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Hepatoprotective activity of laticiferous plant species (*Pergularia daemia* and *Carissa carandas*) from Western Ghats, Tamilnadu, India

V.H. Bhaskar[1] and N. Balakrishnan[2*]

[1]M.P. Patel College of Pharmacy, Kapadwanj, Gujarat, India
*[*2]Department of Pharmacognosy, Technocrats Institute of Technology-Pharmacy, Bhopal(MP), India*

Abstract

The roots of *Pergularia daemia* and *Carissa carandas* have used to treat liver disease and jaundice among the herbal practitioners of Western Ghats Tamilnadu, India.The present study is to evaluate the hepatoprotective effects of the ethanol and aqueous extracts of roots of *Pergularia daemia* and *Carissa carandas* were against ethanol induced hepatotoxicity in rats. The hepatotoxic of ethanol and the hepatoprotective effects of ethanol and aqueous extracts of roots of *P. daemia* (PDEE & PDAE) and *C. carandas* (CCEE & CCAE) were estimated their liver function test, serum lipid profile, levels of lipid peroxidation and the activity of liver antioxidant enzyme glutathione. The PDEE, PDAE, CCEE and CCAE at a dose level of 100 mg/kg and 200 mg/kg produce significant hepatoprotection by decreasing serum transaminase (SGPT & SGOT), alkaline phosphate, bilirubin and lipid peroxidation, while significantly increased the levels of liver glutathione and serum protein. The effects of PDEE, PDAE, CCEE and CCAE were comparable with standard drug silymarin.

Key words: *Pergularia daemia*, *Carissa carandas*, Hepatoprotective, Silymarin, Ethanol

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Introduction

The liver is a versatile organ which is responsible for the metabolism of chemicals and for the regulation of internal chemical environment. Hepatotoxicity may be caused by thousands of synthetic chemicals, drugs, bacteria, fungi, plants and animal toxicants [1, 2]. These agents cause liver damage either by themselves or by getting converted to toxic metabolites. Herbal drugs play major role in the treatment of hepatic disorders. In India a number of medicinal plants and their formulations are widely used for the treatment of these disorders. Ethnopharmacological surveys conducted among herbal practitioners of Western Ghats Tamilnadu, India have revealed a large numbers of laticiferous plant species are used as a

source of herbal therapies. Accordingly we selected two medicinal plants including *P. daemia* and *C. carandas* used to treat liver disease and jaundice.

Pergularia daemia (Forsk.) Chiov. (Asclepiadaceae), known as "Veliparuthi" in Tamil, "Uttaravaruni" in Sanskrit and "Utranajutuka" in Hindi[3], has traditionally been used in the form of whole plant, as an anthelmintic, laxative, antipyretic, expectorant and also used to treat infantile diarrhea and malarial intermittent fever [4-6]. The aerial parts of this plant have been reported to exert various pharmacological activities, including hepatoprotection [7, 8], antifertility [9], anti-diabetic [10], analgesic, antipyretic, and anti-inflammatory [11]. The roots of this plant have been used as an emetic [12], while used to treat gonorrhea [13], asthma, and constipation [14]. Phytochemically the plant has been investigated for the presence of cardenolides, alkaloid, saponins [11] and steroidal compounds [15].

Carissa carandas (Apocynaceae) [3] is an indigenous evergreen shrub or small crooked tree up to 3 m in height with dichotomous branches; light green, elliptic or elliptic–oblong leaves; white or pink, faintly scented flowers in terminal corymbose cymes and ellipsoid; purple or pink and white, normally 8 seeds berries [4]. It is distributed to throughout India in dry, sandy and rocky grounds [6]. *C. carandas* considered being one of the most valuable drugs in various system of medicine and all the parts of this plant highly useful and among them root seems to be much useful. Traditionally it's used in the treatment of scabies, intestinal worms, pruritus, biliousness and used as antiscorbutic, anthelmintic [4, 6]. The various pharmacological activity were reported on this plant like analgesic, anti inflammatory [16], anti pyretic [17], cardiotonic [18], histamine releasing [19] and hepatoprotective [20]. The reported phytoconstituents were fixed oil, volatile oil, resin, alkaloid [6], triterpenoid [21], carissol ,carissic acid and ursolic acid [22, 23].

The previous paper studied the antioxidant properties of roots of *P. daemia* and *C. carandas* [24]. The present pharmacological investigation focus on evaluation of the efficacy of ethanol and aqueous extracts of roots of *P. daemia* and *C. carandas* for their protection against ethanol induced hepatotoxicity.

Materials and Methods

Plant Material

Roots of *P. daemia* and *C. carandas* were collected from Maruthamalai Hills, Coimbatore, India in the month of November 2006 and authenticated by a taxonomist Dr P. Jayaraman, Plant Anatomy research Centre, Chennai, India. The voucher specimen of *P. daemia* (PARC/2007/52) and *C. carandas* (PARC/2007/53) has been preserved in our laboratory for further collection and reference.

Preparation of extracts

Roots of *P. daemia* and *C. carandas* were dried under shade, powdered with a mechanical grinder and pass through sieve no 40. The sieved powder was stored in airtight container and keep in room temperature for the further study. The dried powdered material (500 g) extracted with 95 % ethanol using soxhlet apparatus for about 48 h. The aqueous extract was prepared by cold maceration (72 h.).The solvents were removed from the extracts under reduced pressure by using rotary vacuum evaporator.

Phytochemical Analysis

The PDEE, PDAE, CCEE and CCAE were subjected to identify the presence of various phytoconstituents viz. alkaloids (Dragendroffs test), steroids and terpenoids (Leibermann Burchard test), tannin and phenolic compounds (Ferric chloride test), flavonoids (Shinoda test), amino acids (Ninhydrin test), etc. by usual methods prescribed in standard texts [25, 26].

Animals

Wistar albino rats (150-200 g) used in the present studies was procured from listed suppliers of m/s Sri Venkateswara Enterprises, Bangalore, India. The animals were fed with standard pellet diet (Hindustan lever Ltd. Bangalore) and water *ad libitum*. All the animals were acclimatized for a week before use. The experimental protocols were approved by Institutional Animal ethics Committee after scrutinization (IAEC No. Pcog /16/2008). The animals were received the drug by oral gavages tube. All the animals were care of under ethical consideration as per the CPCSEA guidelines [27] with regular inspections of rats. The laboratory conditions duly undertaken by registered veterinary practitioner.

Chemicals

All the chemicals and solvents were of analytical grade and were procured from Ranbaxy Fine chemicals Ltd., Mumbai, India. The standard drug silymarin was obtained as gift sample from Micro Lbs, India. Standared kits for SGOT, SGPT, ALP and bilirubin were obtained from Span Diagnostics Ltd., India.

Acute toxicity studies

Healthy Wistar albino rats of either sex weighing 150-200 g maintained under standared laboratory conditions were used for acute oral toxicity test according to Organization for Economic Co-operation and Development guidelines 423 [28]. A total of three animals were used which received a single oral dose of (2000 mg/kg) of PDAE, PAEE CCAE and CCEE. Animals were kept overnight fasting prior to administration of PDAE, PDEE, CCAE and CCEE. After administration of extracts the food was withheld for further 3-4 h. Animals were observed individually at least once during first 30 min after dosing, periodically during first 24 h (with special attention during the first 4 h) and daily thereafter for period of 3 days. Observations were done daily for changes in skin and fur, eyes, mucus membrane (nasal), respiratory rate, circulatory signs (heart rate), autonomic effect (salivation, lacrimation, perspiration, urinary incontinence and defecation) and central nervous system (drowsiness, gait, tremors and convulsion) changes.

Evaluation of hepatotoxicity studies on ethanol induced hepatotoxicity rats

The rats were divided into 11 groups of 6 animals (n=6) in each [29].

Group I: Received water (5 ml/kg. p.o) for 21 days once daily, and served as normal control.

Group II: Received water (5 ml/kg. p.o) for 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.

Group III: Received standard drug silymarin (25 mg/kg. p.o.) for 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.

Group IV & V: Received PDAE (100 & 200 mg/kg) 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.

Group VI& VII: Received PDEE (100 & 200 mg/kg) 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.

Group VIII & IX: Received CCAE (100 & 200 mg/kg) 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.
Group X & XI: Received CCEE (100 & 200 mg/kg) 21 days once daily and 40% ethanol (v/v, 2.0 ml/l00 g body wt, p.o.) for 21 days.

Assessment of hepatotoxicity

After 24 h of ethanol administration, the blood was obtained from animals by puncturing retro orbital plexus. The blood samples were allowed to clot for 45 min at room temperature. The serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters including SGOT & SGPT [30], ALP [31], serum bilirubin [32], serum protein [33] and serum cholesterol [34].
After collection of blood samples, the animals were sacrificed under deep ether anesthesia and their livers were excised immediately and washed with ice cold saline and a 10% homogenate prepared in phosphate buffer (pH 7.0). The homogenate was centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was used for the estimation glutathione [35] and lipid per oxidation [36].
Morphological parameters like weight of the animals, weight of the liver have also been used to evaluate the protective effect of the drug. Hepatotoxicity causes loss in liver weight/100 gm body weight of rats [37, 38].

Histopathology studies

A portion of the liver tissue of all the animal groups was excised and was then washed with normal saline. The liver tissues were fixed in 10% buffered neutral formalin for 48h and then with bovine solution for 6 h and were then processed for paraffin embedding. By using a microtome, sections of 5 mm thickness were taken and stained with hematoxylin and eosin. These sections were examined under light microscope using a magnification of 100X [39].

Statistical Significance

The results of the study were expressed as mean ± SEM, n=6. ANOVA [40] was used to analyze and compare the data, followed by Dunnet's [41] test for multiple comparisons.

Results

Acute toxicity study

There was no mortality amongst the graded dose groups of animals and they did not show any toxicity or behavioral changes at a dose level of 2000 mg/kg. This finding suggests that the PDEE, PDAE, CCEE and CCAE were safe in or non-toxic to rats and hence doses of 100 mg/kg and 200 mg/kg, po were selected for the study.

Phytochemical screening

Preliminary phytochemical investigation of the CCEE, CCAE, PDEE and PDAE showed various phytochemicals including alkaloids, glycosides, steroids, flavonoids, saponin, tannin & phenolic compounds, terpenoids, carbohydrates, gums and mucilage.

Effect of CCEE, CCAE, PDEE and PDAE on serum marker enzyme levels

There was a significant elevation in the levels of serum marker enzymes like SGOT, SGPT and ALP content of ethanol intoxicated animals. In contrast, pre treatment with CCEE, CCAE, PDEE and PDAE (100 and 200 mg/kg, po) and silymarin(25 mg/kg, po) exhibited an ability to counteract the hepatotoxicty by decreasing serum marker enzymes.

Effect of CCEE, CCAE, PDEE and PDAE on biochemical parameters

In ethanol treated groups, there was a significant increase in total bilurubin and significant reduction in total protein content. Whereas, pre-treatment with CCEE, CCAE, PDEE and PDAE (100 and 200 mg/kg, po) caused significant reduction in total bilirubin and significant increase in total protein.

Effect of CCEE, CCAE, PDEE and PDAE on antioxidant activity

There was significant increase in MDA content and reduction in GSH of intoxicated animals. Pre-treatment with CCEE, CCAE, PDEE and PDAE (100 and 200 mg/kg, po) and silymarin (25 mg/kg, po) significantly prevented the increase in MDA levels, whereas GSH significantly increased.

Effect of CCEE, CCAE, PDEE and PDAE on liver weight

Ethanol intoxicated group of animals, the weight of the liver was significantly increased, but it was normalized in CCEE, CCAE, PDEE and PDAE (100 and 200 mg/kg, po) treated groups of animals. A significant reduction in liver supports this finding.

Histopathology

Histopathological studies also provided a supportive evidence for biochemical analysis. Histological changes such as steatosis (fatty changes in hepatocytes) and perivenular fibrosis were observed in ethanol treated (toxic) control group. Both the plant extracts were prevented these histological changes.

Discussion and conclusion

The liver can be injured by many chemicals and drugs. In the present study ethanol was selected as a hepatotoxicant to induce liver damage, since it is clinically relevant. Ethanol produces a constellation of dose related deleterious effects in the liver [42]. The majority of ethanol is metabolized in the liver and individuals who abuse alcohol by routinely drinking 50-60 g (about 4 to 5 drinks) of ethanol per day are at risk for developing alcoholic liver disease [43]. Alcohol induced liver damage (ALD) is due to increased lipid peroxidation, impaired antioxidant status, the appearance of free radical adducts derived from fatty acid breakdown and CYP2E1 dependent ethanol metabolism to the 1-hydroxyethyl radical have all been shown to correlate with the development of pathology. In addition, both acute and chronic ethanol administration cause enhanced formation of cytokines, especially TNF-alpha by hepatic Kupffer cells, which have a significant role in liver injury [44-46]. Besides the development of fatty liver (steatosis), another early sign of excessive ethanol consumption is liver enlargement and protein accumulation, both of which are common findings in alcoholics and heavy drinkers [47, 48].

Elevated levels of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) are indications of hepatocellular injury [49]. In the present study PDEE, PDAE, CCEE and CCAE at a dose of 100 mg/kg and 200 mg/kg, po caused a significant inhibition in the levels of SGOT and SGPT towards the respective normal range and this is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by ethanol. On the other hand suppression of elevated ALP activities with concurrent depletion of raised bilirubin level and an increase in the total plasma protein content suggests the stability of biliary dysfunction in rat liver during hepatic injuries with toxicants[50].

Table 1. Effect of CCAE and CAEE on serum enzyme and biochemical parameters in ethanol induced hepatotoxicity in rats

Group/ Dose (mg/kg, po.)	SGOT(U/L)	SGPT(U/L)	ALP(U/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Total Protein (mg/dl)	Serum Cholesterol (mg/dl)
Normal control	168.04 ± 2.80	58.6 ± 2.26	186.0 ± 8.4	0.38 ± 0.06	0.20 ± 0.08	9.57±0.24	8.64±1.51
Toxic control	280.64±8.24*	120.18±7.24*	280.42±6.46*	9.22±4.24*	6.62±0.28*	6.42± 2.46*	22.18±6.44*
Standard (25)	176.16±8.16***	64.08±6.18***	198.20±8.28***	0.54±1.24***	0.32±0.42***	9.24±8.24***	10.18±4.18***
CCAE(100)	204.18±8.24**	80.18±7.24**	208.18±6.28**	0.68±8.42**	0.46±0.82**	7.22± 4.12**	18.42±4.88**
CCAE(200)	192.42±7.24***	72.18±8.26***	204.74±8.52***	0.61±7.66***	0.38±0.44***	7.28±8.42***	16.40±4.28***
CCEE(100)	196.84±8.66**	76.44±9.48**	206.48±8.26**	0.64±8.86**	0.40±0.80**	8.26±9.24**	19.45±4.82**
CCEE(200)	186.28±9.24***	68.24±7.24***	200.18±8.46***	0.58±2.66***	0.36±0.82***	9.16±6.42***	14.22±4.24***

Table 2. Effect of CCAE and CAEE on liver weight, glutathione (GSH) and lipid peroxidation (LPO) in ethanol induced hepatotoxicity in rats

Group/ Dose (mg/kg, po.)	Liver weight (Wt/ 100 g bw.)	Liver homogenate	
		GSH (µmole GSH/g liver tissue)	LPO (µmole MDA/g protein)
Normal control	6.80 ±0.08	9.74±1.19	45.30±8.31
Toxic control	9.12±4.86*	5.88±9.24*	72.82±9.28*
Standard (25)	7.02±2.42***	8.46±8.24***	52.08±8.26***
CCAE(100)	8.28±4.26**	6.84±7.28**	67.24±6.88**
CCAE(200)	8.12±4.88***	7.28±6.82***	67.88±7.28***
CCEE(100)	8.52±4.24**	7.88±8.24**	58.28±6.44**
CCEE(200)	7.46±4.24***	8.80±8.42***	56.24±8.28***

Values are mean ±SEM, n= 6. (One way ANOVA Followed by Dunnet's multiple Comparisons test). *, **, *** denotes statistically significance of P<0.05, P<0.01, P<0.001 compared with standard group.

Table 3. Effect of PDAE and PDEE on serum enzyme and biochemical parameters in ethanol induced hepatotoxicity in rats

Group/ Dose (mg/kg, po.)	SGOT(U/L)	SGPT(U/L)	ALP(U/L)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	Total Protein (mg/dl)	Serum Cholesterol (mg/dl)
Normal control	168.04 ± 2.80	58.6 ± 2.26	186.0 ± 8.4	0.38 ± 0.06	0.20 ± 0.08	9.57±0.24	8.64±1.51
Toxic control	258.42±4.86	88.74±8.26	244.76±8.42	6.44±8.66	2.28±0.48	5.42±8.26	32.00±2.86
Standard (25)	176.28±8.44***	62.76±4.26***	194.28±4.24***	0.42±2.88***	0.42±0.68***	9.82±4.86***	10.84±2.84***
PDAE(100)	188.28±4.28**	69.84±8.22**	200.46±8.22**	0.58±4.62**	0.49±0.98**	8.28±8.48**	16.28±9.48**
PDAE(200)	184.84±4.26***	68.28±6.88***	198.46±8.44***	0.56±2.20***	0.48±0.92***	6.28±8.24***	14.48±8.62***
PDEE(100)	192.86±7.24**	70.42±6.88**	202.46±8.48**	0.58±4.58**	0.50±0.28**	8.18±9.44**	17.86±9.44**
PDEE(200)	180.20±4.24***	66.82±5.24***	196.42±8.28***	0.52±0.48***	0.44±0.86***	9.44±8.66***	12.28±4.66***

Values are mean ±SEM, n= 6. (One way ANOVA Followed by Dunnet's multiple Comparisons test). *, **, *** denotes statistically significance of P<0.05, P<0.01, P<0.001 compared with standard group.

Table 4. Effect of PDAE and PDEE on liver weight, glutathione (GSH) and lipid peroxidation (LPO) in ethanol induced hepatotoxicity in rats

Group/ Dose (mg/kg, po.)	Liver weight (Wt/ 100 g bw.)	Liver homogenate	
		GSH (µmole GSH/g liver tissue)	LPO (µmole MDA/g protein)
Normal control	6.80 ±0.08	9.74±1.19	45.30±8.31
Toxic control	8.24±0.24	3.92±0.28	96.48±8.44
Standard	7.02±0.46***	8.84±0.42***	50.28±9.42***
Lower dose I	8.00±2.42**	7.12±0.64**	58.46±8.26**
Higher dose II	7.20±0.86***	7.42±0.22***	54.96±8.94***
Lower dose I	7.96±0.88**	6.98±0.46**	58.94±9.46**
Higher dose II	7.18±0.82***	7.88±0.68***	52.84±8.64***

Values are mean ±SEM, n= 6. (One way ANOVA Followed by Dunnet's multiple Comparisons test). *, **, *** denotes statistically significance of $P<0.05$, $P<0.01$, $P<0.001$ compared with standard group.

These results indicate that PDEE, PDAE, CCEE and CCAE preserved the structural integrity of the hepatocelluar membrane and liver cell architecture damaged by ethanol which was confirmed by histopathological examination.

Intake of alcohol results in excessive generation of free radicals [51].Free radicals are reactive oxygen species (ROS) are known to cause oxidative damage to number of molecules in cell, including membrane lipids, proteins and nucleic acids. The hepatic cellular injury in ethanol administered animals might be due to increased oxidative stress leading to lipid peroxidation, cell membrane damage and thereby leakage of the cellular enzymes [52, 53].

The thiobarbituric acid assay is the most popular method of estimation of malondialdelyde level, which is an indication of lipid peroxidation and free radical activity. The increase in lipid peroxidation, a degradative process of membraneous polyunsaturated fatty acid has been suggested by the increase in malondialdelyde in ethanol induced toxicity in the liver. The increased lipid peroxidation results in changes in cellular metabolism of the hepatic and extra hepatic tissues, which ultimately leads to the whole cell deformity and cell death [54]. The levels of TBARS in liver tissues of ethanol intoxicated rats were significantly elevated when compared to the level of TBARS in control animals. The administration of PDEE, PDAE, CCEE and CCAE at a dose level of 100 mg/kg and 200 mg/kg showed maximum reduction in TBARS level. The standard hepatoprotective drug Silymarin maintained the decreased lipid peroxidation level to the normal limits in the liver. The results indicate that, the herbal drug *P. daemia* and *C. carandas* have very good hepatoprotective effect in liver damage.

Glutathione Reductase (GSH) is an important source of reducing equivalents during oxidative stress generated by reactive oxygen species. The higher level of ethanol intake develops cirrhosis and liver damage by enhancing lipid peroxidation in the liver. Acetaldehyde the toxic metabolite of ethanol depresses the liver and plasma glutathione level by conjugating with the sulphydryl groups of glutathione [55]. In the present research work, we have observed the decreased level of glutathione in ethanol intoxicated rats. The GSH depletion in hepatic mitochondria is considered the most important sensitizing mechanism in the pathogenesis of alcoholic liver injury. Treatment with PDEE, PDAE, CCEE and CCAE at a dose level of 100 mg/kg and 200 mg/kg significantly improved the level of glutathione both in lever tissues. Similar results also observed with the standard drug Silymarin.

Antioxidant exhibit hepatoprotective activity by blocking the conversion of ethanol to acetaldehyde [56].From the previous studies it was found that PDEE, PDAE, CCEE and CCAE exhibited antioxidant property [24] which may be responsible the hepatoprotective activity of these two drugs.

Also phytochemical reports revealed that the PDEE, PDAE, CCEE and CCAE were found to contain higher concentration of flavonoids and phenolic compounds as well as preliminary phytochemical screening of these extracts showed positive repots of phyoconstituents like flvonoids, terpenoids and glycosides [24]. However, flvonoids [57], terpenoids [58], saponins [59] and glycosides [60] are known to possess hepatoprotective activities in animals.

It can be concluded that PDEE, PDAE, CCEE and CCAE were possess a protective effect against ethanol induced hepatotoxicity in rats, as evidenced by the biochemical and histological parameters. The hepatoprotective effect was more with ethanol extracts of *P. daemia* and *C. carandas* then the aqueous extracts of *P. daemia* and *C. carandas*. Further

studies are in progress for the isolation and characterization of active principle(s) from PDEE, PDAE, CCEE and CCAE for this activity.

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