Govaniadine Ameliorates Oxidative Stress, Inflammation, and Kupffer Cell Activation in Carbon Tetrachloride-Induced Hepatotoxicity in Rats

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ABSTRACT: Liver diseases such as hepatic carcinoma are one of the main health problems worldwide. Herbal drugs are largely used to treat liver injury in the indigenous system of medicine and may provide lead compounds for hepatoprotective drug discovery. The present study is investigated to test the Corydalis govaniana Wall. extract, fraction, and isolate therapeutically active constituents to explore their hepatoprotective, anti-inflammatory, and antioxidant activities. For this purpose, the antioxidant activity of govaniadine, caseadine, caseamine, and protopine was performed by assessing the scavenging events of the stable 2,2-diphenyl-1-picrylhydrazyl. Hepatoprotection of govaniadine was assessed in terms of reduction in serum enzymes (alanine aminotransferase, aspartate transaminase, and alkaline phosphatase) caused by CCl₄-induced liver injury in rats and by histopathological techniques. All the compounds showed significant antioxidant activity with a percentage inhibition of 92.2, 86.7, 85.3, and 79.7, respectively, compared to propyl gallate 90.3%. Treatment with govaniadine reduced the serum enzyme level down to normal levels in the CCl₄-treated group while inhibiting the increase of malondialdehyde, and the induction of superoxide dismutase and the glutathione level was upregulated. Histopathology showed ~47% damage to the liver cells in the CCl₄-treated group; reduction in this damaged area was found to be better upon using govaniadine. Immunohistochemistry results showed that govaniadine as compared to silymarin has exceedingly decreased the inflammation by halting the CCl₄-induced activation of hepatic macrophages. In carrageenan-induced paw edema assay, govaniadine significantly alleviated the edema after 1−5 h at a dose of 20 mg/kg (26.00 and 28.5%), 50 mg/kg (22.05 and 27.0%), and 100 mg/kg (20.02 and 25.30%), respectively. The results of our experiments suggest that govaniadine showed antioxidant and hepatoprotective activity in liver injury. The hepatoprotective function of govaniadine may be associated to the scavenging of the free radical and attenuation of oxidative stress as well as inflammatory responses in the liver. Hence, govaniadine may be a lead compound for the hepatoprotective drug discovery process and further research is needed to find out their molecular mechanism of protection.

1. INTRODUCTION

Liver is the most important organ predominantly responsible for the metabolism of drugs, alcohol, and foreign chemicals; hence, it is vulnerable to injury that results in different liver diseases such as hepatitis, fulminant hepatitis, cirrhosis, and hepatocellular carcinoma. Liver also performs detoxification of variety of chemicals, such as CCl₄, thioacetamide, paracetamol, environmental pollutants, and alcohol. During the process of detoxification, the liver undergoes oxidative stress leading to liver disorders.¹ Despite chemicals and drugs, a number of pathogenic microbes such as bacteria and viruses play a major role in the injury and malfunction of liver. Worldwide, hepatitis is a major cause of morbidity and mortality with an incidence of approximately 50 million. The occurrence of hepatitis is more common in developing countries;² in Pakistan, there is a population of 7.6% suffering with hepatitis B and approximately 4.8% with hepatitis C infection. Complications of persistent liver injury such as liver cirrhosis are challenging adversaries because of unavailability of specific treatment. There are numerous drugs which can cause liver-related morbidity and mortality around the globe.³ Carbon tetrachloride is well-characterized, known, and extensively used in animal model of acute and chronic, oxidative stress-mediated hepatotoxicity.⁴ CCl₄-induced acute and chronic hepatic injury
C. govaniana kidney injury is due to their phagocytic function. 13,14 It has been widely known from experimental studies to investigate the potential of natural products and their bioactive constituents for the treatment of liver injury and their antioxidant activities. 15 CCl4 is responsible for the production of a number of reactive oxygen species via cytochrome P450 thereby initiating liver damage. 8 During the process of CCl4 metabolism, the cytochrome P450 produces free radicals, such as trichloromethyl (CCl3) and lipid peroxide, and causes membrane damage 9 leading to hepatotoxicity. 10,11 Carrageenan-induced inflammation is an extensively studied in vivo model of acute inflammatory response. Inflammation is a basic protective response to various pathological diseases and anticipated to abolish the cause of injury. The inflammatory process is closely connected with the increased expression of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α. 12 A lot of factors can contribute in the progression of inflammation, for example, bacterial, viral, and parasitic infections and allergic reaction. 13 Some physical factors, tissue infarction, irritation, and corrosive chemicals can also contribute to inflammation. A rapid response to injury, microbes, and foreign substances can be a consequence of acute inflammation. However, chronic inflammation is a prolonged process, in which acute injury and pathological condition proceed simultaneously which results in athero-sclerosis, arthritis, cancer, allergies, and autoimmune diseases. 14

Inflammation is either treated with nonsteroidal anti-inflammatory drugs (NSAIDs) or steroid anti-inflammatory-immunity drugs (SAIDs). NSAIDs, are considered to be the potent anti-inflammatory, antipyretic, and analgesic medications with cyclooxygenase (COX) enzyme inhibitory activity. 15,16 SAIDs mainly comprised anti-inflammatory glucocorticoid (GC) drugs such as prednisone acetate and dexamethasone acetate. The GC drugs show anti-inflammatory effects in two ways either to enhance or inhibit inflammatory genes by binding with GC receptors. 17 Although these NSAIDs can help in treating many inflammatory diseases, they have some side effects causing gastrointestinal and liver injury.

Acute liver inflammation is characterized by infiltration of macrophages, T-cells, and neutrophils. 17 Kupffer cells are actively involved in the removal and clearance of microbes from circulation because of their phagocytic function. 13,14 Although Kupffer cells exist at the injured site of liver caused by viruses, bacteria, or chemicals and facilitate the recruitment of other inflammatory cells, 15 the activated Kupffer cells contribute to the development of hepatic injury by releasing both toxic metabolites [reactive oxygen species (ROS)], several cytokines, and chemokines which promote inflammatory responses. 18 Hepatic injury can be treated by hindering the process of oxidative stress that leads to inflammatory responses. 17

Nowadays, a number of hepatoprotective medications have been widely used for the treatment of liver ailments, while several of them have possible adverse effects. Recently, bioactive compounds from medical plants have been considered to be one of the most safe and effective treatment option for hepatic ailments. 19 Several studies reported that antioxidants and anti-inflammatory agents are helpful and beneficial in controlling the progression and development of liver injury. 13,14 The genus Corydalis is native to the Himalayan regions of Pakistan, India, China, and Nepal and is also reported in the Eastern Africa. 17 Corydalis govaniana Wall. is a perennial and glabrous herb and grows in damp and shady places at an altitude of 2400–4800 m. 20 Extracts, fractions, and pure compounds from different species of Corydalis showed biological activities against hepatitis, liver cancer, and other microbes. 21 Crude and pure govanadine isolated from C. govaniana Wall. showed significant anti-leishmanial activity. 22 Roots of this plant have been used against cutaneous infections, syphilis, scrofula, along with diarrhea and dysentery. 23 Several alkaloids have been reported from Corydalis species and were investigated for a number of pharmacological activities such as antioxidant, sedative, and anticancer. 23,24 Alkaloids (tetrahydroprotoberberine) from genus Corydalis are recognized as a novel type of antimalarial agents and dopamine receptor ligands. 26,28 C. govaniana Wall. is used for liver disease by local people living in mountainous region of India and Nepal. 29 Hepatoprotective potential of protopine has been already reported. 30 Isoquinoline alkaloid reported in literature has shown hepatoprotective effects against CCl4-induced hepatotoxicity. 31 An ethnobotanical use, potent hepatoprotective effect of close compounds, and significant antioxidant activity of govanadine in vitro has given an idea to investigate their in vivo hepatoprotective and anti-inflammatory effects. Although scientific research regarding the therapeutic potential of C. govaniana Wall. is required, an endeavor to provide scientific evidence, we tested extracts and pure compounds isolated from C. govaniana Wall. for their antioxidant, anti-inflammatory, and hepatoprotective potential in rats.

2. EXPERIMENTAL SECTION

2.1. Plant Material. The whole plant of C. govaniana Wall was collected from Langtang, Nepal, and identified by Mr. Sanjiv Kumar Rai, a taxonomist at Department of Plant Resources, Thapathali, Kathmandu, Nepal. A voucher specimen, CG-207, has been deposited in Central Department of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

2.2. Extraction and Isolation of Compounds. Extraction, isolation, and structural elucidation of compounds 1–4 have been reported in our previous publication. 32

2.3. DPPH Free Radical Scavenging Assay. The free radical scavenging capacity of these compounds was measured by assessing the scavenging potential of the 2,2-diphenyl-1-picrylhydrazyl (DPPH). 33 Test compounds were allowed to react with a stable free radical of DPPH, prepared in ethanol at a final concentration of 300 mM for half an hour at room temperature. After incubation, the decline in absorption was recorded at 515 nm using the plate reader (SpectraMax-384), while dimethyl sulfoxide was used as the control. All of the chemical reactions were carried out in triplicate to a volume of 200 μL. The results were analyzed with the help of SoftMax Pro software (Molecular Devices, CA, USA) and finally by MS Excel. The percent (%) inhibition was calculated as follows: % Inhibition = 100 − (OD of the test sample/OD of the control) × 100. The experimental outcomes were presented as means ± SEM, as indicated in Table 1. IC50 values were determined by using EZ-FIT, enzyme kinetics software by Perrella Scientific, Inc., Amherst, USA.

2.4. Animals. Male Wistar rats, 200 to 220 g, were kept in individual cages at 22 to 26 °C under 12 h light/dark cycles, with access to chow and tap water ad libitum. All processes
Table 1. DPPH Radical Scavenging Activity of Govaniadine, Caseadine, Caseamine, and Protopine Alkaloid Compounds 1–4a,b

| Compound | % DPPH radical scavenging activitya | IC50 ± SEM (μM) |
|----------|-------------------------------------|-----------------|
| 1        | 92.2                                | 83.7 ± 1.7      |
| 2        | 86.7                                | 75.7 ± 0.9      |
| 3        | 85.3                                | 40.8 ± 0.9      |
| 4        | 79.7                                | 156.3 ± 1.3     |

aPropylgallate was used as standard for radical scavenging activity.

Involving animals and their care were performed according to the procedures approved by the Institutional Ethical Committee for Care and Use of Laboratory Animal (Animal Study Protocol #2016-0002), Department of Biosciences, COMSATS University, in accordance with national guidelines.

2.5. In vivo Hepatoprotective Activity. The experimental animals were allocated into seven groups of six rats each. The normal control (group 1) was injected intraperitoneally with vehicle only (1 mL/kg body weight olive oil); acute liver injury model (group 2) was induced with intraperitoneal injection of CCl4 (1 mL/kg) with 1:1 olive oil; positive control (group 3) was intraperitoneally injected with CCl4 (1 mL/kg) with 1:1 olive oil and silymarin 100 mg/kg/day (oral), for 3 consecutive days before CCl4 treatment and 1 day after treatment; groups 4, 5, 6, and 7 (govaniadine, caseadine, caseamine, protopine treated) were injected intraperitoneally with CCl4 (1 mL/kg) with 1:1 olive oil but also received govaniadine, caseadine, caseamine, and protopine at a dose of 100 mg/kg/day (oral), for 3 consecutive days before CCl4 treatment and 1 day after treatment.

2.6. Assessment of Oxidative Stress Markers. The liver tissues were homogenized in phosphate buffer saline (PBS) (40 mM), at pH 7.4 (Kinematica, Lucerne, Switzerland). Beckman L7-65 Ultracentrifuge (Beckman, Fullerton, USA) was used at 15,000 g for 20 min, at 4 °C for separation of the supernatant. Furthermore, the supernatants were subjected for Cu/Zn superoxide dismutase (SOD) activity, malondialdehyde (MDA), and glutathione (GSH) content. At 550 nm, the absorbance is triggered by these compounds because of radical disassociation from purple to yellow. These compounds isolated from C. govaniana Wall. significantly reduced the DPPH. The results of these compounds expressed in percentage scavenging were provided with a 50 μL of 1% carrageenan solution, injected subcutaneously into the left hind paw. After carrageenan injection, the paw volume was measured at 1–5 h using a digital plethysmometer. The paw swelling was compared with the treated and normal control group, while the reduction in paw edema was expressed as percent inhibition

%Inhibition = (A − B)/A × 100

where A is the paw volume of carrageenan-induced treated group, while B is the treated group at different doses.

2.8. Histology and Blood Biochemistry. 48 h after CCl4 intoxication, blood samples were collected by cardiac puncture and serum was separated for determination of ALT (alanine aminotransferase), AST (aspartate transaminase), AlP (alkaline phosphatase), albumin, and glucose using a dry chemistry analyzer (Roche Diagnostics, Mannheim, Germany). While assessment of the lipid profile (total cholesterol, low-density lipoprotein, high-density lipoprotein, and triglyceride) was done via standard AMP diagnostic kits (Stattoberger Strasse 31b 8045 Graz, Austria), hepatic tissues were removed rapidly and fixed in 10% neutral-buffered formalin for 24 h, dehydrated through a graded series of alcohol, embedded in paraffin, and cut into 6 μm thick sections. The tissue sections were stained with Hematoxylin–Eosin (H&E) staining and were examined under a bright field microscope (Nikon 90i) at different magnifications. The histopathological study of the liver under different conditions was conducted as follows. First, the damaged area was measured in 30 different hepatic sections via NIS-elements software from Nikon, Japan, and then was expressed as percentage of the total area of the section.

2.9. Immunohistochemistry Analysis. 4–6 μm thick hepatic sections were used for immunohistochemistry, as described. The tissue slides were deparaffinized in xylene, dehydrated via graded alcohol, and finally treated with PBS. The hepatic sections were then incubated with primary antibodies for an hour for hepatic macrophages; clone ED1 (diluted 1:100). Subsequently, washing with PBS, the sections were then incubated with the secondary antibody, FITC-conjugated goat anti-mouse IgG (1:100), for 45 min and rewarshed with PBS for few minutes. Finally, the slides were counterstained with DAPI (4’,6-diamidino-2-phenylindole) and mounted. The expression profile and cellular localization of hepatic macrophages/Kupffer cells were evaluated through multichannel fluorescence microscopy (Nikon 90i, Japan).

2.10. Statistical Analysis. The data were expressed as mean ± SEM, and statistical differences at P < 0.05 between the groups were analyzed via one-way ANOVA followed by Dunnett’s multiple comparison tests using SPSS 15.0 software.

3. RESULTS

3.1. DPPH Scavenging Activity of C. govaniana Alkaloids. In this activity, the DPPH, a well-known stable free radical with a deep violet color, reduces to a stable diamagnetic molecule by reacting with any substance that can donate a hydrogen atom to it. The effect of govaniadine, caseadine, caseamine, and protopine alkaloid compounds on the DPPH scavenging property depends on their reaction to donate their hydrogen. The reduction in the DPPH absorbance is triggered by these compounds because of radical scavenging activity via hydrogen donation, which gives a discoloration from purple to yellow. These compounds isolated from C. govaniana Wall. significantly reduced the DPPH. The results of these compounds expressed in percentage scavenging...
of DPPH are presented in Table 1. It was investigated that the scavenging ability augmented with increasing the concentration of the test compounds in the study. The govaniadine showed 92.2% inhibition with an IC$_{50}$ of 83.7 ± 1.7 at a concentration of 60 μg mL$^{-1}$. The other compounds at various concentrations showed that percentage inhibition greater than 50% were found to be significant ($P < 0.05$). Compounds 1−4 (Figure 6) presented significant antioxidant activity (Table 1) at an inhibitory effect of 92.2, 86.7, 85.3, and 79.7%, respectively, while standard propyl gallate showed 90.3% inhibition.

3.2. Alkaloids from C. govaniana—Reduced Histological Changes. In the normal control group, histological analysis displayed well-characterized central vein bordered by hepatic cord of cells with prominent sinusoidal spaces lined by endothelial cells (Figure 1A,B, green arrow). However, after CCl$_4$ treatment, the liver showed necrosis with prominent pale areas around the damaged central vein (Figure 1C,D, red arrow). These areas represent damaged hepatocytes and the existence of mixed inflammatory cells. This specifies that CCl$_4$ only damages the hepatocytes, as it is evident that the free radicals are only produced in the hepatocytes, where it causes membrane damage. Characteristic hydropic degeneration of hepatocytes was obvious in the pale necrotic areas around the central vein. In contrast, healthy hepatocytes (Figure 1A,B, green arrow) were also detected at the vicinity of the necrotic area and could be easily distinguished from damaged cells because of their darker staining characteristic with H/E. Furthermore, histopathological investigation (Figure 2B) revealed ~47% damage in the CCl$_4$-treated group. In contrast, the silymarin-treated group in comparison with the CCl$_4$-treated group indicated reduction ($P < 0.001$) in the necrotic area around the central vein (Figure 1E,F). Upon treatment of govaniadine, the infiltration of inflammatory cells as well as injury to the hepatocytes around the central vein exceedingly reduced (Figure 1G,H). However, treatment with caseadine, caseamine, and protopine has still some injury but it has been protected to some extent with some inflammatory cells surrounding the injured portion of liver, as shown (Figure 1I−N), respectively.

3.3. Alkaloids from C. govaniana—Attenuated Hepatic Enzyme Release. As presented in Figure 2A, CCl$_4$-intoxicated group indicated elevated levels of hepatic enzymes (ALT, AST, and ALP) which signifies the destruction of the hepatocytes, while the silymarin treatment somehow reduced the level of ALT, AST, and ALP. However, (~10%) damage was detected by histopathological analysis (Figure 2B) in the silymarin-treated group. Interestingly, the CCl$_4$ + govaniadine showed 3.5% damage (Figure 2B) at a dose of 100 mg/kg body weight with some sign of necrosis being observed in the central vein region (Figure 1G,H). The caseadine at a dose of 100 mg/kg showed slight improvement in the histology of liver, but still some necrotic cells were present surrounding the central vein (Figure 1I,J); however, it showed good hepatoprotective potential ($P < 0.001$) in comparison with the positive control silymarin at a dose of 100 mg/kg while also reduced the ALT, AST, and ALP enzyme level (Figure 2A) with injury up to 10% (Figure 2B). Similarly, caseamine-treated group revealed protective effects comparable to the positive control silymarin with some sign of inflammation, necrosis (Figure 1K,L), and 13% damage (Figure 2B) surrounding the central vein region. However, treatment with

![Figure 1. Hepatoprotective activity of alkaloids from C. govaniana. Histopathology of liver showing normal (green arrows) central vein, hepatic cords, and sinusoids in the untreated normal control (normal, A,B); pale necrotic areas (red arrows) after CCl$_4$ treatment in the control group (CCl$_4$, C,D); protection by the positive control 100 mg/kg silymarin (CCl$_4$ + silymarin, E,F); protection by govaniadine (CCl$_4$ + govaniadine, G,H) at a dose of 100 mg/kg. Caseadine (100 mg/kg)-treated liver showing some normal (green arrows) and a little injured (red arrows) hepatocytes around the central vein (CCl$_4$ + caseadine, I,J); in caseamine-treated group necrotic areas are evident (100 mg/kg) after CCl$_4$ treatment (CCl$_4$ + caseamine, C,D); however, more injury was noticed in the group treated with protopine (CCl$_4$ + protopine, M,N). Govaniadine protection is remarkably better than that of silymarin. Images were acquired at 10×, scale bar 200 μm, and for 20×, 100 μm scale bar.](http://pubs.acs.org/journal/acsodf)
Figure 2. Quantification of the effects of C. govaniana alkaloids on CCl₄-induced hepatic injury: (A) serum ALT, AST, and ALP levels as markers of hepatic injury under various conditions. Note that the govaniadine indicated complete protection against CCl₄-induced hepatic injury as compared to CCl₄ (**P < 0.001) or CCl₄ + silymarin (**P < 0.001). Caseadine has appreciably reduced the ALP, AST, and ALT levels up to some extent (**P < 0.001), even better than silymarin. Caseamine and protopine have shown some activities but still have shown an elevated level of ALT compared to their counterpart. (B) Percent damage of the liver as evaluated by histology under various conditions. Note that CCl₄ damaged 47% area around the central vein, while 10% damage was seen in the silymarin group; however, govaniadine significantly revealed only 3.5% (**P < 0.001), which were completely absent with higher doses. Similarly caseadine, caseamine, and protopine significantly (**P < 0.001) reduced the damage down to 10, 13, and 19% respectively, as shown in (B).

Table 2. Effect of Alkaloids from C. govaniana on Lipid Profile, Albumin, and Glucose

| treatment groups       | triglycerides (mg/dl) | total cholesterol (mg/dl) | high density lipoprotein (mg/dl) | low density lipoprotein (mg/dl) | albumin (mg/dl) | glucose (mg/dl) |
|------------------------|-----------------------|---------------------------|----------------------------------|-------------------------------|----------------|-----------------|
| Control                | 7.82 ± 0.45++         | 6.13 ± 0.25++             | 3.62 ± 0.21++                    | 2.48 ± 0.32++                 | 2.92 ± 0.11    | 110.33 ± 10.46  |
| 0.5 ml/kg CCl₄        | 11.13 ± 0.58**        | 11.22 ± 0.23**            | 2.83 ± 0.18**                    | 8.42 ± 0.17**                 | 1.8 ± 0.13**   | 152.14 ± 12.29**|
| 50 mg/kg silymarin + CCl₄ | 8.51 ± 0.44++       | 8.72 ± 0.20++             | 3.24 ± 0.23++                    | 2.52 ± 0.28++                 | 1.89 ± 0.08    | 145 ± 8.07      |
| 20 mg/kg gov + CCl₄  | 7.93 ± 0.90++         | 6.94 ± 0.52++             | 3.82 ± 0.28++                    | 2.68 ± 0.29++                 | 2.02 ± 0.21**  | 134.16 ± 15.61**|
| 50 mg/kg gov + CCl₄  | 7.92 ± 0.033***       | 5.570 ± 0.031***          | 3.87 ± 0.031***                  | 2.72 ± 0.030***               | 2.15 ± 0.23**  | 129.16 ± 14.17**|
| 100 mg/kg gov + CCl₄ | 8.200 ± 0.018***      | 5.241 ± 0.021***          | 3.92 ± 0.020***                  | 2.77 ± 0.041***               | 2.25 ± 0.27**  | 126 ± 15.4**    |
| 50 mg/kg caseadine + CCl₄ | 8.10 ± 0.12***       | 6.120 ± 0.011***          | 4.31 ± 0.12++                    | 3.18 ± 0.23++                 | 3.11 ± 0.31**  | 124.10 ± 11.31**|
| 50 mg/kg caseamine + CCl₄ | 8.27 ± 0.10***       | 6.912 ± 0.031***          | 4.92 ± 0.31++                    | 3.71 ± 0.19++                 | 3.72 ± 0.11**  | 132.12 ± 11.31**|
| 50 mg/kg protopine + CCl₄ | 8.29 ± 0.22***       | 7.131 ± 0.063***          | 5.33 ± 0.40++                    | 3.96 ± 0.80++                 | 3.92 ± 0.22**  | 137.23 ± 12.21**|

The results were expressed as the mean values ± standard deviation in each group. ** indicates significant differences comparative to the normal group (P < 0.001), while ++ shows significant difference compared to the model group (P < 0.05 and P < 0.001, respectively).
protopine did not show complete protection (Figure 2B) with 19% injury which is almost much higher than the caseadine- and caseamine-treated groups. Moreover, in all of the tested compounds, only govaniadine-protected the CCl₄-induced hydropic degeneration and necrosis. It was clear from histological observation that the hepatocytes have preserved architecture with densely stained nucleus and clear sinusoids. These results indicate that the hepatoprotective activity of govaniadine has reduced the CCl₄-induced toxicity of hepatocytes which is comparable to the positive control silymarin.

3.4. Effect of Alkaloids from C. govaniana Treatment on Serum Indices. As presented in Table 2, after CCl₄-induced hepatic injury, serum levels of triglycerides, total cholesterol, LDL, and glucose were considerably (P < 0.05) increased with a decrease in the albumin and HDL levels. However, concurrently, the incidence of serum levels in the govaniadine-treated group was considerably lower than compared to the model group (P < 0.05), but they were higher than those in the silymarin group, as shown. Furthermore, the corresponding serum levels in the high-dose govaniadine-treated group were considerably lower.

**Table 3. Anti-Inflammatory Activity of Alkaloids from C. govaniana in Carrageenan-Induced Paw Edema**

| treatment | 1st h | 2nd h | 3rd h | 4th h | 5th h |
|-----------|------|------|------|------|------|
| saline group | 0.225 ± 0.013 | 0.226 ± 0.024 | 0.239 ± 0.012 | 0.211 ± 0.015 | 0.232 ± 0.036 |
| carrageenan group | 0.343 ± 0.017### | 0.373 ± 0.023### | 0.390 ± 0.021### | 0.492 ± 0.054### | 0.443 ± 0.026### |
| aspirin group (150 mg/kg) | 0.251 ± 0.026** | 0.253 ± 0.033*** | 0.250 ± 0.021*** | 0.330 ± 0.052*** | 0.302 ± 0.017*** |
| govaniadine (20 mg/kg) | 0.260 ± 0.022** | 0.285 ± 0.010** | 0.285 ± 0.023** | 0.290 ± 0.013*** | 0.305 ± 0.025** |
| govaniadine (50 mg/kg) | 0.225 ± 0.030*** | 0.270 ± 0.033** | 0.282 ± 0.036** | 0.277 ± 0.033*** | 0.345 ± 0.029** |
| govaniadine (100 mg/kg) | 0.200 ± 0.028*** | 0.253 ± 0.022** | 0.285 ± 0.020** | 0.274 ± 0.040*** | 0.332 ± 0.036** |
| caseadine (50 mg/kg) | 0.270 ± 0.011*** | 0.297 ± 0.020** | 0.291 ± 0.013*** | 0.280 ± 0.030*** | 0.315 ± 0.012** |
| caseamine (50 mg/kg) | 0.298 ± 0.010*** | 0.325 ± 0.032** | 0.313 ± 0.013*** | 0.291 ± 0.025*** | 0.300 ± 0.013*** |
| protopine (50 mg/kg) | 0.325 ± 0.021*** | 0.332 ± 0.013** | 0.327 ± 0.015*** | 0.293 ± 0.011*** | 0.320 ± 0.017*** |

The values were expressed as mean ± SD of paw volume and ANOVA followed by Tukey’s post hoc test. ###P < 0.001 compared to group 1. **P < 0.01, ***P < 0.001 compared to group 2. n = 6 animals per group.

**Figure 3.** Effects of C. govaniana alkaloids on the hepatic oxidative system. MDA (A), GSH (B), and SOD (C) in CCl₄-intoxicated rats. Data were expressed as the mean ± SD, n = 10. ++P < 0.01, compared to the normal control; *P < 0.05, **P < 0.01, when compared to the CCl₄ model group. Group I: normal control; group II: CCl₄ model group; group III: 100 mg/kg silymarin + CCl₄; group IV: 100 mg/kg govaniadine + CCl₄; group V: 100 mg/kg caseadine + CCl₄; group VI: 100 mg/kg caseamine + CCl₄; group VII: protopine + CCl₄.
3.5. Effect of C. govaniana Alkaloids on Oxidative Stress. To investigate the antioxidant potential of govanidine, caseadine, caseamine, and protopine against CCl4-induced oxidative stress, the MDA, GSH, and SOD contents in the liver of different experimental groups were measured. Figure 3 shows the effect of govanidine and other compounds on CCl4-induced oxidative stress. The CCl4-model group revealed significant ($P < 0.01$) elevation of the MDA level. However, the hepatic MDA level was significantly ($P < 0.05$) decreased to some extent by the govanidine at a dose of 100 mg/kg body weight compared to the positive control group as well as other compounds. On the other hand, the CCl4-intoxicated group exceedingly decreased the GSH and SOD levels compared with the normal control group ($P < 0.01$). The activities of GSH and SOD were increased up to some extent by co-administration of test compounds; nonetheless, govanidine significantly ($P < 0.05$) augmented the SOD level in a dose-dependent manner (Figure 3).

3.6. Alkaloids from C. govaniana Alleviates Carrageenan-Induced Paw Edema. As presented in Table 3, carrageenan after 1 h of administration induced significant ($P < 0.001$) paw edema in rats which persisted for 5 h. However, pretreatment with govanidine significantly reduced the paw edema after 1 and 2 h at doses of 20 mg/kg ($P < 0.01$, 26.00 and 28.5%), 50 mg/kg ($P < 0.001$ and $P < 0.01$, 22.05 and 27.0%), and 100 mg/kg ($P < 0.001$, 20.02 and 25.30%), respectively, as shown in Figure 4A–E. All the tested compounds reduced ($P < 0.001$) paw edema after 3 and 4 h of administration at doses of 20 mg/kg (28.5 and 29.0%), 50 mg/kg (28.2 and 27.7%), and 100 mg/kg (28.5 and 27.4%), and their morphological features are clear from Figure 4C,D; however, with these doses after 5 h (20, 21 and 25%) of carrageenan administration, less significant reduction ($P < 0.01$) was observed. Moreover, 150 mg/kg of aspirin exceedingly reduced paw edema after 1 h ($P < 0.01$, 25.1%) and 2–5 h ($P < 0.001$, 25.3, 28.0, 33.4, and 30.2%).

3.7. Immunohistochemistry of Hepatic Macrophages in the Liver Samples. Inflammation of the liver is linked with the triggering of Kupffer cells and their infiltration into hepatic cords, where it secretes proinflammatory cytokines.32 In the normal control group, a small number of immunoreactive Kupffer cells with enlarged nuclei was present in sinusoidal lining (Figure 5A–C). The elongated nucleus of the macrophages was identified with the help of DAPI staining, as shown in Figure 5B. It is evident from double channel fluorescence microscopy that the resident Kupffer cells exhibited elongated shape in the sinusoids (Figure 5C). In the CCl4 intoxicated group, the densely stained activated Kupffer cells were more numerous in number with mixed inflammatory cells infiltrating particularly in the damaged area around the central vein which was evident from the DAPI staining (Figure 5E,F). Silymarin treatment reduced, but not completely, the number of activated Kupffer cells around the central vein compared to the CCl4 intoxicated group which is evident from the DAPI and CD68 staining (Figure 5G–I). Excitingly, govanidine (Figure 6) significantly decreased the number of activated Kupffer cells around the central vein (Figure 5J) to the levels parallel to that of the normal control group, whereas the DAPI staining shown normal morphology of the nuclei (Figure 5K) compared to normal control and CCl4 intoxicated group. Hence, govanidine treatment reduced the number and activity of the sinusoidal Kupffer cells regardless of the CCl4 treatment as supported by the double channel immunohistochemistry (Figure 5L).

4. DISCUSSION

The inflammation process involves multiple factors comprising the triggering of inflammatory cells, secretion of proinflammatory cytokines, and inflammatory mediators. COX-2 enzyme is involved in the production of inflammatory mediator PGE2, which is responsible for the causes of inflammatory symptoms. The inflammatory process is closely associated with the release of proinflammatory cytokines.32 Therefore, the recent study aimed to evaluate the in vivo hepatoprotective and anti-inflammatory effect of C. govaniana biomarkers. CCl4 is one of the widely studied xenobiotic compounds that induce hepatotoxicity; however, carrageenan-induced paw edema is a renowned in vivo model of acute inflammatory response. It is extensively used in experimental models of acute and chronic hepatic injury to investigate hepatoprotective function of different classes of natural products.23 The pathological changes linked with CCl4-induced hepatic damage are almost similar to that of the acute viral hepatitis.24,25

The hepatoprotective drugs in can act in two ways: either to reduce the toxic effect or to normalize the hepatic mechanism which is imbalanced by hepatotoxin.26 Carbon tetrachloride is metabolized to the CCl3 radical in the hepatocytes which is
further converted to the trichloromethylperoxy radical, a highly reactive species by cytochrome P450 enzyme. Covalent binding of the trichloromethylperoxy radical to the macromolecules results in the peroxidative degradation of the membrane. This changes the permeability of plasma membranes, membrane of endoplasmic reticulum, and mitochondria, causing the loss of calcium homeostasis contributing to hepatocytes death through necrosis. Liver enzymes (ALT, AST, ALP, and bilirubin) are released from the ruptured hepatocytes into the blood stream and are conventional indicators of liver injury. Moreover, post-treatment with govaniadine and caseadine, caseamine, and protopine alkaloids has largely reduced the CCl4-induced hepatic damage. The hepatic enzyme levels improved to near normal in all-treated rats indicates that govaniadine, caseadine, caseamine, and protopine alkaloid can alleviate cell membranes and stop enzyme leakage. Precluding the assembly of free radicals and abolishing them and the protection capabilities of this plant against hepatotoxins can be considered for other possible reasons such as for the therapeutic effect of C. govaniana alkaloids.

In the current study, govaniadine, caseadine, caseamine, and protopine pretreatment inhibited paw edema at 4 h after carrageenan injection. Besides, govaniadine controlled the secretion of inflammatory cytokines and inhibited infiltration of neutrophil in cutaneous layers of epidermis in the paw of rats. Similarly caseadine and caseamine have also reduced the inflammatory symptoms to some extent compared to govaniadine. However, treatment with protopine did not halt the swelling and was not effective compared to govaniadine, caseadine, and caseamine in decreasing the inflammatory process. These findings propose that govaniadine, at least in carrageenan-stimulated paw edema, inhibits the release of cytokines and other inflammatory mediators thereby reducing

Figure 5. Effects of govaniadine on Kupffer cells. Immunohistochemistry for Kupffer cells of liver showed normal central vein (A) and hepatocyte nucleus stained with DAPI (B) with clear localization at sinusoidal spaces (C) of the normal control (A,B,C) group and increased migration of Kupffer cells (D) at the site of injury caused by CCl4 treatment (D,E,F). Silymarin treatment (G,H,I) reduced the activation of Kupffer cells to some extent (G). Normal distribution of Kupffer cells treated with 100 mg kg⁻¹ govaniadine (J,K,L). DAPI (nucleus) is characterized by blue color. Magnification 40x and scale bar is 25 μm.

Figure 6. Structure of isolated alkaloid compounds from C. govaniana (1) govaniadine, (2) caseadine, (3) caseamine, (4) protopine.
the redness and swelling of paw edema. CCl₄-treatment caused a noteworthy increase in the normal levels of serum ALT, AST, and ALP. However, these elevated levels of serum enzymes were normalized upon treatment with govaniadine (Figure 3A). Administration of alkaloids isolated from C. govaniana (1) govanadine, (2) caseadine, (3) caseamine, and (4) protopine attenuated the elevated levels of serum enzymes and caused a consequent recovery comparable to the positive control group. Histopathological data also point toward a protective effect of govaniadine against CCl₄-induced hepatic injury (Figure 1G and 2A–D). Histopathological analysis showed hepatocellular necrosis around the central vein and pale color around the injured areas with prominent hydropic degeneration of hepatocytes (Figure 1C,D). The lesions of pericentral, perilportal hepatic cells, and macrophage infiltration in the CCl₄-treated group were ameliorated in rats receiving govaniadine. Elevated levels of enzymes, ALT, and AST are one of the indicators for the loss of cell membrane functional integrity of hepatocytes and cellular leakage.30,31 However, treatment with govaniadine exceedingly alleviated the levels of serum ALT, AST, and ALP toward their normal value which is an important indicator of plasma membrane stabilization and repair of CCl₄-induced hepatic damage. Moreover, ALP elevation is a sign of pathological membrane alteration of hepatocytes and is a marker of hepatobiliary cholestasis and biliary flow.32 Hepatocellular necrosis results in elevation of serum marker enzymes, which are released into the blood stream.33–35 CCl₄-induced serum ALP alteration is in line with increased levels of serum total bilirubin. Effective control of the ALP level in the govanadine treatment group indicates an early improvement of the secretary mechanism of hepatocytes. These biochemical outcomes were further validated by histopathological findings. CCl₄-induced hepatotoxicity leads to the transfer of fatty acids to the hepatic compartment and hence increased triglyceride content in the liver tissues. Concurrently, total cholesterol is increased because of chemical-induced liver injury.36,37 Because of lipid peroxidation, the level of total cholesterol and triglyceride increases after liver injury.38,39 It is noticed that govanadine could efficiently regulate and maintain these liver functional indexes and protect the liver from lipid peroxidation compared to caseadine, caseamine, and protopine alkaloids. It has been assumed that one of the cause of CCl₄-induced hepatic damage is the development of lipid peroxides by CCl₄. Another sign of damage to hepatocytes is lipid peroxidation.40 Being, one of the fundamental organs of the body, the liver plays a vital role in carbohydrate, protein, and lipid metabolism.41,42 In the current experiment, it is observed that CCl₄ can cause increase in the total cholesterol, triglyceride, and LDL levels and decrease in the HDL level. However, decreased protein synthesis and abnormal disruption of cell membrane and phospholipids metabolism might be complex in lipoprotein levels. Thus, there is a need to investigate natural products with antioxidant activity that could inhibit generation of free radicals that are important in the protection of CCl₄-induced hepatotoxicity.43 The body has developed an effective defense system to combat and neutralize the free radical-induced injuries. Such a defense mechanism is accomplished by a set of endogenous antioxidant enzymes against ROS.44,45 The carrageenan-induced inflammation was related to reduce the activities of antioxidant enzyme and lipid peroxidation, which has been shown to control the redox state in the liver.46,47 Moreover, enzymes with antioxidant effects such as SOD, GSH, and catalase are in the cellular defense against reactive free radicals.48 In chemical-induced hepatoxicity, the antioxidant defense system may fail because of the imbalance in the production of ROS, which leads to deregulation of cellular functions, resulting in hepatic necrosis. The CCl₄-treated group reduced the activities of SOD point toward the hepatotoxicity in the rats, while the treated group at a dose of 100 mg/kg of govanadine showed an increased level of SOD, which specifies the antioxidant activity of govanadine. Regarding GSH, it is an indicator of tissue suitability to oxidative stress and the hepatic GSH depletion has been shown to be associated with enhanced CCl₄-induced toxicity.49 In this study, a diminution in the liver GSH level was perceived in the model group. The increase in the hepatic GSH level in the 100 mg/kg of govanadine may be because of de novo GSH synthesis.

Lipid peroxidation is considered as a marker of cellular injury which is also known to induce inflammatory processes. Therefore, MDA echoes the extent of free radicals to facilitate lipid peroxidation of tissue. The lipid peroxide (MDA) level indicates a measure of membrane injury and structural and functional alterations. The carrageenan-induced inflammatory response has been related to the increased production of MDA and the consequential inhibition of GSH, SOD, and GSH in the liver. Similar kind of results were found in some reported studies; an elevation in the MDA content in the edematous paw and in the activities of SOD and GSH in the liver were reduced after carrageenan for 1–6 h.48 In the current study, elevation of lipid peroxide was observed in the CCl₄-treated group. The rise in MDA levels in the liver tissues proposes increased lipid peroxidation that causes tissue damage and failure of protective mechanisms to inhibit the free radical formation.48,49 The results indicated that carrageenan-induced inflammation linked with oxidative stress via lipid peroxidation to halt the cellular antioxidant defense system. Treatment with govanadine, caseadine, and caseamine at different doses significantly reversed these changes. Hence, from the above findings, it is concluded that the possible mechanism of hepatoprotection of govanadine might be because of its antioxidant capabilities.

Phytochemical screening of C. govaniana had shown the presence of alkaloids as major compounds. The presence of these alkaloids contributes to the antioxidant and hepatoprotective activities of C. govaniana. From the above results, it is clear that bioactive alkaloid govanadine has shown dose-dependent hepatoprotective activity, which is comparable to the standard drug silymarin. The results of the current study show that govanadine may be a lead compound for the hepatoprotective drug discovery process, and further study is needed to explore the molecular mechanism of their protection.

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Notes
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