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Hepatodamianol as hepatoprotective constituent of *Turnera diffusa*

Cecilia Delgado-Montemayor[1], Jonathan Perez-Meseguer[1], Ricardo Salazar-Aranda[1], Paula Cordero-Perez[2] and Noemí Waksman[1]*

[1]Universidad Autónoma de Nuevo León, Departamento de Química Analítica, Facultad de Medicina. Av. Francisco I. Madero y Dr. Aguirre Pequeño S/N. Col Mitras Centro. Monterrey, NL. México

[2]Universidad Autónoma de Nuevo León, Unidad de Hígado. Facultad de Medicina y Hospital Universitario, "Dr. José Eleuterio González. Av. Francisco I. Madero y Dr. Aguirre Pequeño S/N. Col Mitras Centro, Monterrey, NL. México

Abstract: It is well known that liver diseases are a major health problem and that there is a lack of hepatoprotective agents. *Turnera diffusa* (damiana) is a plant with a widespread distribution in México, which has many traditional uses, including the treatment of hepatic illnesses. Based on the bioassay-guided fractionation of a methanolic extract obtained from the aerial part of *T. diffusa*, we purified and identified a compound called hepatodamianol (1). This C-glycoside exhibited a four times greater hepatoprotective effect than the widely used hepatoprotective agent silibinin against carbon tetrachloride damage in an *in vitro* model using HepG2 cells. Hepatodamianol produced no cytotoxic effects, and it exhibited a high antioxidant capacity. Therefore, hepatodamianol is a good candidate compound for testing as a hepatoprotective agent in a preclinical trial.

Keywords: Damiana, hepatodamianol, hepatoprotection, *Turnera diffusa*.

INTRODUCTION

The liver is the primary organ that metabolizes foreign compounds, which makes it susceptible to injury and thus the possible development of various pathologies such as hepatitis, fibrosis, cirrhosis and cancer. Liver disease accounts for over two million deaths annually (cirrhosis, viral hepatitis, and liver cancer) and accounts for 4% of all deaths worldwide (1 out of every 25 deaths) (Devarbhavi *et al.*, 2023). In Mexico, hepatic illnesses were the sixth main cause of mortality during 2021, whereas they were the ninth most important cause in 1990 (Secretaría de Salud, 2023). Moreover, epidemiological studies conducted by the National Institute of Statistics and Geography (Mexico) showed that the main diseases in Mexico during 2013 were diabetes mellitus (14.25%), ischemic heart diseases (12.63%), cerebrovascular diseases (5.29%) and liver diseases (4.79%).

Excluding vaccines and interferon α-2b, which can only be used to treat viral infections, modern medicine is limited in terms of the prevention or treatment of hepatic diseases. Conventional or synthetic drugs used for the treatment of liver failure are not effective and they have various side effects (Abdallah *et al.*, 2013). Hence, there is a growing need for safe hepatoprotective agents. The limited number of therapeutic options means that there is considerable interest in searching for active compounds in plants that have traditionally been used for treating liver disorders.

Previous studies have reported that the oxidative stress is closely linked to the pathogenesis of acute liver injury (Ajuwon *et al*., 2014; Tanikawa and Torimura, 2006). Therefore, oxidative stress and the activities of antioxidants are critical issues that need to be addressed in the development of hepatoprotective or therapeutic drugs (Akanitapichat *et al.*, 2010).

Polyphenolic compounds are widely distributed in plants, and they are known to be excellent antioxidants *in vitro*, where they have the capacity to scavenge free radicals and to defend against antioxidants in liver diseases. Therefore, the polyphenolics found in natural products are considered potential sources of new drugs for treating liver disorders. Silymarin, a mixture of flavonolignans obtained from *Silybum marianum* or milk thistle, is a popular herbal extract that is used as a hepatoprotective agent (Shamama *et al.*, 2011).

In Mexico, the use of natural products as herbal remedies is an ancient practice, but there has been little research into the effects of most plants. Several plants have traditionally been used to treat liver diseases in Mexico (Torres-González *et al.*, 2011), particularly *Turnera diffusa* (damiana), which we investigated in the present study.

Several trials have evaluated the antioxidant activity of *T. diffusa*, including the capture of free radicals such as 1-1-diphenyl-2-picrylhydrazyl (DPPH) by thin-layer chromatography (TLC) and spectrophotometry, analyses of the inhibition of xanthine oxidase activity and assays of the total phenol contents. All of these studies have demonstrated that *T. diffusa* extracts have a strong antioxidant capacity (Pérez-Meseguer *et al.*, 2010).

**Corresponding author:* e-mail: nwaksman@gmail.com

In a preliminary survey of medicinal plants from northeast Mexico, the hepatoprotective effect of *T. diffusa* was evaluated using an *in vitro* model based on the induction of damage by carbon tetrachloride (CCl_4), which showed that pre-treatment with a methanolic extract of the aerial part of the plant protected cells from the damage induced by CCl_4 at doses of 10 and 100µg/mL, according to the aspartate aminotransferase (AST) released into the culture medium, the malondialdehyde (MDA) level and the maintenance of cellular viability (Torres-González *et al.*, 2011). Therefore, in the present study, we aimed to isolate the compound(s) responsible for the hepatoprotective effect of *T. diffusa* using bioassay-directed fractionation.

MATERIALS AND METHODS

Plant material

Leaves and stems were collected from several samples of *T. diffusa* (Turneraceae) in different places and at different times of the year between 2008 and 2012. All of the samples were authenticated, and a voucher specimen (No. 23569) has been deposited in the herbarium at the Facultad de Biología, Universidad Autónoma de Nuevo León, Mexico.

Cell lines, chemical and biochemicals

We used the HepG2 cell line ATCC HB-8065. All of the solvents used for extraction and purification were analytical grade, and they were purchased from Fermont (Monterrey, NL, Mexico). HPLC-grade methanol (MeOH) and Alltech® Extract-clean C18 were purchased from Fisher Scientific (Fair Lawn, NJ). TLC was conducted on plates pre-coated with silica gel 60 F_{254} at a thickness of 0.2mm with an aluminium support (Merck, Darmstadt, Germany). Deionized water was obtained from Monterrey Laboratories S.A. de C.V. (Monterrey, NL, Mexico). The mobile phases were filtered through a 0.45 µm nylon filter before use (Waters Corp., Milford, MA). The samples were filtered through nylon acrodiscs (Waters Corp.). *Special chemicals*: Silibinin, DPPH free radical, trypan blue 0.4% for cell culture, MTT, PBS, DMSO tested for cell culture, doxorubicin, methanol-*d4* 100% D, CCl_4 (99.9%) and insulin-transferrin-selenium were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Quercetin was purchased from TCI America (Portland, OR). Dulbecco's modified Eagle's medium (DMEM, Advanced), penicillin G (100 UI/mL)/streptomycin (100µg/mL), L-glutamine 20mM, DMEM: Nutrient Mixture (DMEM/F12), foetal bovine serum (FBS) and trypsin 0.25% (1×) were purchased from Gibco Invitrogen (Carlsbad, CA, USA). Kits for ALT, AST and LDH determinations were obtained from Instrumentation Laboratory (Orangeburg, NY, USA) for use with the ILab 300 Plus system.

Extraction and isolation

Before extraction, the aerial plant parts were dried in the shade at room temperature and then crushed into a fine powder. To determine the antioxidant activity of several *T. diffusa* samples, 5g of the fine powder was extracted with MeOH in a VWR vortex at room temperature (3 times × 15mL × 5min). The extracts were then filtered and evaporated under reduced pressure at 37°C.

For bulk extraction, 500g of the dried selected plant (aerial parts) was crushed into a powder and extracted with MeOH at room temperature (3 times × 800mL × 1h × 200 rpm) with a Heidolph Unimax 1010 shaker. All of the extracts were pooled together and evaporated to dryness *in vacuo* to yield a viscous mass, which weighed 90 g. This syrup was stored under N_2 at 4°C until further use. The chlorophyll contents were eliminated using SPE C-18 cartridges (1000mg/8mL; Alltech) and the samples were eluted with 8mL of 50%, 70% and 100% MeOH. The fraction obtained using 50% MeOH (Fr. SPE MeOH 50%) was the most active. Next, 3.0g of this fraction was subjected to silica vacuum liquid chromatography (VLC) using Cl_2CH_2, AcOEt, AcOEt: MeOHl (1:1) and MeOH as eluents (200mL of each solvent). The fraction eluted with ethyl AcOEt: MeOH (100mg, Fr. VLC) was active, and it was further purified by countercurrent chromatography (CCC). We dissolved 50-100mg of Fr VLC in BuOH: water 1:1 (1mL) and 85 fractions of 1mL were then collected. The active fractions had one principal component (flavonoid mixture) according to TLC and HPLC-DAD. Further purification of these fractions by CCC yielded compound 1 (hepatodamianol). The purity was assessed by TLC, HPLC-DAD and ^{1}HNMR. The proposed structure was established based on the NMR results and comparisons with published results.

Compound 1, hepatodamianol, ^{1}HNMR (MeOD, 400 MHz) flavonoid base δ_H 7.55 (1H, d, H-2′′), 7.5 (1H, dd, H-6′), 6.92 (1H, d, H-5′), 6.6 (1H, s, H-3), 6.3 (1H, s, H-6), sugar moiety δ_H 5.18 (1H, d, H-1′′), 5.06 (1H, d, H-2′′), 4.77 (1H, d, H-1′′′), 4.25 (1H, d, H-4′′), 3.92 (1H, m, H-2′′′), 3.62 (1H, m, H-5′′), 3.27 (1H, bs, H-3′′′), 3.12 (1H, dd, H-4′′′), 2.37 (1H, m, H-5′′′), 1.54 (1H, d, H-6′′) ^{13}CNMR (MeOD, 400 MHz) flavonoid base δ_C 182.6 (C, C-4), 165.2 (C, C-2), 162.7 (C, C-5), 161.9 (C, C-7), 156.3 (C, C-9), 149.7 (C, C-4′), 146.0 (C, C-3′), 122.7 (C, C-1′),119.1 (CH, C-6′), 115.3 (CH, C-5′), 113.6 (CH, C-2′), 104.5 (C, C-10), 103.2 (C, C-8), 102.7 (CH, C-3), 98.6 (CH, C-6), sugar moiety δ_C 205.3 (C, C-3′′), 99.3 (CH, C-1′′′), 79.4 (CH, C-5′′), 78.7 (CH, C-4′′), 77.0 (CH, C-2′′), 74.3 (CH, C-1′′), 71.7 (CH, C-4′′′), 70.6 (CH,C-2′′′), 70.3 (CH, C-3′′′), 69.0 (CH, C-5′′′), 18.2 (CH_3, C-6′′), 16.3 (CH_3, C-6′′′).

HPLC-DAD analysis

HPLC-DAD data were obtained using a Waters 600 series HPLC system, which comprised a Waters 717 plus auto-sampler, high-pressure mixing pump, column oven and Waters 2996 DAD detector. HPLC conditions: column =

AccQTag RP18 (4μm, 3.9 × 150mm; Waters); solvent system, A = H_2O-0.1% trifluoroacetic acid, B = MeOH, where the gradient program started at 70% with A, and it was changed to 30% A after 25min and then held constant for 5 min, before restoring the initial conditions for 5 min at 70%; flow rate = 1mL/min; injection volume = 10μL; sample concentration = 1mg/mL in MeOH.

Countercurrent Chromatography

CCC was performed with an Aligent Technology multilayer coil planet centrifuge for dynamic extraction using a UV infinity 1260 detector, with a teflon column (19 cm, at 30°C and 2100 rpm) and an injection loop of 1 mL. During each separation stage, the column was filled with stationary phase (Hex: H_2O 10:90) and centrifuged at 2100 rpm, where the mobile phase (Hex: BuOH 88: 12) was pumped at a flow rate of 1mL/min for 5 min to ensure that an equilibrium was reached between the two phases. Separation was performed using a quaternary pump at a flow rate of 1mL/min, where the stationary phase employed a gradient starting with Hex: BuOH: H_2O 88:12:0, which was changed to 15: 75:10 after 60 min and then held for 5 min with this mixture. Extraction was performed at a flow rate of 3mL/min with Hex: BuOH: H_2O at 1:9:90.

Free radical reduction: DPPH assay

The antioxidant activities were determined as described by Salazar *et al*. (Salazar-Aranda *et al.*, 2011). Solutions of each extract (compound) in ethanol were serially diluted with additional ethanol to concentrations in the range of 1000 to 1.0μg/mL. The DPPH solution was prepared at a concentration of 125μM in ethanol. Each diluted compound or blank (500μL) was placed in an individual test tube and 0.5mL of DPPH was added. The solutions were stirred and allowed to react in the dark for 30 min, and quercetin was used as a positive reduction control. Spectrophotometric measurements were made at a wavelength of 517nm. The reduction of DPPH was calculated using the following equation.

$$\% \text{ Reduction} = [(\text{Blank} - \text{Sample})/\text{Blank}] \times 100$$

We generated a linear regression curve with the percentage reduction as a function of the concentration of each probe and calculated the concentration that effectively reduced DPPH by 50% (EC_{50}). All of the experiments were performed in triplicate, and the mean and standard deviation were calculated in each case.

Measurement of the hepatoprotective activity in vitro

Cell culture

Cells were grown in DMEM Advanced medium supplemented with FBS (10%), penicillin/streptomycin (1%) and L-glutamine (1%) at 37°C in a humidified atmosphere containing 5% CO_2. The cells in a monolayer at 80–90% confluence were washed with saline solution, trypsinized and then plated (1×10^6 cells per well). The cells were used after attachment.

Cytotoxicity assay (CC_{50})

The cytotoxicities of the extracts and compounds were determined as described by Mossman *et al*. (Mosmann, 1983) with slight modifications. Briefly, 3000 cells were incubated in 96-well plates for 24h at 37°C in a humidified atmosphere containing 5% CO_2. Next, 500 to 0.5μg/mL of the extracts or test compounds was added to each well, before incubating for 48h in the same conditions. After removing the supernatant, each well was washed twice with PBS. The cells were then incubated with 200 μL of MTT (0.5mg/mL in culture medium) for 3 h, before removing the medium and adding 200 μL of DMSO to each well. The plates were mixed, and the absorbances were read at 540 nm in a micro plate reader (Multiskan FC, Thermo Scientific). In each plate, wells were included that only contained cells in the culture medium, and they were treated as 100% viability controls. The positive cytotoxic control was doxorubicin, which was tested at concentrations ranging from 25 to 0.025 μg/mL. Each concentration was evaluated five times on three different plates. Growth inhibition (expressed as a percentage) was calculated based on the absorbance values obtained using the linear regression curve vs the test concentrations. CC_{50} was obtained from the plots as the concentration that inhibited cellular growth at 50%.

Hepatoprotective activity after CCl_4 damage

In order to determine the quantity of CCl_4 necessary to induce damage after 2h, we added 1×10^6 cells to six-well plates and after 12h, the medium was removed, before washing the cells with PBS. The cells were exposed to different concentrations of CCl_4 dissolved in PBS (with 0.05% DMSO), where the concentrations tested comprised 0.1%, 0.3%, 0.4%, 0.5%, 0.75% and 1% CCl_4 (Torres-González *et al.*, 2011; Krithika *et al.*, 2009; Abdallah *et al.*, 2013). After exposing the cells to CCl_4 for 2h, the supernatant was removed and the AST activity was determined and recorded.

The hepatoprotective activity was determined as described by Torres-González *et al*. (Torres-González *et al.*, 2011) with slight modifications. First, 1×10^6 cells were incubated in six-well plates for 12h, washed with PBS and then treated with the extract or test compound, which was dissolved in PBS at 100μg/mL and 50μg/mL, where each concentration was evaluated three times. After incubating for 1h at 37°C in 5% CO_2/95% O_2, the medium was removed and CCl_4 was added. Following further incubation for 2h in the same conditions, the supernatant was collected and the AST activity was measured. AST was quantified using an ILab 300 Plus system and an assay kit (Instrumentation Laboratory). Silibinin was used as the positive hepatoprotection control.

STATISTICAL ANALYSIS

The results were expressed as means ± standard deviation (SD). The data obtained were analyzed using the Student's *t*-test for independent samples with SPSS (v15.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered significant.

RESULTS

Prior to the bioassay fractionation of *T. diffusa*, we tested the antioxidant activities of several specimens collected from different places and at various time of the year. The plant identified as Marín 2008 had the best antioxidant activity and thus it was selected for fractionation (table 1).

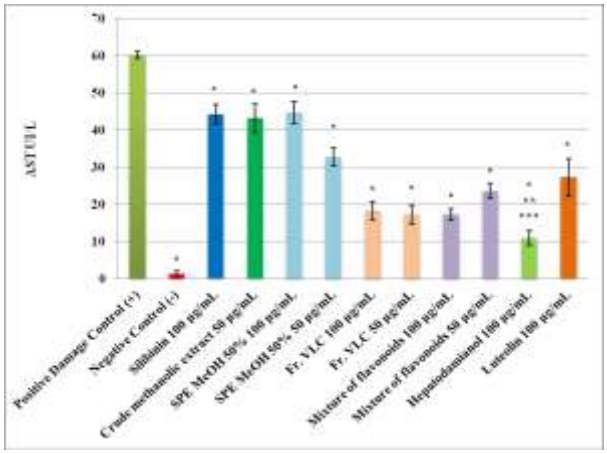

Fig. 1: *In vitro* hepatoprotective effects of the *T. diffusa* extract, fractions and compound 1 in HepG2 cells under CCl_4 damage. Positive damage control (+) = CCl_4 0.4%; negative control (-) = PBS; n = 3. Values are expressed as means ± SD. *: Significantly different compared with the positive damage control, P=0.05. **: Significantly different compared with silibinin, P=0.05. ***: Significantly different compared with luteolin, P=0.05.

To test the use of CCl_4 as a hepatotoxic agent, we first determined the CCl_4 concentration required to cause damage to one million cells during an incubation period of 2 h, where we tested six concentrations based on previous reports (Torres-González *et al.*, 2011; Abdallah *et al.*, 2013; Krithika *et al.*, 2009). We found that 0.4% CCl_4 was the maximum concentration that obtained a proportional relationship between the CCl_4 concentration and the amount of AST released, and thus 0.4% CCl_4 was selected for use in our bioassay. CCl_4 is a non-polar molecule, so we tested the effect of adding 0.05% dimethyl sulfoxide (DMSO) to promote its solubility in phosphate-buffered saline (PBS). We found that PBS with 0.05% DMSO did not cause any damage to cells.

Fig. 1 shows the hepatoprotective activity of the extract. As expected, CCl_4 (damage control) greatly increased the AST level, but pre-treatment with the crude methanolic extract had a significant hepatoprotective effect compared with the control (P<0.05, 28.21% reduction in the AST activity level), which was similar to the hepatoprotective effect obtained using silibinin (26.55%).

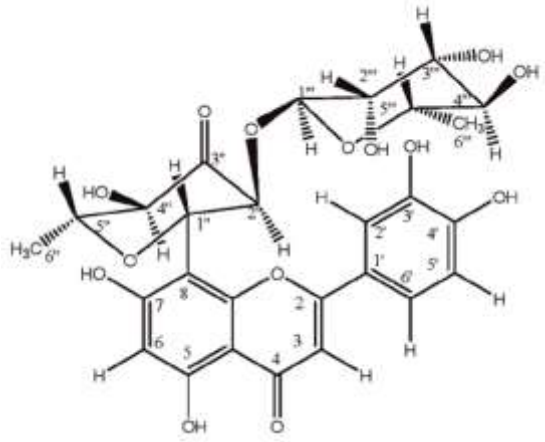

Fig. 2: Structure of Hepatodamianol.

Fig. 1 and table 2 show that the hepatoprotective effect increased, whereas the cytotoxicity decreased, during each fractionation step. The fractionation procedure yielded a compound (1) with superior hepatoprotective effects (81.74% reduction in AST activity; significant difference, P=0.05) compared with silibinin and it was not cytotoxic. Spectroscopic and chromatographic analyses identified this compound as 8-C-β-[6-deoxy-2-O-(α-1-rhamnopyranosyl)-xylo-hexopyranos-3-uloside]-luteolin. The nuclear magnetic resonance spectroscopy (NMR) data were compared with published results (Zhao *et al.*, 2007). The structure of hepatodamianol is shown in fig. 2.

Table 1: Antioxidant activity of methanolic extracts from different samples of *T. diffusa*

Collected in	CE_{50}
Zuazua Sep 2012	48.92 ± 0.27 µg/mL
Zuazua Nov 2012	83.96 ± 0.52 µg/mL
Zuazua Oct 2008	11.01 ± 0.06 µg/mL
Zuazua Nov 2008	11.69 ± 0.48 µg/mL
Marín Oct 2008	11.83 ± 0.02 µg/mL
Marín Nov 2008	10.17 ± 0.07 µg/mL
Dr. González Oct 2008	10.79 ± 0.03 µg/mL
Quercetin	3.01 ± 0.05 µg/mL

Results are expressed as mean ± sd. n=3

DISCUSSION

T. diffusa is a plant that grows throughout Mexico and its traditional uses include the treatment of coughs, diabetes, fever, fungal diseases, gastrointestinal complaints, pain, pulmonary and respiratory diseases, skin disorders and women's health problems. Experimental studies have documented the bioactivity of this plant for some of these

uses. Furthermore, ethno pharmacological studies have reported its use for the treatment of hepatic illness, and there is a preliminary report of its hepatoprotective effects *in vitro*, although no active compound has been identified as being responsible for this activity (Torres-González *et al.*, 2011; Szewczyk and Zidorn, 2014).

Table 2: Results of *in vitro* Cytotoxicity in HepG2 cells

Sample	Cytotoxicity CC_{50} µg/mL
Crude methanolic extract	49.73 ± 5.03
SPE MeOH 50%	>500
Fr. VLC	>500
Mixture of flavonoids	>500
Hepatodamianol	>500
Luteolin	>500
Silibinin	>500
Doxorrubicin	0.14 ± 0.05

Results are expressed as mean ± sd. n=15 at each concentration

Moreover, Brito *et al.* (Brito *et al.*, 2012). Demonstrated that *T. ulmifolia*, a member of the Turneraceae family that grows in Brazil, is a strong antioxidant with hepatoprotective effects in a murine model of harm by CCl_4. Thus, there were significant differences between the enzyme levels i.e., AST and alanine aminotransferase (ALT) in mice treated with a methanolic extract from *T. ulmifolia* compared with untreated mice.

Oxidative stress is one of the main mechanisms involved in the pathology of liver diseases and a close relationship has been reported between antioxidant activity levels and hepatoprotective effects (Hiraganahalli *et al.*, 2012; Rupeshkumar *et al.*, 2012; Brito *et al.*, 2012; Krithika *et al.*, 2009). Thus, natural products with strong antioxidant activities are currently being investigated as possible sources of drugs for use as liver treatments. Considering this, prior to the bioassay fractionation of *T. diffusa*, we tested the antioxidant activities of several specimens collected from different places and at various time of the year. The plant identified as Marín 2008 had the best antioxidant activity, and thus it was selected for fractionation (table 1).

The vast majority of previous hepatoprotection trials using natural products have employed *in vivo* models, especially murine models, but bioassay-guided fractionation requires a model that is easy to perform, inexpensive and fast, as well as other characteristics, which is why we decided to use an *in vitro* model based on a human hepatoma cell line (Kikkawa *et al.*, 2005).

CCl_4 is a widely used and well-characterized source of chemical-induced, oxidative stress-mediated hepatotoxicity in animal models. CCl_4 induces the production of several types of reactive oxygen species (ROS) via cytochrome P450, thereby causing liver injury (Ahmad and Tabassum, 2012; Raj *et al.*, 2010). These ROS can form different radicals that cause lipid per oxidation, thereby leading to membrane damage and changes in enzyme activities, which consequently induce hepatic injury, inflammation, necrosis and apoptosis. However, there have been few reports of its use *in vitro*. We determined that 0.4% CCl_4 was the best concentration to be used in the tests.

Whenever an extract or compound is proposed as a possible new alternative treatment, it is necessary to demonstrate that it is non-toxic. Therefore, we determined the cytotoxicity of the products obtained from *T. diffusa* in the cell line HepG2. Spectrophotometric readings were acquired in the visible region of the spectrum, and before the assay, we determined the effects of the number of inoculated cells per well and the effect of dissolving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in the culture medium (dye), or PBS (colorless solution) (Mosmann, 1983), in order to avoid the possibility of interference with the readings. We selected the maximum concentration of cells that obtained a proportional relationship between the absorbance and the quantity of cells in each well. After dissolving MTT in PBS, there was no proportional relationship between the absorbance and the amount of cells, but when it was dissolved in culture medium (as described previously), the proportional relationship was maintained from 1000 to 3000 cells/well, which is why we tested 3000 cells/well and MTT was dissolved in the culture medium.

The CC_{50} obtained for the cytotoxicity of the methanolic extract in HepG2 cell line (49.73±5.03µg/mL), was similar to the result reported by Avelino *et al.* i.e., CC_{50} = 43.87±7.90µg/mL for the methanolic extract of *T. diffusa* (Avelino-Flores *et al.*, 2014). Although the extract was considered as moderate cytotoxic, the cytotoxicity decreased through the fractionation steps and hepatodamianol resulted non toxic (table 2)

Concerning the hepatoprotective activity, the extract was significative protective compared with the control (fig. 1). It is very interesting to notice that the extract was equally protective against liver damage at concentrations lower than that of silibinin. Given this result, we decided to continue with the fractionation procedure.

Fig. 1 and table 2 show that the hepatoprotective effect increased, whereas the cytotoxicity decreased, during each fractionation step. The hepatoprotective compound isolated (1) showed better hepatoprotective effects compared with silibinin and it was not cytotoxic. This compound, although previously identified from *T.diffusa*, has not been previously isolated form other natural source and it was present in all of the native *T. diffusa* samples that we analyzed; therefore, it was considered a biomarker

of the plant. We named the compound as hepatodamianol. The antioxidant potential of this compound was high according to its DPPH-scavenging activity i.e., CE_{50} 5.56µg/mL and this activity was similar to that of quercetin (positive reduction control) (Pérez-Meseguer *et al.*, 2010).

In 2006, Lima *et al.* (Lima *et al.*, 2006) determined the hepatoprotective activities of several polyphenolic compounds using the HepG2 cell line with *tert*-butyl hydroperoxide as the liver damage agent and lactate dehydrogenase (LDH) as the parameter for assessing damage. They tested luteolin and luteolin-O glycoside as phenolics, where luteolin had a CE_{50} of 5.09µM and its O-glycoside had a CE_{50} of 78µM. Our model was different from that used by Lima *et al.*, but we also evaluated the hepatoprotective action of luteolin, which is the aglycon of compound 1 and although luteolin was active (fig. 1), its activity was considerably less than that of hepatodamianol. In future research, it will be useful to establish the origin of the differences between luteolin and its C- and O- glycosides.

CONCLUSION

In conclusion, our experimental findings confirmed the hepatoprotective effect of the methanolic extract obtained from the aerial part of *T. diffusa*. We employed bioassay-guided fractionation to isolate and characterize the compound responsible for this hepatoprotective effect i.e., a flavonoid C-glycoside called hepatodamianol which protected the liver against CCl_4 damage *in vitro*. The hepatoprotective effects were better than those of the hepatoprotective control silibinin. We also demonstrated the beneficial effects on hepatocellular function due to the presence of strong antioxidant compounds; i.e., flavonoids in general and the isolated hepatodamianol in particular.

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