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The hematological and histological studies for the hepatoprotective-like effect of the hydromethanolic extract and the fractions of *Viola serpens* Wall.

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ABSTRACT

Traditionally, *Viola serpens* has been used in the treatment of several human disorders including liver diseases without any scientific evidence. As the current therapies are not very effective and face challenges of unwanted effects and patient compliance, therefore more effective and safe agents are highly needed. The current study aimed to evaluate the hepatoprotective potential of the crude extract and subsequent fractions of the whole plant in the *in-vivo* model using various hematological and histopathological parameters followed by an HPLC study for the identification of phenolic compounds. Rabbits (1000-1200 g) were used in the study. Paracetamol (2g) was used to induce hepatotoxicity in experimental rabbits. The plant extract was used in two doses (150 and 300 mg/kg body weights) for eight days. The hematological parameters AST, ALT and ALP values were determined along with the histopathology of the liver. Phenolic compounds were identified by high-performance liquid chromatography (HPLC) Agilent-1260 infinity from their retention time, UV spectra and available standards while quantification was done taking the percent peak area. The doses 150 and 300 mg/kg body weight seemed to be more effective. The hematological values and the histopathological slides show the hepatoprotective effect of the plant. Regeneration indicated the presence of nuclei, nuclear cleaning, prominent nucleoli, RBC's, central veins and plates of hepatocytes. The HPLC studies revealed the presence of a number of phenicol compounds. The crude extract and the subsequent fractions of the plant possess strong hepatoprotective activity, providing a scientific rationale for its uses in the treatment of liver toxicities.

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Introduction

Since the beginning of human history. sources of plants, animals and minerals have been used for the cure and prevention of various diseases. Unani, Ayurveda, and Sidha systems of medicine provide cures to diseases through drugs originating from minerals, plants and animals with minimal side effects (1). Plants are the most researched and have been the major and continuous source of drugs used in modern medicine (2,3). It is believed that medicines derived from plant products are safer than their synthetic counterparts (3, 4). Different chemical compounds synthesized by the plants carry various biological activities and also protect against diseases (5).

V. serpense Wall. is one of the important medicinal plants, belongs to the family Violaceae, consisting of twenty-three genera and 930 species (6). Out of the total 930 species about 111were identified and distributed in China and 17 in Pakistan in different localities (7, 8). Height is around 800-3000m mostly in mountains of Northern areas from the sea level (9). It is also distributed in Afghanistan, India, Bhutan, Indonesia, Kashmir, Thailand, Malaysia, Sri Lanka, Myanmar, China and Nepal (10). In folk medicine, it is used as an antipyretic, laxative, emollient and for

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the treatment of jaundice, hepatitis, pneumonia, bronchitis, urinary infections and kidney diseases (11-16).

The phytochemistry of *V. serpens*showed that it contains glycosides, flavonoids, alkaloids, coumarins and tannins (17). It also contains sugar, mucilage, gum, violin and saponins (15). Ascorbic acid, ascorbate oxidase, peroxidase, and catalase have also been reported (18).

The current study was, therefore, designed to evaluate the hepatoprotective potential of the crude extract and different fractions of the whole plant in an animal model using various hematological and histopathological analyses.

Materials and methods

Plant collection

The whole plant (10 kg dry wt.) of *V. serpens* Wall. was collected from the Shangla district of Khyber Pakhtunkhwa, Pakistan during April 2011. The plant was identified by Dr. Mohmmad Ibrar, a taxonomist at the Department of Botany, University of Peshawar, Peshawar with a voucher No.Bot.20158 (PUP) placed in the herbarium of the department.

Extracts preparation for HPLC-UV characterization

HPLC-UV characterization and quantification were carried out according to the reported method [19]. To prepare extract for HPLC analysis 1 gm powdered sample was mixed in methanol and water (1:1; 20 mL; v/v). The mixture was heated at 70˚C for 1 hour in a water bath and centrifuged for 10 minutes at 4000 rpm. After that sample (2 mL) was filtered into HPLC vials through Whatman filter paper. For the identification of phenolic compounds, the High-performance liquid chromatography (HPLC) Agilent-1260 infinity system was used, with basic parts like UVAD (ultraviolet array detector), a quaternary pump, degasser and auto-sampler. The separation was carried out by the Agilent-Zorbax-Eclipse column (XDB-C18). Column gradients system consists of solvent B and solvent C. solvent B composed of deionized water: methanol: acetic acid in the ratio of 180: 100: 20; v/v and solvent C composed of deionized water: methanol: acetic acid in the ratio of 80: 900: 20; v/v. The gradient system was started with solvent B 100%, 85%, 50% and 30% at 0, 5, 20 and at 25 min, and finally, solvent C (100%) started from 30-40 min. Elution occurred after 25 min. The ultraviolet array detector (UVAD) was set at 280 nm for the analysis of phenolic compounds and the chromatogram was recorded from 190-500 n. Identification of phenolic compounds was done using their retention times, UV spectra and available standards while quantification was done taking the percent peak area.

Preparation of plant extract and its fractions

The freshly collected shade dried plant material was powdered and extracted by maceration in 80% methanol for 10 days (3x50 L). The combined methanol extract was filtered with a muslin cloth, evaporated and concentrated under vacuum by using a rotary evaporator at a temperature of 40°C. The viscous extract (1.32 kg) obtained was dissolved in water and partitioned between *n*-hexane, ethyl acetate, chloroform and *n*-butanol fraction by separating funnel. Five different fractions *n*-hexane (44.13g), ethyl acetate (22.7g), chloroform(17g), *n*-butanol (35g) and aqueous (45g) were obtained. The crude extract along with the fractions was investigated for hepatoprotective activity.

Animals and experimental layout

Sixty (60) domestic local mature rabbits (*Oryctolagus cuniculus*) of both sexes were purchased from the local rabbit market and maintained under optimal conditions at the University of Malakand, KPK, Pakistan. The rabbits were fed on chow pellets along with fresh green vegetables and grasses and free access to fresh water *ad libitum*. Before feeding the experimental diet, animals were acclimatized for two weeks. The study was approved by the Departmental Research Ethical Committee (DREC) with reference no: DREC/20160524-1 dated 24/05/2016.

Animals grouping and dosing

The rabbits were divided into fifteen groups, each having four animals. Two doses low (150 mg/kg) and high (300 mg/kg) were tested for the crude extract and fractions. During the experiment, PCM (GlaxoSmithKline) at a toxic dose of 2 g/kg body weight whereas, silymarin 50 mg/kg body weight was used (20).

Group 1, administered with normal saline (5%), served as control.

Group 2 was treated with PCM only.

Group 3 received PCM on day 0, followed by silymarin.

Groups 4, 5 received PCM followed by crude extract at low and high doses, respectively.

Groups 6, 7 received PCM followed by *n*-hexane fraction at low and high doses, respectively.

Groups 8, 9 received PCM followed by ethyl acetate fraction at low and high doses, respectively.

Group 10,11 received PCM followed by chloroform fraction at low and high doses, respectively.

Group 12, 13 received PCM followed by an *n*-butanol fraction at low and high doses, respectively.

Group 14, 15 received PCM followed by an aqueous fraction at low and high doses, respectively.

The experiment was continued for 8 days.

Blood collection and processing

On day 9, animals in all the groups were anesthetized by chloroform inhalation. Blood was collected directly from the heart and transferred to EDTA tubes for serum separation (21). Serum was separated by centrifugation and stored at -20°C till further use. Liver enzymes activity was determined using commercially available kits for aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and alkaline phosphatase (ALP).

Histopathology

To record the protective role of the plant extract and fractions against PCM-induced tissue damage, samples from the liver were collected immediately after killing the animals and preserved in 10% buffered formalin. Tissues were dehydrated in ascending grades of ethanol, cleared with xylene and embedded in paraffin. After making thin slices (3-5µm), samples were stained by Hematoxylin and Eosin (H&E) and photographs were taken by using a camera fitted microscope (22).

Statistical analysis

Data are presented as means ± standard deviation (SD). To compare means the data were subjected to the Tukey Test of Post Hoc Multiple Comparisons in One-way ANOVA. For all this analysis computer software SPSS 16.0 was used.

Results and discussion

Identification of phenolic compounds through HPLC-UV technique

Typical HPL-UV chromatograms of *Viola serpens* methanolic extracts are presented in Figure 1 and a total of seven phenolic compounds (gallic acid, malic acid, ascorbic acid, chlorogenic acid, epigallocatechin gallate, quercetin and morin) were identified in the Methanolic Extract of the plant sample. The Quantification and identification of each phenolic compound with their particular peak position and retention time (Rt) in the chromatogram is presented in Table. All these phenolic compounds were identified with standard phenolic compounds in the *V. serpens* methanolic extract. Quantification of antioxidants compounds was measured by formula:

$$Cx = \frac{Ax \times Cs\left(\mu g/_{ml}\right) \times V(ml)}{As \times Sample\ (wt.in\ g)} \quad (1)$$

Cx= Sample concentration; ***As***= Standard peak area; ***Ax***= Sample peak area; ***Cs***= Standard concentration (0.09 µg/ml). Table 1 indicates that all the phenolic compounds were in the highest concentration. These phenolic compounds might be responsible for the highest antioxidant activity.

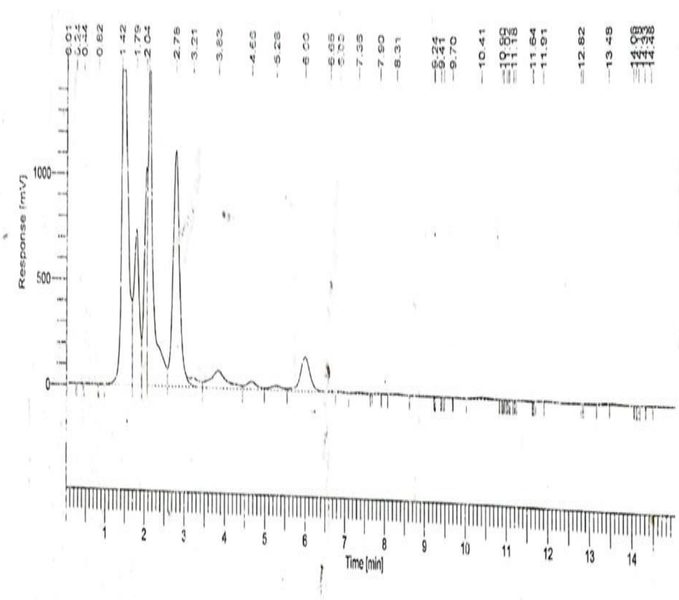

Figure 1. HPLC-UV chromatogram of the crude extract of *Viola serpens*.

Table 1. Identification and Quantification of phenolic compounds in *Viola serpens* plant extract/fractions according to standard reference

Sample Extract	No. of Peak	Retention time (min)	Phenolic compounds Identity	HPLC-UV λmax (nm)	Peak Area of sample	Peak Area of standard	Concentration (μg/ml)
Viola serpens Plant extract	1	2.2	Gallic acid	320	2747648.38	195.44354	12652.6747
	2	2.9	Malic acid	320	52777.25	40.32330	1177.9672
	3	4.6	Ascorbic acid	320	62658.20	22.37612	1261842.6619
	4	6.0	Chlorogenic acid	320	2845612.30	12.92983	198.73.0659
	5	9.3	Epigallocatechin gallate	320	38889.86	7261.4763	4.8200
	6	10.2	Quercetin	320	149888.24	7089.2851	19.0286
	7	12.3	Morin	320	1059.86	2.01066	474.4084

Effect of hematological tests

The ALT values noted in the groups of rabbits treated with PCM alone showed a significant increase (six-folds) than the values noted in the normal saline-treated animals (table 1). Silymarin, a standard hepatoprotective drug has reduced the ALT value by two folds than the PCM value. Crude extract along with all the fractions caused a greater reduction in the value as compared with the PCM value. Chloroform at a dose of 150 mg/kg and ethyl acetate at a dose of 300 mg/kg showed pronounced effects. Whereas the high doses of the crude extract and *n*-hexane showed no significant effects. There was a marked reduction in the AST values of the crude extract along with all the fractions at both the low and high doses in comparison with the PCM values (table 2). However, chloroform at low (150 mg/kg) and high (300 mg/kg) doses, ethyl acetate and *n*-butanol at high doses (300mg/kg) showed a less significant effect on AST values. The rest of the fractions in both doses showed similar values to the standard drug silymarin.

Similarly, there was a marked reduction in the ALP values of all the fractions along with the crude extract at the doses of 150 and 300 mg/kg in comparison with the PCM value. The ALP results showed to be even more pronounced than the silymarin. The values are closer to the values of normal saline and silymarin. The arrangement is given in the decreasing order of their effectiveness i.e. silymarin> aqueous fraction >*n*-butanol > chloroform > crude extract> ethyl acetate >*n*-hexane.

Effect of Histological Analysis

Histological sections of the liver of the rabbits treated with saline solution showed normal tissue architecture with a centrally placed nucleus and foamy cytoplasm of hepatocytes (Figures 2A). No vascular disturbance was noted in the arterial and venous systems. The sinusoidal spaces were neither enlarged nor reduced but of normal sizes.

The hepatocytes of the rabbits treated with PCM alone showed cellular swelling and vacuolation [Figure 2B]. The rounded and sharply demarked boundaries of the vacuoles were suggesting fatty changes. The sinusoidal spaces were much decreased due to increased cell sizes. No vascular changes like congestion or hemorrhages were noted. The crude extract of the plant showed a significant reduction in the PCM-induced damage to hepatocytes. An ameliorative in the toxic effects of PCM on the hepatocytes was noted in the groups of rabbits given crude extract at both low (150 mg/kg) [Figure 2C] and high (300 mg/kg) [Figure 2D] doses. The protective effects were more pronounced at a higher dose. The rabbits treated with *n*-hexane extract of the plant showed a protective effect against PCM-mediated damage to hepatocytes (150 mg/kg) [figure 2E] (300 mg/kg) [Figure 2F]. However, the higher dose of plant extract exhibited little protection as noted in the lower dosed group. Likewise, plant material extracted with chloroform, ethyl acetate and *n*-butanol showed a lesser decrease in liver lesions at higher doses than the lower doses. However, liver histology indicated a significant improvement in the group of rabbits given aqueous plant extract at a higher dose than the lower dose (150 mg/kg).

Table 2. Effect of different solvents extracts of *Viola serpens* Wall on the liver enzymes of the rabbits

Groups	Dose mg/kg	Liver-related parameters with change values		
		ALT	AST	ALP
Normal saline	1 ml/kg	20±4.6	29.8±6.0	30.3±4.3
PCM Control	1000	129±5.3	75.3 ± 18.8	185± 7.8
Standard Silymarin	50	65±2.8***	40±6.9***	81±7.2***
Crude extract	150	76±9***	65 ±18.3***	71.8±10.4***
	300	103 ±1.8	47 ±8.25***	66.3 ± 3.1***
n-hexane	150	53 ±7.53***	44 ± 8.3***	89 ± 11.7***
	300	96 ±9.30	45 ± 2.6***	80.3± 9.5***
Chloroform	150	27 ±7.9***	67.3 ± 4.9**	50±8.6***
	300	68 ±8.2***	82 ±15.4**	70 ±9.5***
Ethyl Acetate	150	66 ±1.9***	66 ± 4.39***	85 ±16.3***
	300	47 ± 4.039***	83 ± 2.5*	62 ±6.7***
n-Butanol	150	68±14.3***	67.3 ± 1.5**	44.3±4.5***
	300	73±3.4***	65 ± 1.96***	45± 33.3***
Aqueous	150	75±6.79***	48 ± 1.9***	39.5±2.4***
	300	62±2.2***	45.5 ±5.3***	34±3.1***

*P<0.05, **P<0.01 ***P<0.001 when compared with PCM treated group change = extract treatment value-PCM toxic value/extract treatment value X100.

The study revealed significant hepatoprotective-like effects of *Viola serpens* crude extract/fractions (150 and 300 mg/kg) in paracetamol-induced liver toxicity in rabbits via biochemical and histological studies.

Exposure of the liver to the drug itself or its active metabolites results either in direct toxicity or may get a chance of immunological reaction (23, 24). Toxic metabolites are the results of about 62% of withdrawn drugs administration. PCM is a commonly used analgesic and antipyretic drug that results in acute centrilobular necrosis and centrizonalheamorrhagic (25, 26). Similarly, 90-95% PCM metabolism occurs through the liver and was excreted through the kidneys (27, 28). In the body, various reactive radicals like hydroxyl radicals, hydrogen peroxide, superoxide anions, nitric oxide, nascent oxygen and lipid oxides generation occur due to certain internal and external factors resulting in disorders like hepatic ailment (29-31). In therapeutic doses of PCM, only 5% of the drug was converted to *N*-acetyl-*p*-benzoquineimine (NAPQI), a highly reactive cytochrome P450 mediated intermediate metabolite (32). Whereas, in toxic doses, it is mostly oxidized by cytochrome P450 enzymes to highly reactive NAPQI (33). Decreased glutathione store or metabolites NAPQI covalently bond to vital proteins, hepatocyte membrane's lipid bilayer and raise the lipid peroxidation (34) which was responsible for mediating liver toxicity. Biochemical parameters (AST, ALT and ALP) with increased levels better reflect the liver injury (35, 36).

In the present study, the liver biomarkers, ALT, AST and ALP values were significantly reduced and comparable to the silymarin treated group in comparison with the values of purely PCM intoxicated groups. This suggests the protection, regeneration, and restoration of the cellular permeability of the plant extract and fractions in the PCM intoxicated rabbit models. The mechanisms involved in this may be the free radical scavenging effect by intercepting the radicals involved in PCM metabolism (microsomal enzymes). Antioxidants are agents that can neutralize the deleterious effects of free radicals. Exogenous support is taken for keeping a balance between oxidants and antioxidants. Plants with antioxidant properties are becoming more and more popular all over the world (37). There is a strong relationship between the and antioxidant activity (38, 39). The antioxidant constituents and the phenolic compounds showed the potential to prevent oxidative degradation of cellular components (39).

The HPLC study showed that the plant contains various phenolic compounds including, Gallic acid, Malic acid, ascorbic acid, chlorogenic acid, epigallocatechin gallate, quercetin and Morin. Phytochemically analysis showed that *V. serpens* contained antioxidant constituents such as ascorbic acid, ascorbate oxidase, peroxidase and catalase (18) along with the phenolic contents identified, which could be the reason behind its hepatoprotective effect against paracetamol-induced hepatotoxicity. Additionally, there was a linear positive correlation between the total phenolic contents and antioxidant capacities of *V. serpens* (40). Moreover, one of the mechanisms in the hepatoprotection may be the presence of phytochemicals such as flavonoids, glycosides, alkaloids, tannins and coumarins present in *V. serpens* plant (17). The scientific reports also indicated the hepatoprotective role of certain flavonoids, triterpenoids and steroids in toxicity (41-43).

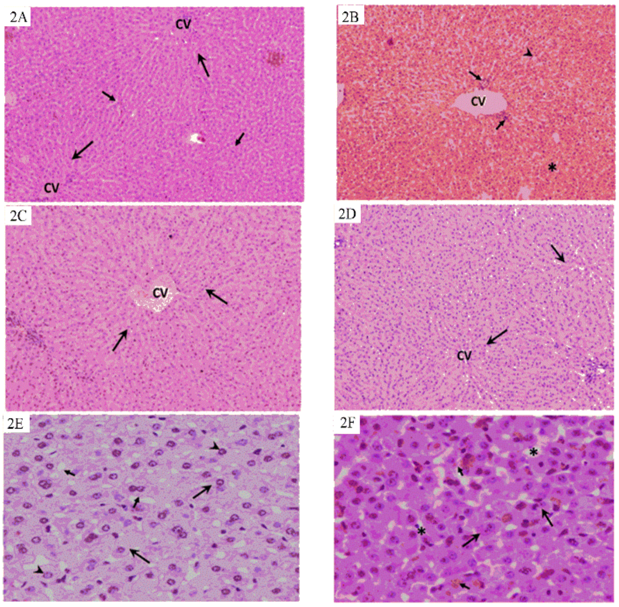

Figure 2. Normal saline-treated liver [2A] showing the normal architecture of central vein (CV), sinusoidal spaces (small arrows), hepatocytes (large arrows) with a centrally placed nucleus and foamy cytoplasm. (100X H&E); [2B]: Liver showing accumulation of lymphocytes (small arrows) around the central vein (CV), fatty changes (small arrow head) and focal area of necrosis (asterisk) with PCM (100X H&E); [2C]: Liver showing regeneration, containing normal liver plates (large arrows) along the central vein with n-hexane 150 mg/kg (H&E) of *Viola serpens*; [2D]: Liver showing normal appearance of central vein and plates of hepatocytes (large arrows) with n-hexane 300 mg/kg of *Viola serpens* (100X H&E); [2E]: Liver showing hexagonal hepatocytes (large arrows) with prominent cell borders (small arrows), nuclei (arrow heads) with nuclear clearing and prominent nucleoli with crude extract of *Viola serpens* at 150 mg/kg (400X H&E); [2F]: Liver showing regeneration of hepatocytes (large arrows) with congestion of sinusoids (asterisks) containing red blood cells (small arrows) with crude extract of *Viola serpens* at 300 mg/kg (400X H&E).

Purely paracetamol treated rabbit groups histopathology showed cellular swelling and vacuolation of the hepatocytes. Fatty changes with swollen vacuoles and decreased sinusoidal spaces due to increased cell sizes have also been indicated. The histological slides of crude extract of the plant both at low and high doses showed significant recovery of the paracetamol-induced toxicity. The mentioned biochemical constituents in the extract showed the presence and recovery of the toxified hepatocytes which is dose-dependent. The histopathology of rabbits treated with the plant fractions showed protective effects. The effectiveness was more at low doses than high doses whereas, the case was reversed in *n*-butanol.

Conclusion

It is concluded from the present study that the crude extract and the different fractions of *V. serpens* Wall. possess strong hepatoprotective activity guided strongly by the HPLC study showing the presence of various phenolic compounds and thus provided a scientific rationale for the uses of the plant in the treatment of liver toxicities. In this regard, a further detailed study regarding phytochemistry and pharmacology is required to ascertain its chemical background.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgment

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Ethical Approval

The study was approved by the Departmental Research Ethical Committee (DREC) with reference no: DREC/20160524-1 dated 24/05/2016.

Statement of Human and Animal Rights

All the experimental procedures were involving animals were according to the standard animal protocol approved by the Departmental Research Ethical Committee of the University of Malakand, Pakistan.

Statement of Informed Consent

As there is no human subject in this study, therefore a statement of informed consent is not applicable.

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