecules from natural or synthetic source, which inactivate the free radicals, are termed as free radical scavenging agent or antioxidants. In this study, the free radical scavenging potential of plant extracts were investigated by various *in vitro* assays like DPPH scavenging assay. The results from in-vitro assays demonstrate the crude extracts of ***Priva cordifolia*** posses the free radical scavenging and antioxidant effect.

CONCLUSIONS

The conclusions drawn from the results of various investigations performed and discussed in earlier chapters are as follows. For the present investigation the medicinal plant were selected based upon the scientifically unexplored traditional medicinal use, namely, *Priva cordifolia.* Preliminary phytochemical analysis, The phytochemical studies concluded that various extracts of *Priva cordifolia* are rich in Glycosides, Phenols, Flavonoids. *In-vivo* atherosclerotic activity, the extracts were tested in high fat diet induced atherosclerosis in Albino rat model. Total cholesterol (TC), low density lipids (LDL), high density lipids (HDL), triglycerides (TG), free fatty acids (FFA) and ratios of cholesterol to lipids were also calculated. The values were tabulated and there was a significant reduction in all the lipids except HDL in extract and standard drug treated groups. Extracts showed dose dependant increase in HDL which is an indicator of good cardiovascular health of rats. The extracts at

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