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Research Article

ANTIOXIDANT, ANTIBACTERIAL AND HEPATOPROTECTIVE ACTIVITIES OF *CISSUS REPENDA* VAHL ON CARBON TETRACHLORIDE (CCL_4) INDUCED LIVER DAMAGE IN BIRD *COLUMBA LIVIA*

D. KARDONG, B. PURKAYASTHA, S. UPADHYAYA*, J. CHETIA

Department of Life Sciences, Dibrugarh University, Assam, India- 786004. Email: sristi_eco@rediffmail.com

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ABSTRACT

Objectives: Drug induced liver damage has been identified as one of the major global diseases in recent years. The plant derived alternative medicines are considered as the most effective solution for combating such liver diseases. In the present study, the antioxidant, antibacterial and hepatoprotective property of *Cissus rependa* Vahl. was evaluated.

Methods: Anti-oxidant activity of the extracts were spectrophotometrically determined using ascorbic acid as standard. Determination of antimicrobial activity followed by Agar well diffusion method and nutrient content by standard laboratory methods. The activity of the two enzymes were measured spectrophotometrically (520 nm) following the DNPH method with minor modification

Results: The study shows that the aqueous extract of the plant leaf contains a good number of phytochemicals such as cardiac glycoside, saponin, alkaloid etc. as well as antioxidant with respect to the scavenging activity against free radicals DPPH (79.79 %) and ABTS (84.6%) which were roughly comparable to that of ascorbic acid (88.2% and 83.0 % respectively). The plant extract also exhibited potent antibacterial activity against *S. aureus* and *P. vulgaris* indicating its bactericidal or bacteriostatic properties. The study also indicates that the oral feeding of aqueous extract of the plant could reduce the CCl_4 induced chronic damage of hepatic tissue in bird (*C. livia*) which was reflected by the decreased level of total soluble protein as well as activity of the enzymes Aspertate aminotransferase (AST) and Alanin aminotransferase (ALT) in both liver and muscle tissues of EX (experimental) group treated with plant extract as compared to that of untreated birds PC (positive control) group which otherwise had highly increased due to CCl_4 induced tissue damages.

Keywords: Antioxidants, *Cissus rependa* Vahl, hepatotoxicity, Aspertate aminotransferas and phytochemicals.

INTRODUCTION

Liver is physiologically one of the most crucial organs in vertebrate. Almost all core pathways for metabolizing protein, carbohydrate and lipid including detoxification of toxic drugs, formation of urea from excess amino acids as well as bile formation etc. are accomplished in the liver. But due to various social customs, viral infection and environmental pollutions, there has been prevalence various health problems in which the WHO has recognized liver damage as one of the major global burdens of diseases in the recent years [1, 2]. It is estimated that about 14-16 million people in South East Asian countries are suffered by virus caused liver diseases. There are three classes of liver diseases, namely-hepatitis, hepatosis and cirrhosis. Most of the liver damages are due to the lipid peroxidation and other oxidative damages in the liver [3]. In practice, the extent of liver damage is assessed by measuring the expression of certain parameters like Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) [4]. Besides these, the depletion of reduced glutathione, the activity of SOD and CAT are also measured to assess the liver damage [5].

Plant has been used to cure various ailments including the liver diseases since ancient times. About 80% of the total world population relies on traditional medicines for their primary health care [2, 6]. Despite tremendous advancement in modern medicines, the folklore medicines with different formulations are still considered as highly effective for liver diseases. In India, more than 87 medicinal plants were used in different combinations in the preparation of 33 patented herbal formulations. These folklore medicines in most cases protect the liver physiology effectively and thus help in regeneration of damaged liver tissue by removing the free radicals [7].

The *Cissus rependa* Vahl belongs to the family Vitaceae is a perennial climber generally grow wild in swamp forest and its tendered leaf is used popularly as traditional vegetable by various tribes of upper Assam and adjoining hilly states of North-eastern part of India [8, 9]. Apart from being a potential vegetable, the *C. rependa* is also claimed to have hepatoprotective as well as in stomachic trouble by local healers [8]. However, no scientific report was found with respect to the phytochemicals, antioxidant properties, antibacterial activities as well as hepatoprotective activities of this traditionally important plant. Therefore, the present investigation aims the qualitative and quantitative estimation of phytochemicals, antibacterial properties as well as hepatoprotective activities of the leaf extract of *C. rependa* Vahl against CCl_4 induced hepatic damage in pegion (*Columba livia*).

MATERIALS AND METHODS

Chemicals and reagents

All the chemicals used in the study were of analytical grade and procured from Merck India Pvt. Ltd.

Collection, processing and preparation of samples from plant materials:

Leaves of *Cissus rependa* Vahl were collected locally from Dibrugarh, Assam (India) during the month of March 2013, shade dried and then powered. Leaf powder was macerated with distilled water for 48 hrs, filtered using Whatman filter paper No.1. The filtrate was then evaporated in aerated oven at 50°C until a dry powdery mass of plant extract was obtained which is kept in refrigerator for further use. These crude extract was dissolved separately in Dimethyl sulphoxide (DMSO) as neutral solvent to make final concentration for biochemical analysis.

Phytochemical Screening

Qualitative and quantitative estimation of phytochemicals and antioxidants

Phytochemical analysis was carried out by following standard methods prescribed by Sadasivam and Manikkam, 2005 [10]. The antioxidants present in the plant material were estimated by measuring the ABTS (at 760 nm) and DPPH (at 517 nm) free radical scavenging activities respectively [11, 12].

The capacity of scavenging free radicals was calculated as scavenging activity (%) =

$$\frac{Absorbance\ in\ control - Absorbance\ in\ sample}{Absorbance\ in\ control} \times 100$$

Where,

Absorbance in control = absorbance of DPPH radical + methanol

Absorbance in sample = absorbance of DPPH radical + sample extract/standard for both the above mentioned equations.

Screening of antibacterial property of *Cissus rependa* leaf extract

Antimicrobial activities of leaf extract were investigated against seven registered bacterial isolates which were obtained from the Microbial Type Culture Collection (MTCC) from Institute of Microbial Technology, Chandigarh (India). These include two gram-positive bacteria, *viz. Staphylococcus aureus* (MTCC3160), *S. epidermidis* (MTCC3615), five gram-negative bacteria, *viz. Proteus vulgaris* (MTCC744), *Bacillus subtilis* (MTCC 441), *B. cereus* (MTCC8750), *E. coli* (MTCC443), and *Enterococcus faecalis* (MTCC439).

The antibacterial property was determined by standard agar well method [13] using aqueous extract of the plant sample dissolved in DMSO (1.0 mg. ml^{-1}). 60µl of the sample was loaded to the well (5mm diameter) bored on the nutrient agar medium in each Petri-plates which was separately inoculated with fresh bacterial culture (cfu = 1.0 x 10^6 ml^{-1}) as above and incubated at 35°C for 48 hrs. The activity was recorded in terms of the Zone of Inhibition (ZOI).

Estimation of crude protein in liver and muscle tissue of pigeon:

The protein estimation was carried out by following the Folin-Phenol method [14]. The data was expressed in terms of mg/g wet tissue ± SD.

Assay of Alanine Aminotransferase (EC 2.6.1.2) and Aspertate Aminotransferase (EC 2.6.1.1) Activity in the tissue extract:

The activity of the two enzymes were measured spectrophotometrically (520 nm) by following the DNPH method with minor modification [15].

Determination of enzyme activity:

The enzyme activity was determined based on the following equation:

$$\frac{(Optical\ density\ of\ sample - Optical\ density\ of\ blank\ tubes) X\ (Dilution\ factor\ of\ the\ enzyme\ extract)}{0.001\ (Unit\ of\ enzyme)\ X\ 0.1\ (Volm.\ of\ enzyme) X\ 30\ (Incubation\ time\ in\ minute)}$$

Where, one unit of enzyme is defined to be 0.001 O.D.

The enzyme activity was expressed as mean of U/ml/min ± SD.

Maintenance of animal models

Live pigeons with average body weight of 200 ± 25 gm were collected from local market and acclimatized under laboratory conditions for 5 days maintaining all the standard protocols as per the guidelines of National Institutes of Health (NIH) and with the approval from the Institutional Ethical committee of Dibrugarh University. The birds were then divided into three groups (A, B and C) of 5-6 members each and distributed in separate poly-acrylic cages with uniform size and other conditions (usual light and dark cycle) except the diet.

CCl_4 induced hepatotoxicity in pigeon

The CCl_4 is a well documented hepatotoxin to induce hepatic damage in living animal [16]. In the present study, the model animals were divided into three groups (A, B and C). The pigeons of group-A categorized as Negative control (NC), was provided with normal food (rice and lentil) and water adlibitum. The group-B marked as Positive control (PC) were injected with CCl_4 at the dose of 1.2 ml/kg using olive oil as vehicle at the ratio of 1:1 for 1 day on day one [1, 17]. The normal diet and other conditions kept same with that of group-A. The group–C marked as the Experimental group (EX) was intoxicated with CCl_4 as described for PC group. On the 2nd day onwards the birds were orally administered with the aqueous extract of *Cissus rependa* Vahl. along with rice and lentil in the dose of 500 mg/kg/day orally for 5 days [18].

All the birds were sacrificed by cervical dislocation on 6th day and tissues were collected separately under aseptic condition and then washed with saline water. The excess saline water was removed by soaking with blotting paper and stored under sterile condition in deep freezer for further use.

Processing of tissues

The ice pellet deposited on the preserved tissues of various treatment was first removed with the help of blotting paper and homogenized in phosphate buffer (pH 7.4) using Biocraft make Teflon headed tissue homogenizer, centrifuged at 5000x *g* for 10 minutes at <4° C and the tissue supernatant was used as the enzyme source.

Statistical analysis

The data of protein estimation and enzyme activity were generated from the record of six replicate for each observation and were subjected to statistical treatment of student's *t*-test (unpaired sample) to determine the significance of results between different animal groups at P< 0.05. The results are expressed as the mean ± SD.

RESULTS

Table 1 shows the preliminary phytochemical screening of leaves of *Cissus rependa* Vahl. The study revealed the presence of bioactive compounds such as alkaloid, protein, amino acid, cardiac glycosides and saponins in the crude water extract of the plant. Other constituents could not be detected with the adopted methodologies.

Table1: Photochemical screening of water extract of *Cissus rependa* Vahl. (qualitative)

Constituents	Result
Tannin	ND
Saponin	+
Flavonoid	ND
Phenol	ND
Cardiac glycoside	+
Anthraquinone	ND
Alkaloid	+
Terpenoid	ND
Steroid	ND
Reducing sugar	ND
Carbohydrate	+
Protein	+
Amino acid	+

(+) indicates presence of constituents; (ND) indicates "Not Detected"

Table 2 shows antioxidant activity in the leaf extract of the plant *C. rependa* vahl. The scavenging activity against DPPH and ABTS free radicals was recorded as 79.79% and 84.6% in crude (500mg ml^{-1}) extract which is comparable with that of ascorbic acid at the same concentration.

Table 2: Free radical scavenging activity of *Cissus rependa* Vahl (500 mg ml^{-1}).

Source	DPPH (%)	ABTS (%)
Cissus rependa	79.79	84.6
Ascorbic acid	88.2	83.0

Table 3 shows the antibacterial property of *C. rependa* in water. Out of 8 bacterial strains studied, only 2 strains namely *S. aureous* and *P. vulgaris* were found to be inhibited by the plant extract. The zone of inhibition (ZOI) including the well diameter (5mm) was recorded to be 10 mm and 8 mm respectively in the 48 hr old bacterial cultures. The results obtained were compared as that of zone of inhibition obtained using standard broad spectrum antibiotic chloramphenicol.

Table 3: Antibacterial activity of *Cissus rependa* Vahl (after 48 hrs. incubation)

Bacterial strains	Zone of inhibition using the plant extract (in mm)	Zone of inhibition using Chloramphenicol (30 mcg) (in mm)
B. subtilis (MTCC 441)	-	12
S. aureus (MTCC 3160)	10	-
S. epidermis (MTCC3615)	-	20
E. faecalis (MTCC439)	-	8
E. coli (MTCC443)	-	-
P.vulgaris (MTCC744)	8	-
B. cereus (MTCC8750)	-	-

Table 4 represents the soluble protein content in liver and muscle tissue of birds under different treatments. The protein content (mg/g) was recorded to be highest 69.52 ± 6.1066 in the muscle tissue followed by 66.34 ± 2.0315 in the liver tissue of PC group which is a highly significant increase from the protein content (mg/g) 49.99 ± 4.1069 and 40.66 ± 4.7234 in the corresponding tissues of NC group. Similarly, a significant decrease in protein content (mg/g) 51.03 ± 2.2655 and 48.01 ± 1.2172 in muscle and liver tissues was recorded in the EX group from PC group.

Table 4: Crude protein content in liver and muscle tissues. Student's *t* test has been performed to the results and results are expressed in mean (mg. g^{-1} wet tissue) ± SD.

Animal group	Liver tissue	Muscle tissue
NC	40.66 ± 4.7234	49.99 ± 4.1069
PC	66.34 ± 2.0315	69.52 ± 6.1066
EX	48.01 ± 1.2172	51.03 ± 2.2655

Table 5 shows the ALT and AST activity in liver and muscle tissues. A similar trend with respect to the activity of enzyme ALT and AST was recorded in two different tissues of three groups of bird. Between the two enzymes, muscle tissue shows higher ALT activity than liver tissue and vice versa in all three groups of birds The activity of the two enzymes was recorded to be significantly increased in the tissues of PC group while compared with that of the NC and EX groups. Maximum activity (U/g wet tissue/min.) was recorded to be 260.11 ± 14.3634 for ALT and 262.46 ± 14.5522 for AST in muscle tissue and liver tissue respectively in PC group. Thus, lowest activity (U/g wet tissue/min.) for ALT (51.923 ± 9.8522) and AST (93.71 ± 19.205) were noted in the liver tissue of EX group and muscle tissue of NC group.

The total protein content and enzyme activity of all three groups of the birds showed highly significant values that were different from each other.

Table5: ALT and AST activity in liver and muscle tissues. Student's *t* test has been performed to the results and results are expressed in mean (results are in mean U/g wet tissue/min. ± SD).

Animal group	ALT activity Liver tissue Mean ± SD	Muscle tissue Mean ± SD	AST activity Liver tissue Mean ± SD	Muscle tissue Mean ± SD
NC	80.92 ± 10.5346	128.35 ± 17.4722	137.2 ± 17.3576	93.71 ± 19.205
PC	153.58 ± 9.5886*** LT= 0.65	260.11 ±14.6347*** LT= 0.506	262.46 ± 14.5522*** LT= 0.754	188.19 ± 10.3819*** LT = 113
EX	51.923 ± 9.8522*** LT= 0.75	147.06 ± 8.6372*	110.64 ± 7.4067** LT= 0.127	114.08 ± 8.0162* LT = 0.037

Values are mean ± SD; n=6 in each group. Values are statistically significant at * P≤ 0.05 (significant) ** P≤ 0.01 (more significant); *** P≤ 0.001 (highly significant). LT = Levene's Test for Equality of Variances (Higher the value less is the level of significance).

DISSCUSSION

The list of plant having hepatoprotective property is very long. However, emphasis has been given to use plant derivatives which are highly potential against drug induced liver damage that involves free radical formation [7].

The results obtained from the present study provide certain informations with respect to the phytochemical constituents, antioxidant property, antibacterial activity and the hepatoprotective potentiality of the plant *Cissus rependa* Vahl. Since the plant is edible with sour taste and could be eaten raw or cooked in water, the present investigation was conducted with the leaf extract prepared directly in distilled water to avoid the effect organic solvent.

As evident from the phytochemical analysis (Table 1 and 2), the leaf extract of *C. rependa* Vahl contains a good number of phytochemical which serve as antioxidants and attributed to the free radical scavenging property of the plant [19, 20]. But, to our surprise, the flavonoid was not detected under the present experimental protocol which may be due to sample extraction procedure. However, the presence of free radical scavenging property in high amount in the plant extract (Table 2) also supported by its rich phytochemicals that imparts antioxidant activity and contributes to curing of various diseases [21, 22]. The free radical scavenging property of present observation may be attributed to the alkaloid, saponin and cardiac glycoside which were detected in conspicuous amount [23]. The plant extracts in various solvents exhibit potential antibacterial activity [24]. The aqueous extract of the plant exhibited antibacterial activity against the test organisms (Table 3). Out of seven test strains, only two strains namely *S. aureus* (gram +ve, inhabit the respiratory tract is a facultative pathogen) and *P. vulgaris* (gram –ve, inhabit the human intestine and urinary tract is an opportunistic pathogen) were found to be inhibited by the leaf extract (10 mm and 8 mm respectively). The results obtained were found to be encouraging as compared to that of chloramphenicol. Literature suggests that gram + ve (*S. aureus*) is more susceptible organism against various plant extracts than gram –ve (*P. vulgaris*) bacteria [25]. The study also showed that gram + ve and gram – ve bacteria exhibit different susceptibility to different solvent extract of medicinal plants which have been described as due to the

morphological differences in cell wall between the two bacterial types. However, Silymarin from blue capitulum's seeds has been found to inhibit both *P. vagaries* and *S. aureus* which corroborates our result (Table 3). The present study also revealed that the crude water extract of *C. reppenda* is quite effective [6] against the two bacterial strains at the concentration of 1 mg ml^{-1} (60 µg/ well) which is higher than the result mentioned in a previous study on methanolic extract of *C. occidentalis* (400 mg ml^{-1} that produced 5-11 mm inhibition zone) against *E. coli* [26]. The activity against both types of bacteria observed in the present study may be due bacteriostatic rather than bactericidal property of the plant extract. Again, aqueous extract of some plants do not show antimicrobial activity and these differences in plants has been described as due to spatial and temporal variations of plant species [26, 27]. In another studies, it was reported that the effective antibacterial activity of water extract of *C. occidentalis* is due to its ability to induce membrane leakage for various positive ions [26].

Hepatoprotective activity of the extract was carried out on liver tissues of pigeon collected from local market considering similarity of the physiological system of avian system to that of mammals and the availability factor of the specimen in the locality in absence of lab grade animal husbandry. CCl_4 is the most commonly used toxin to induce damage in the liver tissue [28]. The hepatotoxic effect of CCl_4 is largely due to the caused by generation of free radical $*CCl_3$ in cytochrome P450 which rapidly reacts with oxygen to form a trichloromethyl-peroxy radical ($*CCl_3OO$). The later causes lipid peroxidation and initiates membrane damage of the hepatic cells that leads to expression and release of more marker enzymes and protein to the serum [3, 29, 30, 31]. It has been shown that the phytochemicals like flavonoids, polyphenols saponins and glycosides serves as scavengers of free radicals produced by the CCl_4 activity and prevents peroxidative damage of cell membrane [23, 32]. The total protein estimation is also useful in hepatoprotective study as its increased level indicates severe mobilization of tissue protein due to non viral liver cell damage. On contrary to a previous study [33], our findings indicates that the total soluble tissue protein and the activity of AST and ALT were significantly increased in the liver and muscle tissues of PC group as compared to that of NC and EX group (Table 4). The results obtained from the present study provide two significant informations. Firstly, the total soluble protein was increased significantly in both liver and muscle tissues of CCl_4 treated PC group (66.34 ± 2.0315 mg. gm^{-1}in liver and 69.52 ± 6. mg. gm^{-1} in muscle tissue) which was kept at significantly lower level (48. 01 ± 1.2172 mg. gm^{-1} in the liver and 51.03 ± 2.2655 mg. gm^{-1} in muscle of EX group) indicating protective role of the *C. reppenda* extract against liver cell damage that otherwise caused by CCl_4. In a previous work, it has been shown that *A. catechu* exerts its hepatoprotective effects through synthesis of new proteins and accelerated detoxification by virtue of its antioxidant property [10]. The differences in two observations may be due to consideration of different tissues viz. whole liver homogenate in the present study and the blood serum in case of the previous study. On the other hand, differential response to the CCl_4 effect by two different physiological systems can't be ruled out. However, more elaborate study is needed to address the issue. Secondly, a similar trend was observed with respect to the ALT and AST activity in which the PC showed highest enzyme activity in both tissues as compared to the respective tissues in the NC and EX group. The activity of the two enzymes in both tissues of EX group was kept at significantly low level (ALT: 51.923 ± 9.8522l; AST: 110.64 ± 7.4067 for liver and ALT: 147.06 ± 8.6372; AST: 114.08 ± 8.0162) due to protective effect of *C. reppenda* Vahl extract despite CCl_4 administration. The Levene's Test for Equality of Variances indicates that the enzyme activities in both tissues of EX group are closer to the respective values of the NC group than that of PC group. Reports on hepatoprotective activity of various plant extracts are numerous [**3, 20]**. The present findings also indicate that the extract of *C. reppenda* in water is quite effective against CCl_4 induced liver damage **[11]**. In most of these studies, the extent of liver damage were assessed with respect to the enhanced activity of AST, ALT and ALP enzymes in the blood serum which diffused out from the liver due to the CCl_4 induced lipid peroxidation leading to the damage of the hepatocyte cell membrane **[19, 33]**. Since the present study was carried out in the whole tissue homogenate and not in blood serum, the findings indicate that CCl_4 may have enhanced the expression of gene for these enzymes apart from its cell membrane damaging effect. However, more elaborate research is needed for elucidation of the exact mechanism of this finding.

CONCLUSIONS

The screening of plants with hepatoprotective potential is an essential practice to combat the ever-increasing trends of hepatotoxins in the polluted environment. On the basis of the findings in the present study, the *C. reppenda* Vahl which is frequently used as nonconventional, traditional knowledge based vegetable by various tribes and communities of Assam is an excellent hepatoprotective property enriched with antioxidant activity. The presence of mild antibacterial activity against selected strains of gram +ve as well as Gram-ve bacteria may indicate its novelty in phytomedicinal applications. So, the plant could be prescribed as a highly valuable food plant for human consumption.

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