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Original Article

Preliminary study on antioxidant and hepatoprotective activity of Clerodendrum phlomidis (L) against CCl_4 intoxicated albino rats

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ABSTRACT

Ethanolic extract of leaves, root and stem of *C. Phlomidis* (Verbenaceae) were prepared by continuous hot percolation. The content of total flavonoid for each extract was determined by aluminium chloride colorimetric method and the percentage content of flavonoids were determined as quercetin equivalent. The potential of drug against free radicals is evaluated by in- vitro DPPH radical scavenging assay. Hepatoprotective activity was investigated on albino rats and carbon tetrachloride (CCl_4) was used to produce hepato- toxicity. Ethanolic extract of leaves showed the maximum activity, which is comparable to the standard silymarin, while other parts were found to be less significant.

1. INTRODUCTION

Taxons of *verbenaceae* family possess various pharmacological activities. *Clerodendrum* species have been used in Indian and Chinese traditional medicine for ages. Traditional “Vaidhyas” in Vidarbha region (Maharashtra) prescribes fresh leaf juice of *C. Phlomidis* with curd for treatment of liver diseases. A decoction of *C. Phlomidis* leaves are used alongwith other plants for inflammation, and is effective in treating bronchitis, headache, weakness, drowsiness and digestive problems [1]. In vitro and in- vivo studies of dried leaf extracts of this plant have demonstrated antioxidant activity [2]. The plant extract also showed a significant reduction in gastrointestinal motility in charcoal meal test in rats. The results obtained establish the efficacy and substantiated the folklore claim of the plant as an anti-diarrheal agent [3].

The diterpenoid compounds from *Clerodendrum* spp. are effective at lower concentration to induce anti-feedency and growth inhibition in Earias vitella and Spodoptera litura [4]. Although *C. Phlomidis* has been used in traditional medicine for various properties, however, its hepatoprotective property has not been documented well. This paper attempts to justify the traditional claims of Vaidhyas and provides basis for the exhaustive utilization of this medicinal plant in therapeutics.

C. Phlomidis is a fairly common shrub of arid plains, low hills and deserts of Pakistan, Sri Lanka, Burma and India. It is frequently found in Vidharbha region of Maharashtra. It is about 1.5- 3 m tall, stem ashy-gray, branches pubescent. Leaves are opposite, ovate to rhomboid-ovate, 1.5- 5 cm long and 1- 3 cm broad, entire to sinuate-crenate, subacute-obtuse, petiole up to 2.5 cm long, densely hirsute. Flowers are creamy-white or pale yellowish in colour. Dried roots and flowers of *C. Phlomidis* yielded aL-rhamnopyranosyl (1-2)-2-D-glucopyranosyl-7-0- naringin-4-0-a and D-glucopyranoside-5-methyl ether 2,4-trihydroxy,6-methoxy chalcone-4,4-a-D Diglucoside [5]. Chalcone glycoside, together with pectolinarigenin, 7-hydroxyflavone and 7-hydroxyflavanone 7-O-glucoside have been isolated from the flowers and leaves of *C. Phlomidis* [6].

2. EXPERIMENTAL

2.1 Procurement of Plant Material

The fresh leaves of plant were collected from the Wardha District, Maharashtra with the help of local villagers in the month of March- April, 2006. The plant was authenticated by the Plant Taxonomist, Dr. C.D. Zanwar, J.B. Science College, Wardha (MS) and a specimen was deposited with herbarium number KAS/ 217.

2.2 Preparation of Extract

Dried leaves stem and roots of *C. Phlomidis* were extracted by soxhalation with ethanol. The extracts were concentrated under reduced pressure with the help of a rotary vacuum evaporator. Each concentrated extract was then evaporated to dryness. The dried extracts were diluted with suitable amount of normal saline and used in experiments.

2.3 Preliminary phytochemical screening

Determination of Total Flavonoid

The content of total flavonoid was determined by aluminium chloride colorimetric method [7]. The content (%) of flavonoid was determined as quercetin equivalent. 1.0 ml of plant extract (0.1 gm /10 ml) in the solvent (Stock solution SS) was mixed with 2.0 ml $AlCl_3$ (2 % w/v) in methanol and the solution was made up to 25 ml with methanolic solution of acetic acid (0.5 % v/v) (Probe solution PS). At the same time 1.0 ml of SS was made up to 25.0 ml with methanolic solution of acetic acid (Contrast solution CS). The absorbance of PS against CS was measured at 420 nm after 30 min. The result expressed as total flavonoid content (TFC), calculated as quercetin using equation:

$$TFC = \frac{A_{420nm} \times \text{Dilution factor}}{E_{(1\%,1cm)} \times \text{Weight of extract (gm)}}$$

Where, $E_{(1\%,1cm)}$ of Quercetin $AlCl_3$ complex is 500.

2.4 Animal Care

Adult male Wistar rats of age 5-8 weeks, weighing 100-160 g were used. The animals were maintained in the Institute's animal house under standard laboratory conditions like, light (12/24 h) and temperature (24 + 1 C°) with commercial pellet diet and water ad libitum. The animals were acclimatized to laboratory condition for seven days before commencement of experiments fasted overnight just before the experiment, but were allowed free access to water. . All the procedures and protocols of the experiments were reviewed and approved by IAEC, Barkatullah University, Bhopal (CPCSEA Reg. No. 444/01/C/CPCSEA/25/07/2001)

2.5 Acute oral Toxicity Study

The acute oral toxicity study was performed as per the Organization for Economic Cooperation and Development (OECD) guidelines no. 425 on Wistar albino rats (140-200 g). Three overnight fasted rats received the extract of dose 2000 mg/kg, b.w. Extract was given in the form of suspension with 0.5% carboxy methyl celluloe in distilled water by oral gavage. Each animal was observed individually for any toxicity sign of gross changes like convulsion, tremor, circling movement, depression, and mortality after dosing, for 24 hours, with special attention given during the first 4 hours. All observations were systematically recorded with individual records being maintained for each animal.

2.6 *In-vitro* antioxidant activity by DPPH method

The free radical scavenging activity was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) method [8]. 0.1 mM solution of DPPH was prepared in methanol, and 1 ml of it was added to different concentrations of *C. Phlomidis* extract (100, 200, 300, 400 and 500 μg/ml) in the test tube and final volume of 3 ml was made with methanol. The mixture was shaken vigorously and allowed to stand at room temperature for 30 minutes. Absorbance of the resulting mixture was measured at 517 nm against methanol as blank, by using a UV-visible spectrophotometer (Systronics, 2203, Japan). Each sample was then measured in triplicate and results were represented as mean. The ascorbic acid was used as a standard antioxidant in this method. Percentage of DPPH free radical scavenging activity (FRSA) was determined as follows:

$$\%\ (FRSA) = \frac{\text{(Absorbance of control – Absorbance of test sample)}}{\text{Absorbance of control}} \times 100$$

2.7 *In-vivo* Carbon Tetrachloride (CCl_4) induced Hepatotoxicity [9]

Randomization, grouping and dosing of animals

The protocol was followed as described by Mukherjee, PK. with some modification. Male albino rats weighing 100-160 g. was taken. Animals were divided into 6 groups of six rats each. Group I was kept as normal receiving only normal saline orally for 15 days. Group II (Toxicant) were given same treatment as Group I for 7 days and on 8th day they were given intra- peritonial dose of toxin solution 1.25 ml/kg body weight followed by same treatment upto 15 days. Group III, IV and V were given the extract of *C. Phlomidis* leaves, roots and stem respectively (300 mg/kg bw, oral) for seven days, on 8th day single dose of 1.25 ml/kg bw of CCl_4 was administered i.p., and treatment was continued up- to 15 days with respective extracts. Animals of VI group (standard) was protected with silymarin, (7 mg/kg bw, oral) for 7 days before intoxication by CCl_4 and from 8th day again treated with silymarin up- to 15 days.

2.8 *Evaluation of effect of C. Phlomidis against Carbon Tetrachloride (CCl4) induced Hepatotoxicity*

2.8.1 Analysis of serum for liver function test

Blood samples were collected, allowed to clot and the serum was separated by the centrifugation at 3000 RPM at for 15 minutes and stored at 4ºC for further biochemical analysis. Different biochemical enzymes for liver function test like serum alanine aminotransferase (SGPT), aspartate aminotransferase (SGOT), alkaline phosphatase (SALP) and bilirubin. The enzyme assay was carried out by Reagent Kits maintained by Span Diagnostics Ltd. and the procedures were essentially those described in the literature available with kits.

2.8.2 Histoarchitechture

The gross liver was examined, weighed and stored in 10 % formalin and were processed for paraffin embedding using the standard micro technique [10]. A section of the liver (5 μm) stained with hemotoxylin and eosin was observed microscopically for histopathological studies.

Statistical Analysis

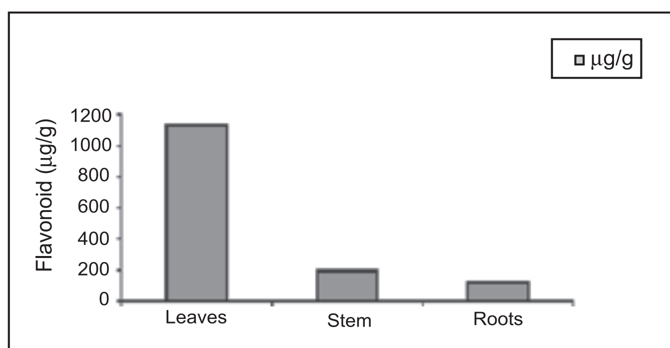
The data obtained in present investigation were analyzed by the mean ± S.D. Values were calculated for each group. The data were analyzed by one way analysis of variance (ANOVA) by

unpaired student's t- test. A value of $p < 0.05$ was considered as significant. "Graph Pad Insat Demo version" and "ez- Anova" were used for analysis of data.

3. RESULTS AND DISCUSSION

The total flavonoid content was expressed as quercetin equivalent, flavonoid content (Graph 1).

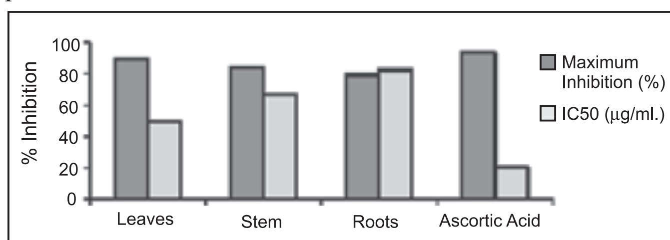
Graph 1. Total flavonoid content of different parts of *C. Phlomidis*.



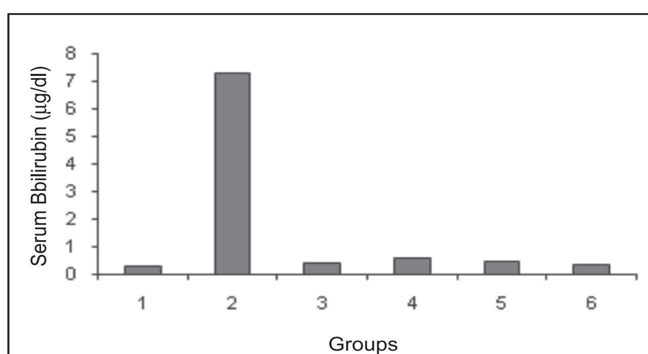
DPPH radical scavenging model is widely used to investigate the antioxidant activity of several plants. The proton radical scavenging action has been known as important mechanism of antioxidant. DPPH was used to determine the proton radical scavenging activity of *C. Phlomidis* (Graph 2).

SB levels were significantly higher in animals receiving CCl_4 (Group II) and were within the normal limits in III, IV, V & VI groups (Graph 3), which received CCl_4 and extract of leaves, stem and roots of *C. Phlomidis* and silymarin respectively which shows that the extract of leaves has the most significant hepatoprotective activity than roots and stem.

Graph 2. DPPH free radical scavenging activity of different parts of *C. Phlomidis*.



Graph 3: Effect of different parts of *C. Phlomidis* on serum bilirubin



Histopathological studies revealed centrizonal and focal necrosis and ballooning in the liver of CCl_4 intoxicated rats. (Fig. 1A &1B) Group III receiving the extract of leaves of *C. Phlomidis* showed no signs of ballooning. (Fig. 1C) Mild ballooning with sinusoidal dilatation and binucleate cells was observed in groups IV (Fig. 1D) and group V (Fig. 1E) treated with extracts of roots and stem of *C. Phlomidis* respectively, while binucleate cells spread throughout the liver sections. The rats treated with silymarin syrup appeared to be normal as in Group I. CCl_4 causes acute free radicals mediated hepato-toxicity. The metabolism of CCl_4 may deplete the liver cells [11]. An evidence of hepatic injury is leakage of cellular enzymes into the plasma [12]. The increased level of SGOT, SGPT, ALP and bilirubin may be interpreted as a result of the liver cell destruction or changes in membrane permeability indicating the severity of hepatocellular damage [13, 14, 15]. The rise in SGPT and SGOT activity is almost always due to hepatocellular damage [16]. An increase in ALP reflects the pathological changes in biliary flow [17]. Pretreatment of animals with various extracts of *C. Phlomidis* reduced the increased activity of these enzymes in serum (Table 3), caused by CCl_4. A determination of serum bilirubin represents a parameter for the evaluation of hepatic function and any abnormal increase in the bilirubin level indicates hepatobiliary dysfunction and malfunctioning of hepatocytes [18].

There are evidences that triterpenoids, flavonoids may be effective antioxidants in a wide range of chemical oxidation systems, demonstrated by their ability to scavenge various free radicals in aqueous and organic environment [19]. Recent studies have suggested that flavonoids may protect free radical induced damage [20-22]. *C. Phlomidis* contain flavonoids, terpenoids, phenolic acids and other phenolic compounds (Table 1), and their presence may be a cause of its hepatoprotective activity. The free radical scavenging capacity may be the possible mechanism of hepatoprotective activity (Table 2).

Table 1. Total Flavonoid content (%) of leaves, stem and roots of *C. Phlomidis*.

Flavonoid Content	Mean ± SD (µg /g)
Leaves	1130 ± 11.3
Stem	194 ± 10.5
Roots	118 ± 7.1

Table 2. DPPH free radical scavenging activity of different parts of *C. Phlomidis*.

Extract	Maximum Inhibition	IC_{50} (µg/ ml)
Leaves	89.76 ± 1.21	49.87
Stem	84.03 ± 1.50	67.19
Roots	79.16 ± 1.04	82.53
Ascorbic Acid	93.45 ±0.78	20.13

4. CONCLUSION

In the present investigation, it was observed that SGOT, SGPT, ALP and Serum Bilirubin levels were significantly reduced in the animals receiving plant extracts and CCl_4 than those given CCl_4 alone, indicating that the degree of hepatic cell damage was of lesser magnitude in plant extract treated groups. The observed alterations of serum parameter level to hepatic damage was confirmed by histopathological studies, which showed that, animals challenged with CCl_4 had centrizonal necrosis, focal necrosis and ballooning in the liver. In animals treated with different plant extracts, there was no noticeable hepatocellular necrosis, only mild ballooning and binucleate cells was observed. Binucleate cells in liver are indication of hepatic cell regeneration. These observations point towards a hepatoprotective activity of test plant in the experimental model as evidenced by both biochemical and histopathological studies. The leaves were found to be most effective, in comparison to other two extracts. The results of study indicate towards the antioxidative potential and hepatoprotective activity of *C. Phlomidis* against CCl_4 induced hepatic necrosis in rats and rationalizes its use as a constituent of various herbal hepatoprotective formulations.

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Conflict of Interest

None

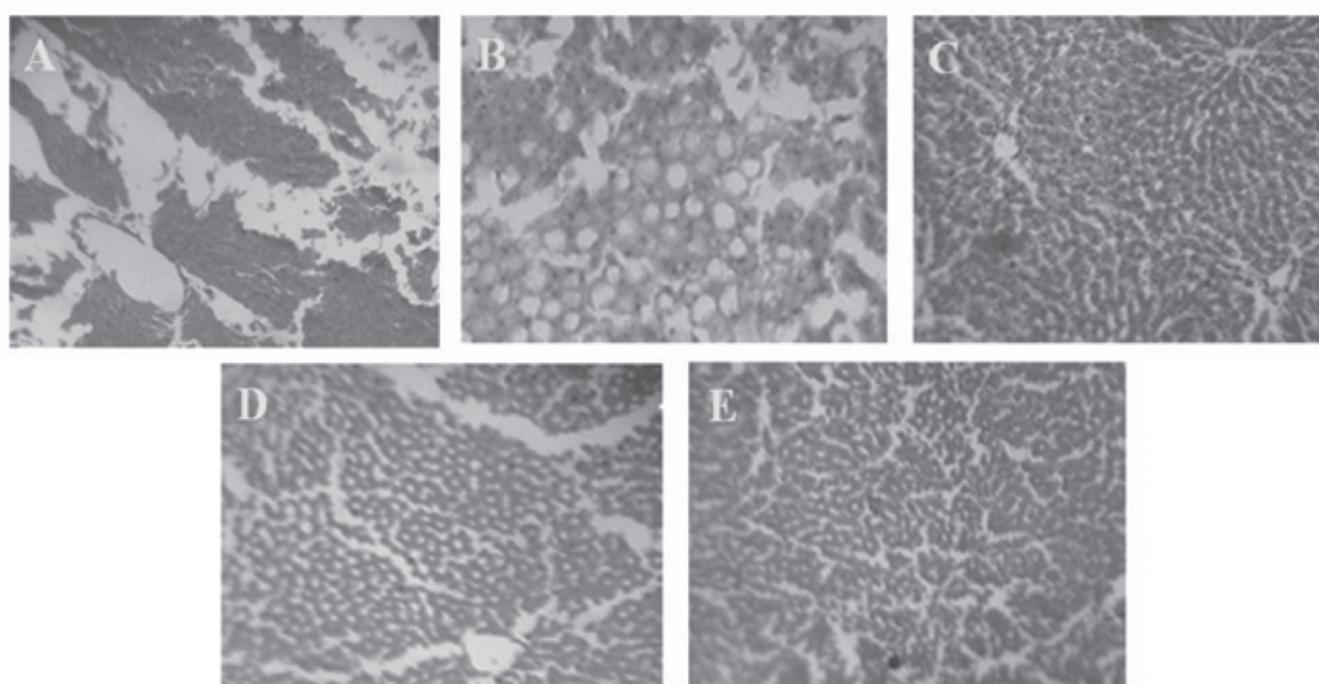

Fig. 1. Section of liver tissue of intoxicated rat (Group II) showing centrizonal and focal necrosis (A) and ballooning (B), Section of liver tissue of leaf extract treated (Group III) rat showing no signs of necrosis and ballooning (C), Root extract treated rat (Group IV) showing mild ballooning with sinusoidal dilatation and binucleate cells, and stem extract treated rat (Group V) showing mild ballooning with sinusoidal dilatation.

Table 3. Effect of Ethanolic Extracts leaves of *C. Phlomidis* on Serum Enzyme Level and Bilirubin Level of Albino Rats.

Groups	SGPT (IU/L)	SGOT (IU/L)	SALP (IU/L)	SB(mg/dl)
Group I	75.33 ± 4.96	84.01 ± 10.25	463.33 ± 14.52	0.27 ± 0.06
Group II	197.33 ± 9.16	294.50 ± 9.76	788.80 ± 13.29	7.3 ± 0.08
Group III	81.50 ± 3.45**	101.77 ± 11.04*	469.06 ± 10.33**	0.39 ± 0.01**
Group IV	88.33 ± 8.33*	154.74 ± 3.14**	581.54 ± 14.54	0.60 ± 0.02**
Group V	84.65 ± 2.74**	104.05 ± 8.34*	497.86 ± 13.91	0.45 ± 0.03*
Group VI	78.85 ± 7.50*	91.33 ± 7.50**	473.77 ± 12.90**	0.33 ± 0.04*

Values are expressed as mean±SD. n = 6 rats in each group. *P<0.01, ** P<0.05 compared to control group.

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