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Original Research

Safety of *Desmodium adscendens* extract on hepatocytes and renal cells. Protective effect against oxidative stress

Céline François[1], Mourad Fares[1], Claudio Baiocchi[2], Jean Michel Maixent[3]

[1]INSERM U927, Faculty of Medicine, University of Poitiers & CHU la Miléterie, Poitiers, France, [2]Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy, [3]PROTEE Laboratory, Université du Sud Toulon-Var, La Garde Cedex, France

Address for Correspondence: Pr. J.M. Maixent Laboratoire PROTEE-EA 3819-EBMM-Batiment R, Université du Sud Toulon-Var, BP 20132, 83957 LA GARDE Cedex, France. E-mail: jmmaixent@gmail.com

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ABSTRACT

Aim: The increased consumption of traditional medicinal plants has been driven by the notion that herbal products are safe and efficient. The purpose of this study was to evaluate the safety and the protective effect of a hydro alcoholic extract of *Desmodium adscendens* (DA) on liver (HEPG2) and kidney (LLC-PK1) cells. **Materials and Methods:** A hydro alcoholic extract of DA was used. HEPG2 or LLC-PK1 cells were treated with different does of DA, and viability test (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS]), cytotoxicity assay lactate dehydrogenase (LDH release) and study of the cell morphology were used in order to determine effects of DA on these two cells. **Results:** A viability test (MTS), a cytotoxicity assay LDH release and a study of the cell morphology revealed that pretreatment with 1 mg/ml or 10 mg/ml DA did not alter viability or LDH release in HEPG2 or LLC-PK1 cells. However, DA at the dose of 100 mg/ml significantly decreased cell viability, by about 40% ($P < 0.05$). Further, MTS studies revealed that DA 1 mg/ml or 10 mg/ml protected LLC-PK1 cells against a glucose-induced oxidative stress of 24 h ($P < 0.05$). **Conclusion:** Hence, the lowest concentrations of DA (1 mg/ml and 10 mg/ml) were safe for HEPG2 and LLC-PK1 and protective against an oxidative stress in LLC-PK1 cells. These data suggest that DA extracts used as a traditional herbal as food health supplements should be used at the lowest dosage.

KEY WORDS: Cytoprotection, *Desmodium adscendens*, HEPG2, LLC-PK1, glucose-induced stress, toxicity

INTRODUCTION

In a number of cultures, the traditional use of plants has been the mainstay of health maintenance. The effect of these plants or extracts are known for a multitude of beneficial effects, which include antibacterial, antiviral, anti-diabetic, or antioxidant, and have been the subject of numerous studies [1,2].

Desmodium adscendens (DA) (Sw.) DC. is a perennial plant from the *Fabaceae* family that commonly occurs in tropical areas of Africa, South America, Asia, Australia and Oceania. It has been used for many years because of its pharmacological properties and valued in folk medicine practices. Traditionally, DA is used as a wild vine (decoction) used in the Amazon rainforest of Peru, in South American countries and in the west coast of Africa. An aqueous extract of the leaves has been used for pain, fever and also epilepsy [3]. In Africa, DA is also frequently used to treat diseases linked to problems of smooth muscle contraction like asthma [4]. In Brazilian traditional medicine, the leaves of DA are used to treat a wide range of conditions that include gonorrhea, diarrheas, body aches, excessive urination, and ovarian inflammations. In France it is traditionally used as a food health supplement for its hepatoprotective effect since it was demonstrated that DA has a positive effect against hepatic infection *in vivo* [5]. Recently, publications on DA are focused on chemical composition of DA from Ghana and Nigeria. Based on thin layer chromatography, Pothie *et al.* [6] characterized the main compounds in DA: Flavonoids, triterpene, saponins, amines and alkaloids. Baiocchi *et al.* [7] used the high resolution mass spectrometry to quantify saponins and alkaloids. Flavonoids during an antioxidant activity-guided isolation were quantified in plant material from Africa using high performance liquid chromatography with diode array detector, mass spectrometry and multi-dimensional nuclear magnetic resonance spectroscopy [8]. This last chemical characterization identified the isovetixin-2''-0-xyloside (flavone C-glycosides)

as the main compound in an ethanol extract similar to the plant dietary supplement found in France and Belgium. The European regulation since 2006 requires for dietary supplements the characterization of all chemical compounds present in plant extracts. One pharmacovigilance case on a desmodium extract was declared to the Directorate for Competition Policy, Consumer Affairs and Fraud Control and the ANSES. An acute hepatotoxicity was associated in one woman to the consumption of this plant extract in the same time as other 5 medicine drugs (ANSES). Very few publications are available on the safety of DA, and only the acute toxicity of the plant has been evaluated in a study of potential neurological defects [9]. DA is used in France and Belgium as a food health supplement, in a liquid form issued of an alcoholic extraction, but its potential harmful effect has not been assessed to our knowledge on hepatocytes.

The purpose of this study was to evaluate the safety of a hydro-alcoholic extract of DA on hepatocytes and renal cells. The cytoprotective effect following oxidative stress on renal cells was also evaluated.

MATERIALS AND METHODS

Plant Material and Extract

The plant extract used in this study is a hydro alcoholic extract of the aerial parts of African *DA* with an extraction ratio of 1:1. 1 g of liquid extract corresponds 1 g of dry plant. This liquid extract was provided by the Nutergia Laboratory and represents the extract the concentrated liquid formula of the product sold in France and Belgium as a food dietary supplement.

Cell Culture

A pig kidney epithelial cell line (LLC PK 1) and a *Human liver hepatocellular carcinoma cell* line (HepG2) were obtained from American Type Culture Collection (Rockville, MD). They were grown in T-75 flasks in medium 199 (M 199, LLC-PK1) or Dulbeco's Modified Eafle's Medium (HepG2) supplemented with 10% fetal calf serum (Gibco) and penicillin (100 U/ml)/streptomycin (100 μg/ml) (Sigma-Aldrich, France) at 37°C in a 5% CO_2 humidified atmosphere as we previously described [10]. When cell cultures reached about 80% confluence, cells were removed using 0.25% trypsin in ethylenediaminetetraacetic acid (Gibco) and sub-cultured into 6 or 96 well plates. At confluence, cells were serum-starved for 24 h prior to experimentation.

Viability Test

MTS is a calorimetric assay based on the ability of viable cells to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, France) to formazan. The quantity of formazan produced, as measured by 490 nm absorbance, is directly proportional to the number of viable cells in culture. Cells were seeded in 96-well culture plates (5×10^3 cells/well). After the serum-starvation period, the medium was carefully removed and replaced by 100 μl of various concentrations of DA extract (1 mg/ml, 10 mg/ml, 100 mg/ml) for a 24 h incubation period. The cells were then incubated with 20 μl MTS tetrazolium compound for 2 h at 37°C. During the incubation, the MTS salt is metabolically reduced only by viable cells into an insoluble colored formazan, and the absorbance/optical density was read with a Victor 1420 Multilabel Counter (WALLAC, USA) at a single wavelength of 490 nm.

Lactate dehydrogenase (LDH)-Cytotoxicity Assay

15×10^4 cells/well were cultured in 6-well culture plates. The serum-starvation period was followed by 24 h of incubation with the different concentrations of DA extract, dimethyl sulfoxide (DMSO), or triton X-100. The culture supernatants were collected and the cells were lysed in a phosphate buffered saline 1X/0.1% triton solution and scraped. Then the cell suspension was sonicated for 30 s and centrifuged (1000 g, 10 min). LDH measurements were performed in supernatants and cell suspensions, using the Roche IFCC method (Roche/Hitachi Modullar P) at the University Hospital of Poitiers.

Cell Morphology

Phase contrast microscopy was conducted using an Olympus CKX 41 (Olympus, France) and Q-Capture Imaging Pro sofware Program (Olympus, Canada).

Cytoprotection

Cells were seeded at 3.10^5 cells/ml in 96-well plates, grown to 80% confluence, and synchronized by a 24 h incubation in the STARV medium at 37°C, followed by a 24 h incubation with the different concentrations of DA extract. Then cells were treated with 30 mM D-glucose for 24 h and the MTS assay was performed.

Statistical Analysis

Results are expressed as means ± standard error mean of five experiments. Statistical analysis was performed using analysis of variance (ANOVA) and then the Dunnett's post-test (GraphPad Prism®, GraphPad Software, San Diego, CA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

In this study, we assessed cell viability and LDH release after treatment of two cell lines (HepG2 and LLC PK1) with a DA extract for 24 h at three different doses (1, 10 or 100 mg/ml) and after triton ×100 treatment [Figure 1f].

Pictures of cell morphology changes are illustrated by Figure 1.1 for hepatocytes and by Figure 1.2 for renal cells. Control conditions included the vehicle DMSO, control cells without DMSO and triton for the chemical alteration of the cell shape. Figure 1.1 and 1.2 showed an alteration in cell shape only after a treatment with 100 mg/ml compared with untreated

cells and those exposed to two other doses of DA extract. This alteration of cell shape was found in the two cell lines (hepatocyte and renal cells) at the same concentrations of DA extract [Figure 1.1 and 1.2e].

Figure 2a and b shows the results of the assessment of cell viability obtained in HepG2 and LLC-PK1. The DA extract at 100 mg/ml significantly decreased ($P < 0.05$) cell viability by about 40% compared with control group with and without DMSO. The treatment of this cell line with 1 mg/ml or 10 mg/ml of the extract showed a trend to increase the cell viability between 14% and 22% although not statistically significant.

The results obtained in LLC PK1 and HepG2 cells show that the extract of DA at 100 mg/ml induced a significant decrease ($P < 0.05$) of cell viability by about 35% compared with control.

The release of LDH in the culture medium of HepG2 and LLC-PK1 after a treatment with DA extract at 100 mg/ml significantly increased cell injury ($P < 0.05$) by 7% and 16% compared with control, respectively. Treatment of cells as shown in Figure 3a and b with the DA extract at 1 mg/ml or 10 mg/ml show no significant increase in LDH release irrespective of the cell line used.

Protective Effect in LLC-PK1 Cells

After treatment with the DA extract for 24 h at different doses (1, 10 or 30 mg/ml) in LLCPK-1 cells, an oxidative stress was induced by incubating the cells for 24 h in serum-free culture medium saturated with D-glucose (30 mM), and cell viability was measured using MTS assay. The results presented in Figure 4 show that glucose-induced oxidative stress decreased significantly cell viability by about 60% ($P < 0.05$), and this decrease was prevented by pretreatment with 1 mg/ml or 10 mg/ml of DA ($P < 0.05$). In fact, at the dose of 10 mg/ml of DA extract, the observed cell viability was undistinguishable from that of control cells. Pretreament with 30 mg/ml of DA extract did not result in protection against oxidative-stress-induced decreased in cell viability.

DISCUSSION

Herbal medicines have become a popular form of therapy and patients who are self-medicated with herbs for preventive or

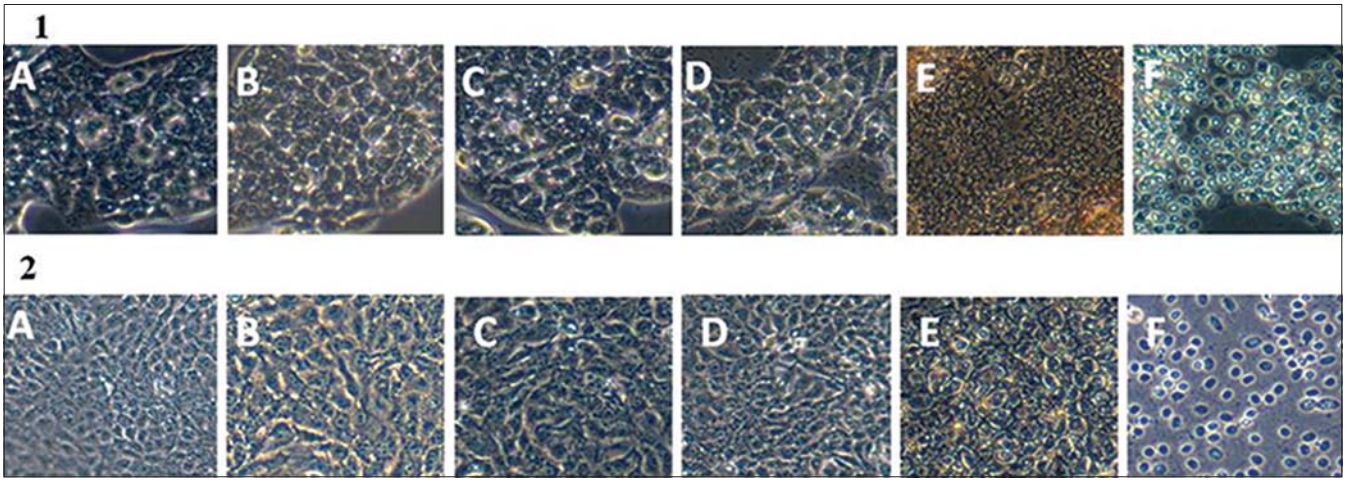

Figure 1: Morphological changes observed under light microscopy. Magnification ×10. HepG2 (1) and LLC PK1 (2), (a) dimethyl sulfoxide, (b) control, (c) 1 mg/ml desmodium extract, (d) 10 mg/ml desmodium extract, (e) 100 mg/ml desmodium extract, (f) triton ×100

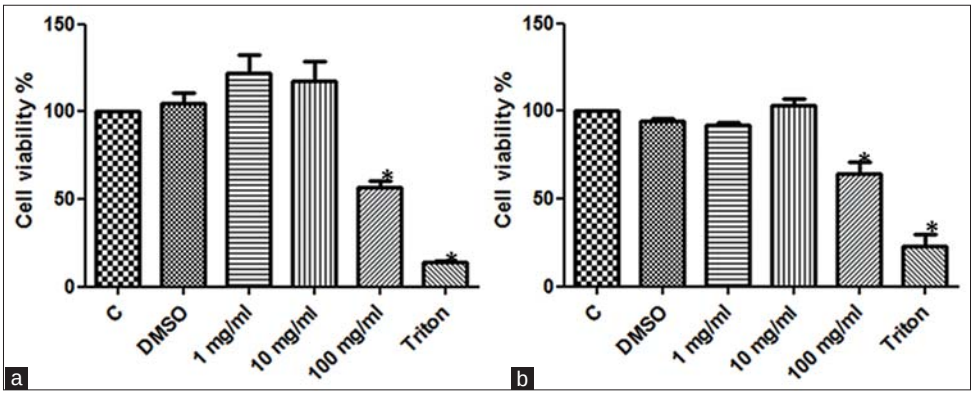

Figure 2: Effect of *Desmodium adscendens* (DA) extract on cells growth. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, (a) Cell viability of HepG2 cell line, (b) Cell viability of LLC PK1 cell line incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml), dimethyl sulfoxide and triton were used as negative and positive controls respectively. Data are represented as mean ± standard error mean of six determinations. *$P < 0.05$ versus control

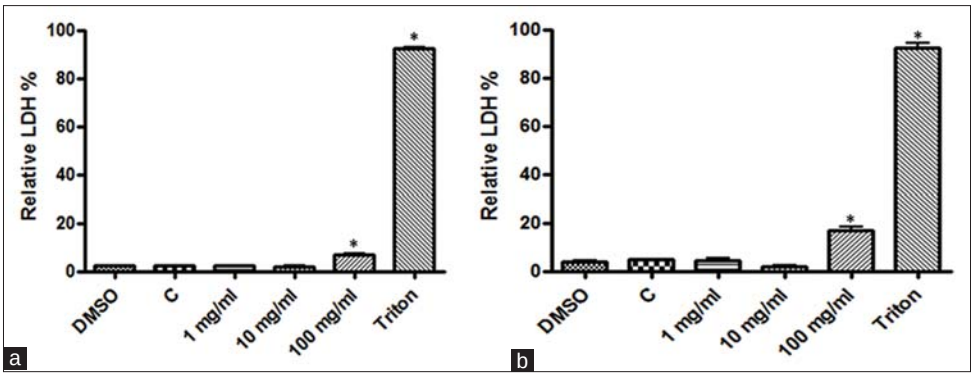

Figure 3: Effect of *Desmodium adscendens* (DA) on cell injury assessed by the lactate dehydrogenase (LDH) release, (a) LDH release of HepG2 cell line, (b) LDH release of LLC PK1 cell line incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml), dimethyl sulfoxide and triton were used as negative and positive controls, respectively. Data are represented as mean ± standard error mean of six determinations. **P* < 0.05 versus control

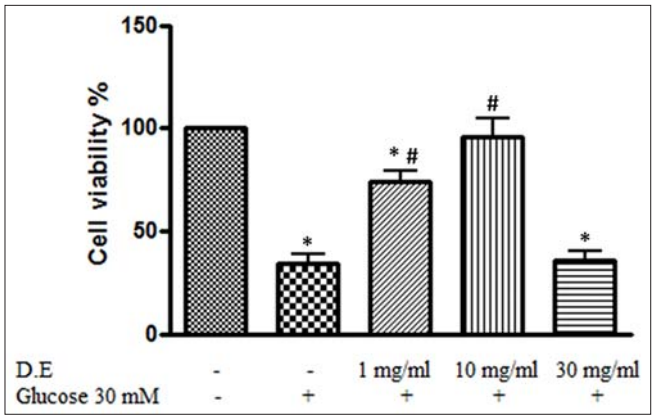

Figure 4: Protective effects of *Desmodium adscendens* (DA) extract against oxidative stress on LLC-PK1 cell line. Cell viability of LLC-PK1 cell line was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Oxidative stress was induced high glucose (30 mM) and incubated for 24 h without or with DA extract 1, 10 or 100 mg/ml). Data are represented as mean ± standard error mean of six determinations. **P* < 0.05 from control

therapeutic purposes may assume that these products are safe because they are natural. However, any product can become toxic if not used correctly [11]. Hence, the objective of this study was to evaluate the potential toxicity of a hydro-alcoholic extract of DA. We used two well-defined cells lines to appreciate potential risks of liver and renal damages [12,13]. The safety of DA extract on HepG2 and LLC-PK1 ranged from 1 mg/ml to 10 mg/ml of DA extract as suggested by the absence of changes in cell viability or cell morphology following 24 h of exposure to these concentrations. This was correlated to a lack of increase in LDH release in both cell lines.

On the other hand, our data suggest that the concentration of 100 mg/ml of DA extract significantly decreased cell viability of both HepG2 and LLC-PK1, and was associated with a significant increase of LDH release. Whether this toxicity could be attributed to the amount of alcohol used in the plant extract remains to be determined. Indeed, alcohol has been shown to increase the number of apoptotic cells [14,15] and lead to the induction of intracellular enzymes like alcohol deshydrogenase and the production reactive oxygen species (ROS) [16]. Consistent with the observed effect, ethanol cytotoxicity is dose-dependent and occurs within 24 h *in vitro* [17]. The effect of ethanol should be assessed as vehicle on our cells. The amount of ethanol in the DA extract should be measured. The dose of 100 mg/ml of DA extract is not safe despite the alcohol vehicle is known. This dosage should be used with caution.

The cytoprotective effect of this extract was also examined by using a glucose-induced oxidative stress [18,19]. Since the cytotoxicity studies discussed in the previous paragraph revealed that the dose of 100 mg/ml was not safe for the cells, a lower concentration of 30 mg/ml was used in this set of studies, in addition to the concentrations of 1 mg/ml and 10 mg/ml. Concentrations of 1 mg/ml and 10 mg/ml of DA efficiently decreased glucose-induced oxidative stress. A total recovery of cell viability at the dose of 10 mg/ml during the 24 h of exposure suggested a very efficient antioxidant activity. Hence, the concentrations of 1 mg/ml and 10 mg/ml were safe and also cytoprotective. This cytoprotective effect of low concentrations of DA against glucose-induced oxidative stress is to put in parallel with the detection of these antioxidant properties. In fact, it was shown that a hydroalcoholic extract of DA had scavenging antioxidant activities, inhibiting the ROS generation induced by H_2O_2 [20]. Cellular test, 2,2-diphenyl-1-picrylhydrazyl (DPPH) test and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic) (ABTS) have shown the scavenging antioxidant effect of an hydroalcoholic extract of leaves of DA [20]. The results of the DPPH and ABTS tests, expressed in mg of vitamin C equivalents per g dry weight, were 8.47 and 12.83, respectively. ROS levels measured by flow cytometry revealed, at a concentration of 25 mg/ml, a decrease of 83% of ROS level generated by exogenous H_2O_2. In the present study, pretreatment with 30 mg/ml of DA did not provide any protection. The fact that the two lower concentrations of this study have a protective effect, contrary to the dose of 30 mg/ml, may also be ascribed to ethanol. Actually, ethanol treatment

was also shown to impair antioxidant levels *in vitro*, resulting in ROS generation and increased oxidative stress [21]. Thus, low doses are sufficient to obtain a protective effect, while at higher concentrations, oxidative stress driven by alcohol in the extract seems to annihilate the protective activity of DA. The generation of ROS by exogenous H_2O_2 was shown to be inhibited by a pre-treatment with the DA extract in a similar way to our study [9].

In conclusion, lowest concentrations (1 mg/ml and 10 mg/ml) are safe for the cells and protective against an oxidative stress. DA extracts used as a traditional medicine as food health supplements in Europe for its efficient on stress in human should be used at the lowest dosage.

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AUTHOR CONTRIBUTIONS

The author's responsibilities were as follow/MF, JMM study design and writing the manuscript; CF and MF conduct the cell culture and supplementation. All authors participated in the study and take responsibility for the content of this report. None of the authors had a personal or financial conflict of interest.

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