Investigational
Medicinal Chemistry & Pharmacology

Research Article Open Access

Aqueous extracts of *Desmodium adscendens* (Fabaceae) possess *in vitro* antioxidant properties and protect hepatocytes from Carbone tetrachloride-induced injury and Hepatitis C Virus infection

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Abstract

Background: *Desmodium adscendens* (DA) is an herbaceous plant found in Africa and South America and used in traditional medicine against liver-related diseases. In the present study, the aqueous decoctions from DA were tested for their antioxidant and hepato-(protective and curative) properties on CCl_4-induced acute injury on primary culture rat hepatocytes. We also investigated its inhibitory effects on hepatitis C virus (HCV) using genotype 1b subgenomic replicon systems and cell-culture derived HCV particles (HCVcc).

Methods: Five chemical antioxidants assay were used for the evaluation of antioxidant properties of aqueous extract of DA. Hepato-(protective and curative) activities of DA against CCl_4-induced hepatotoxicity in primary rat hepatocytes were assessed by measuring cell viability, alanine aminotransferase (ALT) activity leakage into the incubation medium and malondialdehyde (MDA) content as markers of lipid membrane peroxidation. Antiviral activity against hepatitis C virus (HCV) was performed using HCV genotype 1b sub-genomic replicon cell line LucUbiNeo-ET cells and HCV cell-culture derived particles (HCVcc) by measuring luciferase activity and indirect immunofluorescence respectively. Antioxidant activity was assessed, and cytotoxicity and oxidative injury were determined by assessing cell viability by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and leakage of ALT, a cytosolic enzyme. LucUbiNeo-ET cells were used as HCV genotype 1b replicons systems and were incubated with various concentrations of plant extract. The antiviral activity was assayed by measuring the luciferase activity in cell lysates by reporter gene assay.

Results: Average yield of 4.16 ± 0.44 million cells/g of liver and viability of 90 ± 1.29 % were obtained. Hepatocyte viability was not affected by increasing concentrations of plant extracts. Moreover, treatment of cells with plant extracts at concentrations of 1-1000 µg/mL before and after oxidative injury provided a cytoprotective effect to the cells by increasing the percent of cell viability, reducing ALT leakage and decreasing the formation of malondialdehyde and an antioxidant capacity was noted. A strong activity was observed with *D. adscendens* harvested before flowering (DA1) compared to its activity after flowering (DA2). DA1 extract was tolerated by LucUbiNeo-ET cells up to the concentration of 500 µg/mL after 24 h of incubation. DA1 extract at 25 µg/mL produced a significant (p<0.01) decrease in HCV infection compared to the DMSO-treated group.

Conclusions: These findings indicate that *Desmodium adsccendens* aqueous extracts possess promising hepatoprotective effects against CCl_4-induced liver injury and HCV infection. These support its traditional use for the management of some liver diseases.

Keywords: *Desmodium adscendens*, Hepatotoxicity; Primary rat hepatocytes; HCV; Carbon tetrachloride.

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Citation on this article: Chuisseu PDD, Galani BRT, Younang NCK, Kouam AF, Simo BFN, Tchana AN, Seron K, Dubuisson J, Tiegs G, Kouamouo J, Moundipa PF. Aqueous extracts of *Desmodium adscendens* (Fabaceae) possess in vitro antioxidant properties and protect hepatocytes from Carbone tetrachloride-induced injury and Hepatitis C Virus infection. *Investigational Medicinal Chemistry and Pharmacology (2020) 3(1):36; Doi: https://dx.doi.org/10.31183/imcp.2020.00036*

Invest. Med. Chem. Pharmacol. (IMCP) ISSN: 2617-0019 (Print)/ 2617-0027 (Online)

Background

The liver is one of the most important organs in the body and is primarily involved in the metabolism of endogenous and exogenous agents. It plays an important role in eliminating and detoxifying substances that are harmful to the body. During the detoxification of xenobiotics, reactive oxygen species (ROS) are generated, which initiate a series of reactions of lipid peroxides within the hepatocytes, and subsequently cause oxidative stress, cell death and liver disease, such as hepatocellular carcinoma, viral and alcoholic hepatitis and so on [1,2]. Liver disease is a major health problem worldwide, with high endemicity in developing countries [3]. Liver injury induced by chemicals, drugs, and viruses is a well-recognized toxicological problem. The pathogenesis of the damage is multifactorial, ranging from inflammation and oxidative stress to immunological and apoptotic reactions [4]. Although, there is a great advance in understanding the molecular pathology of liver injury, there are still only limited drugs capable of stimulating liver function, offering hepatic protection against damage or contributing to the regeneration of liver cells. In the search of new antihepatotoxic agents, herbal medicine is emerging as an alternative and became a solution of local, regional and global importance.

Carbon tetrachloride (CCl_4), a potent environmental hepatotoxin, has been served as a model compound for the study of hepatotoxicity and the cellular mechanisms behind oxidative damage and further was used to evaluate the therapeutic potential of drugs and dietary antioxidants [5]. Primary culture isolated hepatocytes provide the opportunity to evaluate the effects by direct interactions of the studied compounds [4].

Desmodium adscendens (Fabaceae) is an herbaceous plant found in Africa and South America, where a decoction of leaves and stems is used in traditional medicine for various indications, including the management of asthma and livers related diseases [6]. In the rainforest region, preparations from *D. adscendens* (DA) are used to treat digestive system disorders or abdominal and back pain [7]. Hydro alcoholic extract of DA moreover presents a safety against Human liver hepatocellular carcinoma cell line (HepG2) and protects them from a possible lesion [8]. It has been found that the main secondary metabolites in *D. adscendens* include flavonoids (rutin, vitexin, and isovitexin), triterpene saponins, amines, and alkaloids. Saponins are located mainly in leaves, flavonoids – flowers, and alkaloids – in leaves and stems. The qualitative composition of *D. adscendens* depends on the place of its cultivation [9,10] In the present study, the aqueous decoction from DA was tested for its antioxidant and hepato-(protective and curative) properties on CCl_4-induced acute injury on primary culture rats liver hepatocytes. We also investigated its antiviral properties using sub-genomic replicons and HCVcc systems.

Methods

Animals and reagents

In this study, *Wistar albino* rats of both sexes were used. The animals were maintained in a standard diet and water *ad libitum*. They were housed in propylene cages and exposed to 10-12 h of daylight cycle. All reagents purchased from Sigma Chemicals Company (Hamburg, Germany) and Prolabo (Paris, France) and used in this study were of analytical grade.

Adult male and female *Wistar albino* rats were used in this study. They were housed in plastic cages bred in the animal house of the Department of Biochemistry (University of Yaounde I) and placed on a semi-synthetic diet (LAVANET, Bockle, Cameroon) with free access to the tap water ad libitum. All reagents used in this study were of analytical grade and purchased from Sigma Chemicals Company (Hamburg, Germany) and Prolabo (Paris, France).

Ethical considerations

All procedures in this study followed the Cameroon National Veterinary Laboratory guidelines based on the Guide for the care and use of laboratory animals [11] and were received the visa of the Laboratory of Animal Physiology of the Faculty of Sciences, University of Yaoundé I– Cameroon.

Plant material collection and preparation of crude extracts

Freshly leaves of *Desmodium adscendens* were collected in the locality of Yaoundé (Cameroon) before flowering (November 2012) and after flowering (May 2013). The botanical identification of the plants was done at the Cameroon National Herbarium, where voucher specimens are kept under the reference number 10259/SRF Cam. The leaves were thoroughly washed, rinsed with distilled water, dried at room temperature and grounded. The powder obtained (100 g) was extracted by decoction with 3 L of distilled water for 1 h. After cooling, the mixture was filtered with Whatman grade 1 filter paper and the filtrate was dried by lyophilisation to obtain a dark green residue which constitutes the aqueous extract of *D. adscendens*, named DA1 and DA2 respectively for the leaves harvested before and after flowering.

Chemical antioxidant studies

For the following assays, plant extracts and ascorbic acid (positive control) were tested in triplicate at the final concentrations of 0.01; 0.1; 1; 10 and 100 µg/mL.

2,2-Diphenyl-Picryl-Hydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging activity of the plant sample was evaluated as previously described by Moyo et al. [12]. A volume of 3.1 mL of DPPH solution (40 µg/mL) in pure methanol was mixed with 50 µL of the test sample (plant extract or ascorbic acid) to reach the desired concentration. Then, the mixture was incubated at room temperature in the dark for 30 min, and the absorbance recorded at 517 nm against the blank (DPPH solution). Control samples were prepared with the same volume containing the lowest concentration (0.01 µg/mL) of plant extract or ascorbic acid. The percentage of DPPH scavenging was calculated according to the following equation:

Scavenging activity (%) = $100 \times [A_{control} - A_{sample}]/A_{control}$ where:

$A_{control}$: absorbance of control; A_{sample}: absorbance of sample.

Hydroxyl (OH°) radical scavenging assay

Hydroxyl radical scavenging assay was performed according to the method described by Su et al. [13]. Hydroxyl radicals were generated by the Fenton reaction of Fe^{2+} and H_2O_2. The reaction mixture consisted of 0.7 mL of $FeSO_4$ (3 mM), 1 mL of H_2O_2 (1 mM), 1 mL of distilled water, 50 µL of the test sample, and 0.4 mL of sodium salicylate (10 mM). The mixture was then incubated at 37 °C for 1 h, and the absorbance recorded at 562 nm against a blank containing all reagents except sodium salicylate. The scavenging activity was calculated using the same equation described above for the DPPH radical scavenging activity assay.

Reducing ability assay

The reducing ability of plant samples was assessed as described previously by Varshneya et al. [14]. In the test tube, 50 µL of the test sample, 1.1 mL of phosphate buffer (0.2 M; pH 6.6) and 1 mL of potassium ferrocyanide $[K_3Fe(CN)_6]$ (0,25 %) were added and mixed. After incubation at 50 °C for 20 min, 1 mL of trichloroacetic acid (10 %) was added to the mixture before centrifugation (1000 rpm, 10 min). The supernatant (1 mL) was mixed with 200 µL of ferric chloride $[FeCl_3]$ (0,02 %) and the absorbance of the green solution obtained was measured after 10 min at 700 nm against a blank containing all reagents where $FeCl_3$ was replaced by 200 µL of phosphate buffer.

Total antioxidant capacity assay

The determination of total antioxidant capacity was done by the phosphomolybdenum method as described by Blažeković et al. [15]. The tubes containing the test sample (Extracts) (50 µL) and reagent solution: 0.6 M sulfuric acid (1 mL), 28 mM sodium phosphate (1050 µL), and 4 mM ammonium molybdate (1050 µL) were incubated at 95 °C for 90 min. After cooling the mixture at room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

Inhibition of rat liver lipid peroxidation assay

The inhibition of lipid peroxidation by plant extracts and ascorbic acid was determined according to the thiobarbituric acid method. The ferrous chloride/Hydrogen peroxide complex ($FeCl_2$–H_2O_2)was used to induce lipid peroxidation in liver homogenates according to the method described by Su et al. [13]. Each test sample (50 µL) was mixed with 1 mL of a 10 % liver homogenate, and then, 50 µL of $FeCl_2$ (0.5 mM) and 50 µL of H_2O_2 (0.5 mM) were added. The mixture was incubated at 37 °C for 60 min, and then, 1 mL of trichloroacetic acid (15 %) and 1 mL of thiobarbituric acid (0.67 %) were added, and the mixture was heated to 100 °C for 15 min. After centrifugation (3000 rpm, 5 min), the absorbance of the supernatant was recorded at 532 nm. The percentage of inhibition was calculated according to the same equation described above for the DPPH radical scavenging activity assay.

Anti-hepatotoxic activity of plant extract

Isolation of primary rat hepatocytes

The rats were anesthetized using ketamine at the dose of 87.5 mg/kg of body weight and hepatocytes were isolated *in situ* by liver perfusion with collagenase according to the protocol described by Gandin et al. [16]. Hepatocytes were washed and suspended in Dubelcco's Modifield Eagle's Medium (DMEM) supplemented with glutamine (2mM), $NaHCO_3$ (0.5 g/L), penicillin (100 IU/mL), streptomycin (100 µg/mL), fungizon (5 µg/mL) and 10% fetal bovine serum. Cell viability was calculated immediately after isolation by the trypan blue exclusion test [17]. The yield of hepatocytes isolation was $4.16 \pm 0.44 \times 10^6$ cells/g of liver and cell viability percentage of 90.5 ± 1.29. After isolation, the hepatocytes were suspended in the culture medium (DMEM) at the concentration of 0.2×10^6 viable cells/mL.

Cells treatment: General procedure

In this study, carbon tetrachloride (CCl_4), plant extract and silymarin (used as reference compound) were diluted in DMSO in order to obtain a final concentration of 0.2% DMSO in the medium. Freshly isolated primary rat hepatocytes suspension (approximately 0.2×10^6 cells/mL) in triplicate were distributed into 1.5mL Eppendorf tubes labeled as control, CCl_4, and test (silymarin or plant extract + CCl_4). Thereafter, cells were incubated at 37 °C into a CO_2 incubator (5% CO_2, 95% air) in the absence (control group) or presence of CCl_4 alone (CCl_4 group), or in presence of CCl_4 and silymarin or plant extract (test group) for 6 h. Afterward, supernatants and cells were collected for biochemical analysis (cell viability, cell plasma membrane integrity and lipid peroxidation assessments)

Effect of plant extract on cell viability

Cells were treated with plant extract at the final concentrations of 1; 10; 100 and 1000 µg/mL. After 6 h, cells were harvested by centrifugation (720×g, 5 min, 4°C) and aliquots of supernatants were used for biochemical analyses.

Determination of the toxic concentration of CCl_4 to be used

Cells were treated with CCl_4 at the final concentration of 1, 2, 4, 6, 8, 10, 12, 15 and 20 mM. The mixture was incubated for 6 h and cells were harvested by centrifugation (720×g, 5 min, 4 °C) for cell viability measurement and the half lethal concentration (LC_{50}) of CCl_4 was determined and served as a toxic indicator concentration for the next experiment.

Concentration-response study of the hepato-(protective/curative) activity of plant extract

The plant extract and silymarin were tested at the final concentration of 0.01; 0.1; 1; 10 and 100 µg/mL in pre-treatment (protective) and in post-treatment (curative). In the pre-treatment test, cells were treated with plant extract or silymarin for 30 min before the addition of CCl_4 (LC_{50}). In the post-treatment test, cells were incubated in the presence of CCl_4 (LC_{50}) for 30 min before the addition of plant extract or silymarin. After 6 h, cells were harvested by centrifugation (720×g, 5 min, 4°C) and aliquots of supernatants were used for biochemical analyses.

Biochemical assays

Cell viability

Cell viability of rat hepatocytes was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay [18]. After incubation, cells were washed with 500 µL of phosphate-buffer saline and 300 µL of MTT dissolved in PBS at a concentration of 0.5 mg/mL was added. After 90 min of incubation,

tubes were centrifuged (720xg, 4 °C, 5 min) and the MTT solution was discarded by aspiration. The remaining formazan crystals were dissolved with 300 µL of acidified isopropanol. Optical density was then read at 560 nm using a microplate reader (Microreader V-320). Cell viability in each test group and the toxicant group was expressed as a percentage of the control group. Cells in the control group were considered as 100% viable.

Cell plasma membrane integrity assessment

Hepatocyte plasma membrane integrity was assessed by measuring the enzyme Alanine Aminotransferase (ALT) activity in the medium [19]. Briefly, 360 µL of ALT substrate (phosphate buffer 0.1M pH 7.4 containing 0.2 M L-alanine and 2 mM α-ketoglutarate) was added to 40 µL of cellular supernatant. The mixture was homogenized and maintained at 37 °C for 30 min. Then 200 µL of 2,4-Dinitrophenyl hydrazine solution was added and the mixture maintained at room temperature for 20 min. Finally, 2mL of 0.4M NaOH was added and the optical density was read 30 min later at 505 nm (SHIMADZU-UV-120-01). ALT activity was determined by using the standardization curve established with sodium pyruvate.

Lipid peroxidation assessment

Lipid peroxidation was also assessed and the formation of malondialdehyde (MDA) in the cells was measured by the thiobarbituric acid (TBA) method [20]. Briefly, 500 µL of cellular supernatant, 100 µL of Triton X-100 solution (0.2% Triton X-100 v/v in phosphate buffer saline), 125 µL of trichloroacetic acid (20% w/v), and 500 µL of TBA (0.67% w/v) were mixed. The mixture obtained was heated at 100 °C for 20 min in a boiling water bath. After cooling for 5 min in cold water, the mixture was centrifuged (3000 × g, 15 min, 4 °C). Finally, the absorbance of supernatant collected was taken at 532 nm. MDA concentration was determined by using molar extinction coefficient (ε_{MDA} = 1.56 × 10^5 $M^{-1}.Cm^{-1}$).

Anti-HCV Bioassays

The effects of *D. adscendens* plant extracts on HCV were assessed *in vitro* using subgenomic replicons and HCVcc systems.

Huh-7 derived cell lines (LucUbiNeo-ET) stably transfected with an HCV subgenomic replicon, Con 1 and containing the luciferase gene of the firefly *Photinus pyralis*, were used as replicon systems. Cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen GmbH, Karlsruhe, Germany) supplemented with 10% fetal bovine serum, 100 IU/mL of penicillin, 100 µg/mL of streptomycin and 0.25% geneticin G418 using culture flasks in a humidified atmosphere 20% O_2 and 5% CO_2. Cells were incubated until 95% confluence and sub-cultivated every 3 days. LucUbiNeo-ET cells were seeded in 24-well plates at a density of 1 x 10^5 cells/well at 37 °C in a humidified atmosphere of 5% CO_2. Cells were incubated in the presence or absence of Telaprevir 1 µM or DA1 extract at 250, 500 and 1000 µg/mL. After 24h of incubation, the medium was removed and cells washed once with 500 µL of 1 x PBS. The luciferase activities were measured as previously described [21] . Briefly, after 24 h of incubation, the medium was discarded and cells were washed once with 500 µL of 1x PBS and lysed with 120 µL of cell culture lysis reagent (CCLR, 1/5 dilution) (25mM Tris-phosphate (pH 7.8), 2mM DTT 2mM 1,2-diaminocyclohexane-N, N, N0, N0-tetraacetic acid, 10% glycerol, 1% Triton®X-100). After an incubation period of 10 minutes on ice, the lysates were transferred into 1.5 mL microtubes and centrifuged for 1 minute at 14,000 rpm, at 4°C. Subsequently, lysates were ice-placed and supernatants were collected into fresh microtubes. The luciferase activity was measured using the luciferase assay reagent (LAR). Bioluminescence was instantly detected and quantified using a luminometer (Tecan® infinite M200). Luciferase values were normalized to the protein content of each sample.

Cell-derived infectious HCV particles (HCVcc) were produced by the transfection of Huh-7 cells with the JFH1-CSN6A4 plasmid as previously reported [22]. Stock solutions with a titer of 6 x 10^5 focus forming units/mL were aliquoted and stored on -80 °C until use. Virus titers were determined by indirect immunofluorescence assay using anti-E1 monoclonal antibodies. Briefly, Huh-7 cells were seeded at 6000 cells/well in 96-well plates and incubated overnight at 37 °C in an atmosphere of 5% CO_2. The day after, the supernatant was removed and 100 µL of the viral inoculum containing DA aqueous extract at 25 µg/mL or Epigallocatechin-3-gallate (EGCG) was added to the cells and incubated for 2 h at a multiplicity of infection of 1. After 2 h, the inoculum was replaced by 100 µL of fresh medium containing only DA1 extract or Boceprevir and incubated for 28 h. After this time, cells were washed with PBS, fixed in ice-cold methanol and used for immunofluorescence analysis. Briefly, cell nuclei were stained with 1 µg/mL of DAPI. HCV-infected cells showed focus formation that appeared as clusters of E1- positive cells. The foci were counted, and confocal images were recorded on an automated confocal microscope (IN Cell Analyzer 6000, GE Healthcare Life Sciences) using a 20X objective as described previously by Calland et al. [23]. During quantification, four fields per well were recorded randomly. Each image was then analyzed using Columbus image analysis software (PerkinElmer). Nuclei were first segmented, and the cytoplasm region was extrapolated based on DAPI staining. Infection rates were scored as the ratio of the infected cells to total cells. The infection rate of DMSO- treated cells was taken as 100%, and the relative number of infected cells was then calculated as a percentage of the number of infected DMSO-treated cells. The number of cells per well and multiplicity of infection (MOI) were calculated to obtain 30 to 40 % infected cells at 28 h post-infection.

Statistical analysis

The results are expressed as mean ± SD. Data were analyzed by a single-way analysis of variance (ANOVA) followed by a Dunnett Multiple Comparisons test, using GraphPad prism 5.0 software. A difference was considered statistically significant at $p<0.05$.

Results

In vitro antioxidant activities

DA extracts concentration-dependently inhibited DPPH radical

The antioxidant effect of DA extracts on DPPH radical is shown in Figure 1. Our findings indicate that vitamin C, used as a positive control, concentration-dependently scavenged DPPH radical with about 98% inhibition at 100 µg/mL. A similar activity was also found with DA1 and DA2 with a lesser extent. Both extracts led to about 8% inhibition (7.59% for DA before flowering (DA1) and 8.69% for DA after flowering (DA2) when tested at 100 µg/mL. The EC_{50} of vitamin C was 4.90 ± 0.10 µg/mL while the extracts had an EC_{50} > 100 µg/mL.

Hydroxyl (OH°) radical scavenging activity of DA extracts

The effects of DA extracts on HO° radical are presented in Figure 2. As shown in this figure, the OH° scavenging activity by DA extracts was significantly lower than that of vitamin C. DA1 and DA2 exhibited a concentration-dependent activity, with the better activity observed at the dose of 100 µg/mL. The EC_{50} of the extracts were > 100 µg/mL.

Reducing abilities of DA extracts

The reducing powers of ferric ions by aqueous extracts of *D. ascendens* are presented in Figure 3. At 100 µg/mL, DA1 and DA2 showed a reducing ability significantly (p<0.001) higher than in the control group by 0.55 and 0.28, respectively. A concentration-dependent effect was observed with DA1 at 10 and 100 µg/mL while DA2 only exhibited a significant effect at 100 µg/mL. Vitamin C displayed a concentration-dependent inhibition with an IC_{50} < 10 µg/mL, which suggests that the reference compound was found more active than plant samples.

Total antioxidant capacity of DA extracts

The total antioxidant capacity of DA extracts expressed as ascorbic acid meq/g extract of the extract is presented in Figure 4. According to this figure, all these extracts have a notable antioxidant capacity on the reduction of molybdate. Vitamin C, DA1 have respectively an IC_{50} of 1.11 ± 0.09 µg/mL and 47.32 ± 12.38 µg/mL while the IC_{50} of DA2 is > 100 µg/mL

Inhibition of rat liver lipid peroxidation assay

Figure 5 shows the effects of DA extracts on the *in vitro* inhibition of the lipid peroxidation induced in rat liver homogenates. At 100 µg/mL, DA1 and DA2 exhibited respectively 14.66 and 11.28% inhibition. Compared to vitamin C (IC_{50} 13.28 ± 0.55 µg/mL), both extracts have a moderate inhibition of lipid peroxidation (IC_{50} > 100 µg/mL) as shown in the figure.

In vitro hepato- (protective/curative) activities

Effect of DA extracts on cell viability and ALT leakage

The hepatocyte viability in the pretreatment with plant extracts (Figure 6a) is not affected by increasing concentrations of DA1 extracts, unlike DA2 which significantly decreases the viability of hepatocytes from 100 µg/mL. Low concentrations (1 and 10 µg/mL) of DA1 and DA2 significantly decrease the release of ALT in the culture medium (Figure 6b). However, at high concentrations (1000 µg/mL), all the extracts negatively affect the membrane integrity characterized by the increase in the activity of ALT.

Determination of toxic concentration of CCl4 using effect on cell viability and ALT leakage

Incubation of hepatocytes in the presence of CCl_4 at a concentration range of 1–20 mM caused a marked decrease of cell viability (Figure 7a) and an increase of ALT leakage (Figure 7b) into the incubation medium after 6 h. The LC_{50} was found to be 3.90 ± 1.28 mM, and 5 mM was used as the toxic concentration in this work.

Effect of pre-treatment with plant extracts before CCl_4 poisoning on hepatocyte viability, ALT activity, and MDA level.

Incubation of cells with CCl_4 (5 mM) for 6 h resulted in a significant decrease in cell viability (p<0.01) (Figure 8a), increase in ALT leakage (p<0.001) (Figure 8b) and MDA formation (p<0.001) (Figure 8c) in toxicant group. Pretreatment of hepatocytes with the plant extracts (DA1 and DA2) or silymarin before intoxication induces a concentration-dependent protective and inhibitory effect on cell viability, ALT leakage, and MDA production.

Hepatocytes pretreated with the DA1 and DA2 extracts (figure 8a) show a better significance (p<0.05) of the increase in cell viability at a concentration of 1 µg/mL, compared with the CCl_4 group. The effects on ALT activity (Figure 8b) corroborate these observations. Indeed, DA1 and DA2 induce a better reduction (p<0.001) of ALT at this concentration. Besides, at this same concentration, DA1 and DA2 significantly (p<0.001) decrease the level of MDA (Figure 8c).

Silymarin, a hepatoprotective reference compound, exhibits a significant increase (p<0.05) in cell viability from the dose of 1 µg/mL and a significant decrease (p<0.001) in the release of ALT and MDA production.

Hepatocurative effect of plant extracts after CCl_4 poisoning on hepatocyte viability, ALT activity, and MDA level.

The intoxication of hepatocytes with CCl_4 and treatment with plant extracts and silymarin induced the same effects on these parameters as indicated earlier. Cell viability percentage, ALT activity and MDA concentration in the incubation medium are presented in figure 9.

Figures 9a and 9b respectively represent the measurement of cell viability and ALT activity when the hepatocytes are preincubated in the presence of CCl_4, then treated with plant extracts.

In the hepatocurative therapy, silymarin (standard) and DA1 and DA2 extracts significantly (p<0.001) and dose-dependent increase the viability of hepatocytes (Figure 9a). This result corroborates their effect on the activity of ALT which decreases (p<0.001) according to the increasing concentrations of these extracts and silymarin (Figure 9b). The best activity was observed at a concentration of 100 µg/mL. However, Silymarin, DA1 and DA2 extracts significantly (p<0.01) decrease in a concentration-dependent manner the level of MDA (Figure 9c).

Anti-HCV activities

Desmodium adscendens-mediated inhibition of HCVcc infection

Considering the hepatoprotective activities of *D. adscendens* on oxidative stress, we hypothesized its effects on HCV infection. To confirm this hypothesis, we infected Huh-7 cells with HCVcc particles in the presence or absence of DA1 extract for 30 h and quantified the expression of glycoprotein E1 in treated cells by indirect immunofluorescence assay as described in the methods section. As shown in Figure 10, DA1 extract at 25 µg/mL significantly (p<0.01) reduced HCV infection in HCVcc systems as compared to the DMSO-treated group. A more pronounced response was found with Boceprevir and EGCG used as standard compounds.

Inhibition of HCV replication by DA1 aqueous extract

To evaluate the effect of DA1 extract on HCV replication, we used LucUbiNeo-ET cells which contain an HCV subgenomic RNA

replicon and express the luciferase enzyme as a reporter system. Figure 11 shows a significant (p<0.01 and p<0.001) reduction in luciferase values at the different tested concentrations of DA1, comparatively to the DMSO-treated group, indicating, therefore, an efficient inhibition of HCV replication. However, this decrease was lower than those of Telaprevir 1 µM used here as a positive control.

Discussion

In the present study, we evaluated the capacity of *D. adscendens* to protect primary rat hepatocytes against CCl_4-induced acute injury and its ability to mediate inhibition of HCVcc infection. Since the changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis [24], CCl_4-mediated hepatotoxicity was chosen as the experimental model. It has been known that oxidative stress and the generation of free radicals play a critical role in CCl_4-induced liver injury [4]. The mechanism by which CCl_4 causes cell oxidative injury involves that cytochrome P-450 system transforms CCl_4 into CCl_3 and the latter is transformed into a more reactive CCl_3O_2 which causes lipid peroxidation, disturbs Ca^{2+} homeostasis and leading to cell death [25,26]. CCl_4-induced hepatic injury is commonly used as an experimental method for the study of hepatoprotective effect of medicinal plants [27]. The use of rat hepatocytes culture reduces the number of animals sacrificed for hepatoprotective activity study [28]. Furthermore, isolated hepatocytes with high yield have become a valuable tool for assessing the possible protective effect of drugs. It has been shown that certain hepatotoxins such as carbon tetrachloride cause a reduction in cell viability as well as an increase in certain markers [27,28]. Indeed, there is on the one hand the increase in the level of transaminases (ALT), cytosolic enzymes of which their elevation in the extracellular medium is a sign of alteration of the cell membrane integrity, and on the other hand MDA increase concentration, which indicates a membrane lipid peroxidation [27,28]. The ability of a hepatoprotective drug to reduce the injurious effects, or to preserve the normal hepatic physiological mechanisms which have been disturbed by a hepatotoxin, is an index of its protective effects [27].

Overall, in this study, there is a high yield of isolated hepatocytes and general modifications through the evaluation of markers, thus reflecting satisfaction with the standardization of our chosen experimental model. Indeed, CCl_4 was used at a concentration of 5 mM. This concentration was found to be toxic for 6 h exposure of hepatocytes leading to about 50% decrease of cell viability and a great increase of ALT leakage. This indicates a loss of hepatocytes membrane integrity and MDA formation evidencing oxidation of lipid membrane.

In the pre-treatment of hepatocytes with *D. adscendens* extracts, we have noted protection against CCl_4 induced hepatotoxicity as shown in Figures 8a, 8b, and 8c. Here, at the concentration of 1 µg/mL, the cell viability was maintained while significant (p<0.01) decreased in MDA formation and inhibited ALT leakage were noted. In the post-treatment of hepatocytes with *D. adscendens* extracts, after poisoning with CCl_4, we also have a restoration/protection as shown in Figures 9a, 9b, and 9c. Here, at the concentrations of 1, 10 and 100 µg/mL, the cell viability was also maintained while significant (p<0.01) decrease in MDA formation and inhibited ALT leakage were also noted. The variation in bioactivity between the plant harvested before flowering (DA1) and that harvested after flowering (DA2) could be due to the composition of secondary metabolites which vary according to the seasons. Indeed, environmental factors such as light, temperature, soil water, soil fertility and salinity impact on biosynthesis and accumulation of secondary metabolites of plants, and therefore their pharmacological efficacy [29,30] .

In both pre-and post-treatment tests, these results suggest that DA extracts protect the liver through anti-oxidative effects. These effects were confirmed by the antiradical activities of DA1 and DA2 extracts, their reducing capacities (ferric ions and molybdate) and the inhibition of lipid peroxidation. These findings are in line with previous studies that demonstrated the potential of plant extracts to protect the liver from CCl_4-induced hepatotoxicity by inhibiting lipid peroxidation and improving antioxidant activity [31,32]. The place of the oxidative stress in the pathogenesis of viral hepatitis and particularly in hepatitis C virus infection has been previously documented [33,34] It has been shown that HCV nucleocapsid protein increases the oxidative stress in the liver [35] and that some proteins of the replication complex (NS3 and NS5A) increased calcium uptake by mitochondria and caused oxidation of mitochondrial glutathione, leading to increased ROS [36]. Therefore, it was important for us to assess the antiviral properties of DA extracts on HCV. Our study identified for the first time, *D. adscendens* as an inhibitor of HCV infection and replication (Figures 10 and 11), suggesting thereby a possible effect of this plant on different steps of the HCV life cycle including entry, replication, and assembly. Further studies would be necessary to clarify the exact anti-HCV mechanism of this natural plant extract and fully characterize the potentially active secondary metabolites.

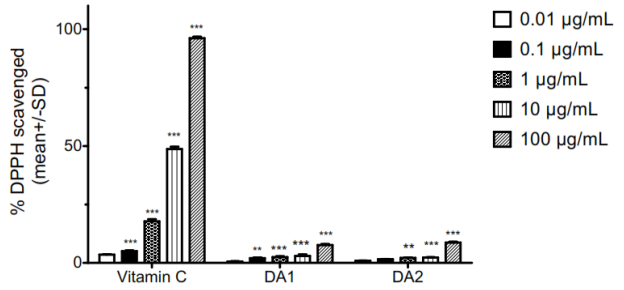

Figure 1: Comparative DPPH scavenging activities of plant extracts (DA1 and DA2) and ascorbic acid (vitamin C), known as DPPH radical scavenger. Values are mean ± SD of two independent experiments in triplicate. **P<0.01 and ***P<0.001 compared with 0.01 µg/mL group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

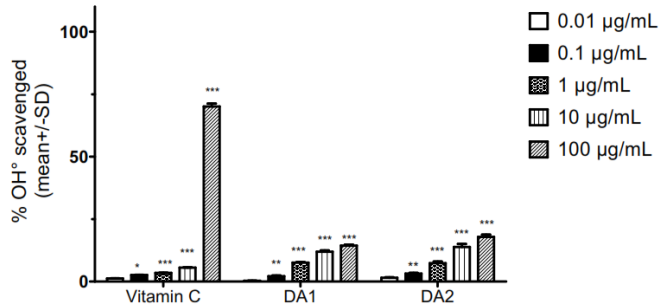

Figure 2: Hydroxyl (OH°) radical scavenging activities of plant extracts (DA1 and DA2) and vitamin C. Values are mean ± SD of two independent experiments in triplicate. *P<0.05, **P<0.01 and ***P<0.001 compared with 0.01 µg/mL group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

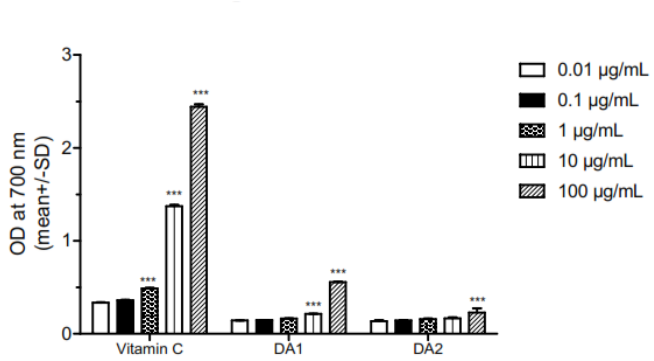

Figure 3: Reducing ferric ion ability by DA1, DA2 and ascorbic acid. Values are mean ± SD of two independent experiments in triplicate. ***P<0.001 compared with 0.01 µg/mL group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

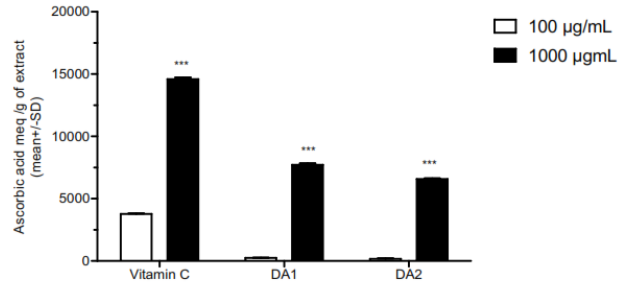

Figure 4: Total antioxidant capacity (Molybdate reduction) of plant extracts (DA1 and DA2) and vitamin C. Values are mean ± SD of two independent experiments in triplicate. ***P<0.001 compared with 100 µg/mL group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

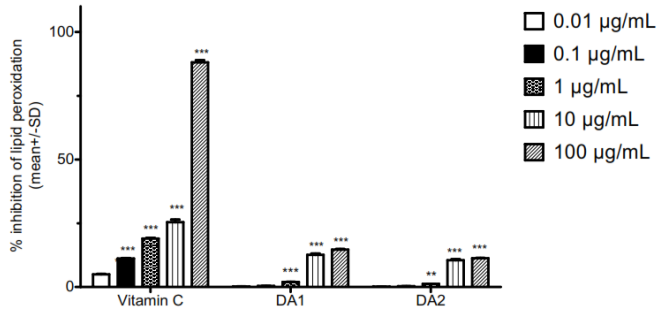

Figure 5: Inhibition of lipid peroxidation by DA1, DA2 and ascorbic acid. Values are mean ± SD of two independent experiments in triplicate. **P<0.01 and ***P<0.001 compared with 0.01 µg/mL group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

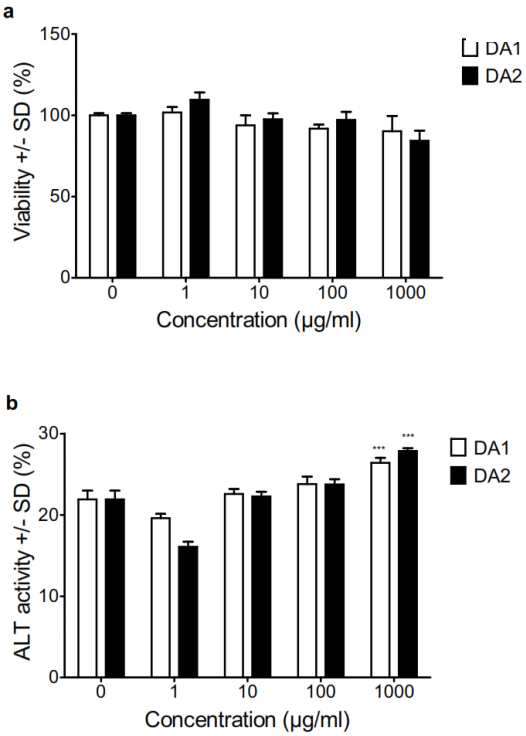

Figure 6: Effect of plant extracts (DA1 and DA2) on cell viability (a) and ALT leakage (b). Values are mean ± SD of two independent experiments in triplicate. P < 0.001, compared with control (0 mM). DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

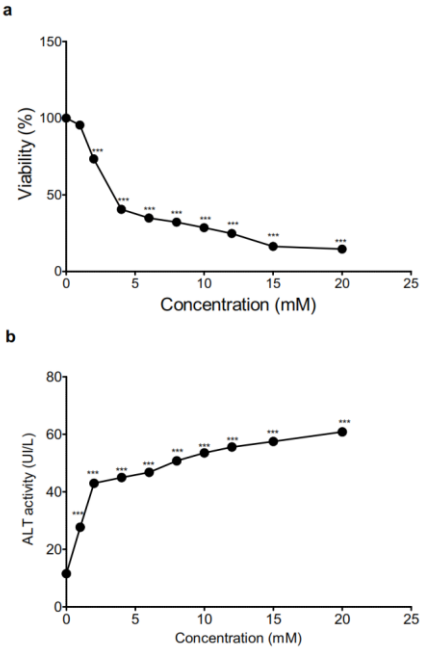

Figure 7: Effect of carbon tetrachloride concentrations on (a) cell viability indicating dose respond toxicity of CCl_4 and (b) ALT leakage indicating disruption of membrane integrity. Values are mean ± SD of two independent experiments in triplicate. P < 0.001, compared with control (0 mM). DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

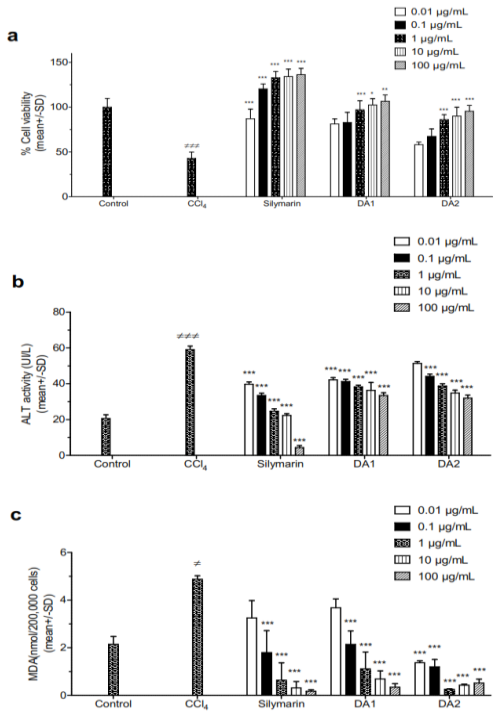

Figure 8: Effect of DA1 and DA2 extracts in pretreatment on the hepatocytes viability (a), ALT leakage (b) and lipids membrane oxidation (c). Values are mean ± SD of two independent experiments in triplicate. ≠≠P<0.01 and ≠≠≠P<0.001 compared with control group; *P<0.05, **P<0.01 and ***P<0.001 compared with CCl_4-intoxicated group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

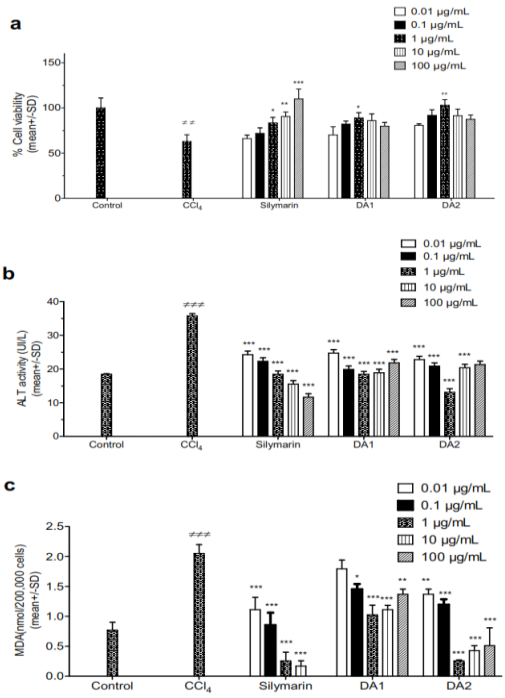

Figure 9: Effect of plant extracts (DA1 and DA2) in post-treatment on the hepatocytes viability (a), ALT leakage (b) and lipids membrane oxidation (c). Values are mean ± SD of two independent experiments in triplicate. ≠P<0.05 and ≠≠≠P<0.001 compared with control group; *P<0.05, **P<0.01 and ***P<0.001 compared with CCl_4-intoxicated group. DA1 and DA2 represent the aqueous extracts of *D. adscendens* harvested respectively before and after flowering.

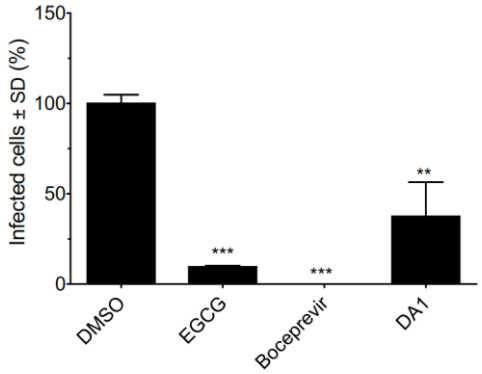

Figure 10: DA1 extract inhibits HCVcc infection. Huh-7 cells were inoculated with HCVcc in the presence of EGCG 50 µM or DA extract at 25µg/ml and incubated for 2 h. The inoculum was removed and replaced with a fresh medium containing DA1 extract and incubated for 28h. DMSO was used as normal control. Boceprevir at 0.5 µM was added only during the post-infection step for their effect on replication. Data were normalized to the DMSO control, which is expressed as 100% infection. Values are mean ± SD of two independent experiments in triplicate. Results are presented as percent viability calculated against untreated cells (*P<0.01 and ***P<0.001). DA1 represent the aqueous extracts of *D. adscendens* harvested respectively before flowering.

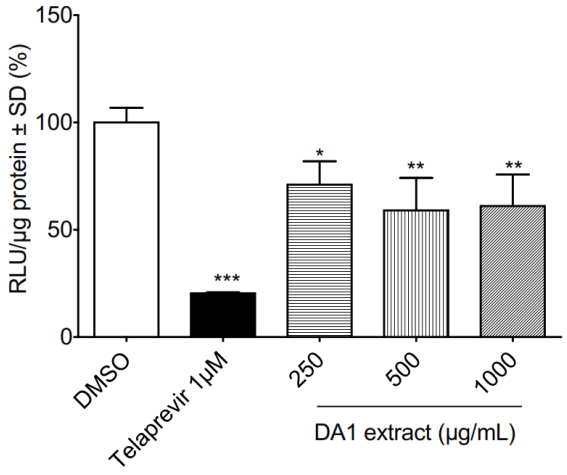

Figure 11: Inhibition of HCV replication by *D. ascendens* aqueous extract in LucUbiNeo-ET cells. LucUbiNeo-ET cells were treated with different concentrations of DA1 extract or Telaprevir 1 µM or 0.1% DMSO and analysed for relative luciferase activities after 24-h incubation. Values are mean ± SD of two independent experiments in triplicate. Results are presented as percent viability calculated against untreated cells (*P<0.05, **P<0.01 and ***P<0.001). DA1 represent the aqueous extracts of *D. adscendens* harvested respectively before flowering.

Conclusions

Based on the results of this work, we can conclude that the aqueous extracts of *D. adscendens* have a hepatoprotective potential against CCl_4-induced damage in primary rat hepatocytes. Inhibition of oxidative stress and the capacity to protect membrane integrity by this plant extract seems to be the key mechanisms of its activity. Besides, DA has revealed anti-HCV activity in cell culture, which supports the traditional use of this plant in the management of viral hepatitis.

Subsequent studies will allow us to identify potentially active constituents and clarify their role in anti-HCV activity.

Additionally, the analysis of the influence of DA in the differentiation of stem cells into hepatocytes would be also explored in order to complete information on the biological activity of this plant.

Abbreviations

List of abbreviations:
ALT: Alanine aminotransferase
CCl_4: Carbone tetrachloride
DA: *Desmodium ascendens*
DMEM: Dubelcco's Modifield Eagle's Medium
DPPH: 2,2-Diphenyl-Picryl-Hydrazyl
EGCG: Epigallocatechin-3-gallate
HCV: Hepatitis C virus
HepG2: Human liver hepatocellular carcinoma cell line
LAR: Luciferase assay reagent
MDA: Malondialdehyde
MOI: Multiplicity of infection
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
ROS: Reactive Oxygen Species

Authors' Contribution

PDDC, JK, and PFM designed the experiment; PDDC, BRTG, NCKY, AFK, and BFNS carried out the experiments; PDDC, BRTG, and AFK wrote the manuscript; KS, JD, and GT provided reagents; PDDC, ANT contributed to plant collection and provided some facilities for the study; PDDC and BRTG analyzed data; PFM supervised the work.

Acknowledgments

The authors are thankful to the general team manager of the *Université des Montagnes* which provides the necessary general supervision and material and logistical support for the realization of this work. We also thank the Cameroon National Herbarium for the identification of plants.

Conflict of interest

The authors declare that they have no competing interests.

Article history:

Received: 19 March 2020
Received in revised form: 07 April 2020
Accepted: 08 April 2020
Available online: 08 April 2020

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