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Preliminary phytochemical screening and Evaluation of hepatoprotective activity of ethanolic extract of whole plant of *Evolvulus alsinoides* using CCl_4 induced model in experimental animals

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Abstract

Present study was conducted to evaluate the preliminary phytochemical screening and hepatoprotective activity of whole plant of *Evolvulus alsinoides*. The whole plant was defatted with petroleum ether and then extracted with 90 % ethanol. The phytochemical screening was done for ethanol extract using standard procedures. Acute toxicity was done using OECD 423 guidelines and the extract was found to be practically non-toxic upto a dose of 1500mg/kg b.w. when given orally. Hepatoprotective activity was evaluated using Carbon tetra chloride induced model in rats. In CCl_4 induced mothod of 90% Ethanolic extract of whole plant of *Evolvulus alsinoides* (75 & 150mg/kg b.w.) produced significant dose dependent hepatoprotective acti vity. Histopathological studies could be carried out to assess the degree of damage. The hepatoprotective effect of the aforesaid extract was substantiated by pentobarbital sleeping time experiment in mice. The effect of the extracts at 150 mg/kg was compared to that of the reference drug, Silymarin (50 mg/kg). *In vitro* antioxidant studies were conducted to confirm the antioxidant mechanism involved in their hepatoprotective activity in CCl_4 -induced in rats. The presence of flavonoids could be responsible for hepatoprotective activity.

Keywords: Evolvulus alsinoides; phytochemmical screening; hepatoprotective; flavonoids; antioxidnant

Introduction

Hepatotoxicity is defined as injury to the liver that is associated with impaired liver function caused by exposure to a drug or another non-infectious agent. Hepatotoxic agents can react with the basic cellular components and consequently induce almost all types of liver lesions. Toxins and drugs are among the basic etiopathogenetic agents of acute liver failure in Western countries. Nevertheless, chemical toxins (including acetaminophen, carbon tetrachloride, galactosamine and thioacetamide) are often used as the model substances causing experimental hepatocyte injury in both in vivo and in vitro conditions. Despite the fact that hepatic problems are responsible for a significant number of liver transplantations and deaths recorded worldwide, available pharmacotherapeutic options for liver diseases are very limited and there is a great demand for the development of new effective drugs [1].

Herbal-based therapeutics for liver disorders has been in use in India for a long time and has been popularized world over by leading pharmaceuticals. Despite the significant popularity of several herbal medicines in general, and for liver diseases in particular, they are still unacceptable treatment modalities for liver diseases. The use of natural remedies for the treatment of liver

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diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines [2].

The curative properties of medicinal plants are mainly due to the presence of various complex chemical substances of different composition, which occur as secondary metabolites. They are grouped as alkaloids, glycosides, flavonoids, saponins, tannins; carbohydrates & essential oils. Any part of the plant may contain active components. The medicinal action of plants are unique to particular plant species or groups of plants and are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct [3].

Abundant natural substances, inorganic chemicals which are absorbed through intestinal tract, nasal route or by parenteral route are metabolized by liver. Cytochrome P450 reductase enzyme system which is present in the smooth endoplasmic reticulum of liver facilitates the metabolism of diverse substances. Over dosage or excess use of the drugs, chemical agents, microcystins etc generally referred as hepatotoxins which cause hepatotoxicity. Hepatotoxicity is the main reason for most of the drugs to be withdrawn from market [4]. Some of the pharmaceutical, chemical products and the waste materials are hepatotoxins which enter into the human body. One of the potent hepatotoxic agents is CCl_4, when it is ingested it gets circulated to all organs because of its high lipid solubility. On ingestion of toxic doses it causes blockage in the lipoprotein synthesis and produces fat accumulation in the liver because these lipoproteins carry the triglycerides away from this organ. The synthesis of proteins is reduced and there is a rapid decline in the levels of cytochrome P450 as well as glucose 6-phosphatase and the sequestration of Ca^{2+} ions by Ca^{2+} ATPase is reduced by the endoplasmic reticulum, as a result there is an increased intracellular Ca^{2+} concentration [5].

A number of studies have shown that the plant extracts having antioxidant activity protect against CCl_4 hepatotoxicity by inhibiting lipid peroxidation and enhancing antioxidant enzyme activity [1].

Evolvulus alsinoides (Family Convolvulaceae) is a herb from Ayurveda for the treatment of fever, cough, and cold as well as for venereal diseases, azoospermia, asthma and bronchitis [6, 7], adenitis and dementia and liver problems [8, 9]. This plant is reported to have a nootropic and psychotropic effect [10], topical application is said to promote hair growth, and is also referred to as Drawf morning glory. It is used commonly in modern days to promote vitality and memory similar to Ashwagandha, Bacopa monnieri, and Jatamansi [10] and is reported to be somewhat tasteless. However, there is no scientific report or verification of the use of this plant in the treatment of these conditions. Evolvulus alsinoides (EEEA) has been initiated in our laboratory and here we report the preliminary result of studies on acute toxicity and hepatoprotective effects on experimental models.

Materials and methods

Plant Material

The whole plants of *Evolvulus alsinoides* were collected in Tirupathi, Andhra Pradesh, India, and authenticated by the renowed botanist from the Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. A voucher specimen (No. SHM 01/12) has been preserved in our laboratory for future reference. The plant material were dried under shade and then powdered with a mechanical grinder and stored in airtight container. The dried powder material of the whole plant was defatted with petroleum ether and the marc thus obtained was then extracted with ethanol in a soxhlet apparatus. The solvent was completely removed under reduced pressure and a semisolid mass was obtained (EEEA, yield 15.67 %). The dried EEEA was suspended in normal saline and used for the present study.

Phytochemical Analysis

The crude ethanolic extract of herbal plant was subjected to the following chemical tests for the identification of various active constituents like alkaloids, carbohydrates, steroids, glycosides, saponins, flavonoids, tannins, phenolic compounds, triterpenoids, proteins, aminoacids and fixed oils [11–16].

Chemicals

Silymarin was used as a standard hepatoprotective agent and was obtained as a kind gift from Micro Labs., Ltd., Hosur (Bangalore), India; Methanol, Carbon tetrachloride, Ethanol, Ferric chloride, Ferrozine, Tween-80 were obtained from S.D Fine Chemicals, Mumbai; Olive oil is obtained from Seven Ships, Hyderabad and Normal Phase Pre-coated Chromatographic Plates were purchased from Merck, Germany. Analytical kits Serum Glutamic Oxaloacetate Transaminase (SGPT), Serum Glutamic Pyruvic Transaminase (SGOT), Alkaline phosphate (ALP) Total Bilirubin (TBL), Total protein (TPL) and Albumin (ALB) by manual methods were purchased from Span, Diagnostics Ltd., Surat, India. The biochemical analytical kits for colorimeter were purchased from Merck Specialties Private Limited, Mumbai, India. All other chemicals and solvents used were of analytical grade.

Experimental animals

Healthy Wistar A lbino rats, weighing 200-220g were procured from the Teena Biolabs Pvt. Ltd. (Reg, no. 177/99 CPCSEA),

Hyderabad, Andhra pradesh. Animals were housed at CPCSEA approved animal house of Vaagdevi Institute o f Pharmaceutical Sciences, (1533/PO/a/11/CPCSEA) Warangal. The animals were kept under standard laboratory condition (12 hr light and 12 hr dark cycle) and had free access to commercial pellet diet (Vyas labs Ltd, Hyderabad, India) with water *ad libitum.*The animal house temperature was maintained at 25 ± 2^0 C with relative humidity at (50 $\pm$15%). The study was approved by the Institutional Animal Ethical Committee of Vaagdevi Institute of Pharmaceutical Sciences, (14/03/2012). Ethical norms were strictly followed during all experimental procedure.

Acute Oral Toxicity Study

The acute oral toxicity procedure was followed by using OECD 423 guidelines. The acute toxic class method is a stepwise procedure with 6 animals of a single sex per step [17]. The different doses of EEEA (5, 50, 300, 2000 mg/kg body weight) were administered orally to the rats. After the oral administration of test, animals were observed individually at least once during the first 30 minutes and periodically during the first 24 hours, with special attention given during the first 4 hours and daily thereafter, for total of 14 days.

Hepatoprotective Activity: In the present study, all animals were kept on same diet for 7 days. Seven days after acclimatization, the rats were divided into five groups, each group consisting of six animals. Group-I served as a control and received 1ml/kg of 1 %w/v tween-80 in distilled water p.o. for seven days. Group-II t reated with vehicle (1 ml/kg of 1% w/v tween-80 in distilled water p.o.) daily for seven days followed by CCl_4 on the seventh day. The Group-III animals were administered with 50 mg/kg of standard drug Silymarin p.o. for seven days followed by CCl_4 administration. Group IV- V test groups were treated in the similar way using ethanolic extract of *Evolvulus alsinoides* (EEEA) 75, 150 mg/kg, respectively followed by CCl_4 administered p.o on the seventh day [18].

All the rats were anaesthetized with thiopental sodium (60 mg/kg intraperitoneally), 36 hrs after administration of CCl_4, blood was collected from the retro-orbital plexus of rats, by inserting a fine capillary gently in the inner angle of the eye. The capillary is slided under the eye ball at 45^0 angle and over the bone socket to rupture the fragile venous capillaries of the ophthalmic venous plexus. The passage is about 10 mm. The tip of the capillary is slightly retracted and the blood was collected in a tube from orbital cavity with the capillary. After collecting the desired volume, capillary is removed with simultaneous release of pressure by fore finger and thumb. Any residual blood droplet around the eyeball is wiped off by dry cotton wool. After blood collection, the blood samples were allowed to coagulate at room temperature for at least one hour. Serum was separated by centrifugation at 3000 rpm for 30 minutes and then analyzed for TB, SGPT, SGOT, ALP, TP and ALB levels [19–22]. The animals were then dissected, the livers were carefully removed, washed with 0.9% saline solution and preserved in formalin solution (10% formaldehyde) for histopathological studies.

Determination of sleeping time: Pentobarbitone-induced sleeping time was carried out in Swiss albino mice. A 50% v/v CCl_4 in olive oil at a dose of 1.5 ml/kg/p.o was used as the toxic substance for inducing liver damage.

Group-I served as a control and received 1 ml/kg of 1% TWEEN-80 in distilled water p.o. for seven days. Group-II served as a toxic control and were given 1% TWEEN-80 for seven days followed by 1.5 ml/kg of CCl_4 (50% v/v CCl_4 in olive oil) only on the 7^{th} day p.o. The Group-III (Standard) animals were administered with 50 mg/kg of Silymarin p.o. for seven days followed by 1.5 ml/kg of CCl_4 (50% v/v CCl_4 in olive oil) only on the 7^{th} day p.o. Groups IV, V were treated in the similar way to that of Group III (Standard) using ethanolic extract of *Evolvulus alsinoides* (EEEA) 75 mg/kg, 150 mg/kg a respectively.

All the various groups of animals were given Pentobarbitone 60 mg/kg i. p. 2 hrs after administration of CCl_4. The time between loss of righting reflex and its recovery was recorded [23].

Histopathological Studies: After the animals were sacrificed, livers were taken out and washed with normal saline (0.9%). Then, 2-3 pieces of approximately 6 mm^3 size were cut and fixed in phosphate buffered 10% formaldehyde solution. After embedding in paraffin wax, thin sections of 5 mm thickness of liver tissue were cut and stained with haemotoxylin-eosin stain.

In-vitro antioxidant studies:

DPPH Free Radical Scavenging Assay: The antioxidant activity was determined on the basis of a free radical scavenging activity of stable 1,1-Di Phenyl-1, 2-picryl hydrazyl (DPPH) radical. 0.5 ml of the different concentrations of the extract was mixed with 2 ml of DPPH solution, 0.5 ml of TRIS Buffer and 2 ml of methanol. Ascorbic acid was used as a standard and prepared in a manner similar to the test. All the samples were mixed thouroughly and allowed to stand in a dark for 30 min. T he absorbance was measures at 517 nm using methanol as a blank. All determinations were made in triplicate and t he percentage inhibition was calculated using the following formula [24] .

% Inhibition = (Control – Test)/ Control X 100

Metal chelating activity: The chelation of ferrous ions by extracts was estimated by method of Dinis *et al*., 1994 [25].

Briefly, 50 μl of 2 mM $FeCl_2$ was added to 1 ml of different concentrations of the extract (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. All determinations were made in triplicate and t he percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula

% Activity = [(A0- As)/ As] X100

where A0 was the absorbance of the control, and As was the absorbance of the extract/ standard. Na_2 EDTA was used as positive control.

Results and discussion

Powder of dried whole plant *Evolvulus alsinoides* was extracted and a total of 70gm of extract having black color was obtained. The percentage yield of the plant was found to be 15.67%. The phytochemical investigation of the crude extract revealed the presence of flavonoids, carbohydrates, triterpenoides, glycosides, proteins, alkaloids, amino acids, tannins and phenolics and absence of fixed oils and saponins.

Acute toxicity

The ethanolic extract of whole plant of *Evolvulus alsinoides* was found to be safe at the maximum tested dose of 1500mg/kg body weight by oral route. After 24 hours animals was found well tolerated. There was no mortality and no signs of toxicity. A general behavioural, neurological and autonomic profile was found to be normal and all the concentrations of extracts were found to be safe. Thus $1/10^{th}$ of the maximum tolerated dose i.e; 150 mg/kg was taken as effective dose. The study was conducted using two doses i.e; 75mg/kg b.w p.o. and 150mg/kg b.w p.o.

Hepatoprotective activity

In recent years, many studies have been undertaken with indigenous medicinal plants in an attempt to develop safe and effective herbal drug for the treatment of liver disorders. In the present study, the mechanistically hepatoprotective model viz. CCl_4, was used for liver damage induction, to investigate whether the selected plant extract could decrease efficiently the toxicity produced by the hepatotoxicants.

The results of the present study demonstrate that the various biochemical changes produced in serum as well as liver and histological changes of liver by CCl_4 toxicity were prevented or reversed by administration of EEEA (Table 1).

The CCl_4 administration to rats leads to marked elevation in the levels of serum ALT, AST and ALP. This might be due to the release of these enzymes from the cytoplasm of hepatic cells, into the blood circulation rapidly after rupture of the plasma membrane and cellular damage [26], resulting from the CCl_4 -induced lipid peroxidation [27]. Treatment with EEEA at 75 and 150 mg/kg b.w.p.o. significantly reduced the level of these marker enzymes in CCl_4 treated rats in hepatoprotective study. The decrease in the levels of these enzymes may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by CCl_4 (Table 1) [28]**.**

In CCl_4 induced hepatotoxicity, elevated serum TB level is due to defective excretion of bile by the liver indicates the loss of integrity of the liver and necrosis. This leads to increase in the binding, conjugating and excretory capacity of hepatocytes, which is proportional to the erythrocyte degeneration rate [29]**.** At both the test doses EEEA showed a significant depletion in the serum bilirubin level suggesting the possibility of the extracts ability to stabilize biliary dysfunction of rat liver during injury with CCl_4 in prophylactic and curative studies.

In CCl_4 hepatotoxicity, a depression in total protein occurs due to the disruption and dissociation of polyribosome on endoplasmic reticulum leading to defective protein biosynthesis [30, 31]**.** In hepatoprotective study, EEEA at 75 and 150 mg/kg doses, increased the serum TP and ALB levels with varying degree of significance. This may be due to the promotion of the assembly of ribosomes on endoplasmic reticulum to facilitate uninterrupted protein biosynthesis. The overall observation made on the biochemical parameters revealed that the extract have better hepatoprotective activity with the higher dose i.e. 150 mg/kg b.w.p.o., comparable to that of the reference drug, Silymarin (50 mg/kg).

The histopathological profile of the liver of CCl_4 administered rats revealed drastic alterations in histoarchitecture showing centrilobular necrosis, fatty changes, broad infiltration of lymphocytes and vacuolization. In hepatoprotective studies; EEEA at both test doses showed definite signs of protection and recovery against CCl_4 injury respectively. The effects of the extracts were proportionate with the doses.

The liver is made up of hepatocytes and specialized cells called Kupffer cells interspersed with sinusoids. It is supplied with branches of bile duct. The normal hepatocytes have intact plasma membrane while in the event of viral infection/disease state or when drugs or chemicals affect liver cells, there will be changes in the permeability of plasma membrane and disruption of cells is caused by excessive formation of fibrotic tissue eventually leading to necrosis.

Table 1 Hepatoprotective activity of ethanolic extract leavesof EvolvulusAlsinoides (EEEA) on different biochemical parameters in CCl_4 induced liver damage in rats.

Groups	SGPT (U/ml)	SGOT (U/ml)	ALP (U/L)	TB (mg/dL)	TP (gm/dL)	ALB (gm/dL)	CHOL (mg/dL)
Normal	43.25±5.46	48.6±3.61	18.66±1.51	2.41±1.06	8.75±0.29	2.58±0.22	79.3±4.87
Toxic Control (CCl_4)	152.4±12.31	131.7±12.76	78.59±8.15	7.25±0.37	2.55±0.14	1.11±0.06	148.3±3.19
Standard (Silymarin)	69.75±3.39***	75.23±5.09***	43.08±2.41***	2.75±0.29***	7.09±0.09***	2.45±0.19***	99.74±2.39***
EEEA 75	107.8±10.56**	107.9±12.7	60.31±4.52*	5.09±0.05*	3.92±0.44*	1.7±0.05*	132.9±3.07*
EEEA 150	87.07±5.84***	85.20±5.57***	50.99±3.81***	3.63±0.26***	5.95±0.4***	2.2±0.11***	108.4±3.999***

V alues expressed as Mean ± SEM (n=6). Significant*(P <0.05), ** (P<0.01), *** (P<0.001) compared with standard and toxic group.

Histopathological studies could be carried out to assess the degree of damage. This is done by staining the fine section of liver isolates and examining under the microscope.It is evident from the results that the biochemical and histopathological observations of the studies were complementing each other. This confirms that ethanolic extract of whole plant *E. alsinoides* (EEEA), have hepatoprotective effect in CCl_4– induced hepatotoxicity in rats by their ability to stabilize cell membranes, scavenge free radicals and antioxidant properties.

The hepatoprotective effect of the aforesaid extracts was substantiated by pentobarbital sleeping time experiment in mice. The duration of pentobarbital-induced sleep in intact animals is considered as a reliable index for the activity of hepatic microsomal drug metabolizing enzymes (MDME) [32]. The pentobarbital is metabolized by hepatic MDME to inactive metabolites and any drug with an inhibitory effect on MDME is expected to prolong pentobarbital-induced sleep time. The damage conferred by CCl_4 on hepatocytes as well as on the hepatic MDME causes a loss of drug metabolizing capacity of the liver, resulting in prolongation of pentobarbital induced sleep time [33]. This indicates the hepatoprotective potential of the test extracts against CCl_4 -induced damage to hepatocytes including MDME. The effect of the extracts at 150 mg/kg was compared to that of the reference drug, Silymarin (50 mg/kg) (Table 2).

Table 2 Effect of EEEA on pentobarbitone induced sleeping time in CCl_4 intoxicated rats

Group	Dose (mg/kg)	Sleeping time in min
Normal	..	61.33
Toxic control (CCl^4)	..	136.59
Standard (Silymarin)	50	78.18
EEEA 75	75	91.73
EEEA 150	150	88.68

In vitro antioxidant studies were conducted to confirm the antioxidant mechanism involved in their hepatoprotective activity in CCl_4 -induced in rats. It has been shown that protective agents exert their action against CCl_4 -induced liver injury by impairment of CCl_4 mediated lipid peroxidation, either through decreased production of free radical derivatives [34] or due to the antioxidant activity of the protective agent itself [35] **.** In DPPH method, test extract exhibited a concentration dependent DPPH radical scavenging activity. The maximal activity was found at the highest concentration used i.e., 1000 µg/ml. *In vitro* ion chelating study also supported the free radical scavenging activity of the test extract. It exhibited a concentration dependent protection against ion chelation as evident from the ameliorated MDA levels in liver microsomes. The results suggest that the hepatoprotective activity of the ethanolic extract may be due to the inhibition of the ion chelation resulting in the enhancement of the resistance of the hepatocyte membrane to CCl_4 -mediated injury. Hence, the bioactive compounds (EEEA) were found to possess antioxidant properties. The results of the study substantiate the role of antioxidant properties of the ethanolic extract in showing their hepatoprotective effect in CCl_4 -induced hepatotoxicity in rats (Table 3 and 4).

Table 3 DPPH radical scavenging activity of EEEA

Concentration (µg)	% Inhibition
Control	..
5	6.31
10	13.91
15	35.2
20	45.15
25	61.52
30	69.36
Ascorbic acid (25µg)	84.56

Table 4 Metal chelating activity of EEEA

Concentration (µg)	% Inhibition
Control	..
20	16.83
40	23.66
80	42.44
160	68.05
320	70.45
Na_2 EDTA (50µg)	79.27

It is well known that plants or their extracts contain a number of chemicals belonging to the different classes, most of them are of pharmacological importance such as flavonoids, steroids,

triterpenoids and their glycosides, etc. Hence, the extract was subjected to preliminary chemical analysis to identify the classes of compounds present in them. The results of the study revealed the presence of carbohydrates, flavonoidal glycosides and steroidal/triterpenoidal compounds in the compound (EEEA). It is well documented that flavonoids are strong antioxidants and posses hepatoprotective activity, eg. quercetin, ascorbic acid and rutin [36]. Further, the literature reveals that the plants containing steroids and triterpenoids can control liver diseases [37]. Therefore, the hepatoprotective activity of the bioactive extract may be attributed to the above classes of compounds present in the respective bioactive extract.

From the overall results, it can be concluded that the indigenous medicinal plant selected for the investigation viz. *Evolvulus alsinoides* possess hepatoprotective activity. The ethanolic extract of this plant (EEEA) exhibited hepatoprotective activity against CCl_4 induced liver injury in rats. The mechanism of action behind the hepatoprotective activity of the aforesaid extracts may be multifactorial. It could be due to; (1) prevention of the process of lipid peroxidation through free radical scavenging, (2) stabilization of the hepatocellular membrane, (3) prevention of inflammation, (4) inhibition of cytochrome P-450 activity and (5) enhancement of protein biosynthesis. However, comprehensive investigations are required to elucidate the exact mechanism of action, to evaluate its efficacy and toxicity in other models, and to carry out pre-formulation studies for developing it as a safe and effective herbal protectant.

Conclusion

Present study reveals the hepatoprotective activity of 90% ethanolic extract of whole plant of *Evolvulus alsinoides* on Carbon tetrachloride induced model. The extract was found to be practically non-toxic upto a dose of 1500mg/kg b.w. when given orally. In CCl_4 induced method the extract (75 & 150mg/kg b.w.) produced significant dose dependent hepatoprotective activity. T he presence of flavonoids could be responsible for hepatoprotective activity, is more likely to be involved in the reaction with the proteins of the layer tissues and thereby showing the activity.

Having confirmed the hepatoprotective activity of *Evolvulus alsinoides* on liver , it deserves further studies to identify which exact chemical constituents of the ethanolic extract mediated the specific observed effects and investigate their mechanism, as also the isolation and characterization of active principles responsible for hepatoprotective activity.

Authors' contributions

SHM, SHF and ST designed and performed the experiments. SHM wrote this manuscript, KP and GP read and corrected the manuscript. All authors read and approved the final manuscript.

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