Antioxidant and hepatoprotective activity of *Hamelia patens* extracts

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Abstract: *Hamelia patens* is widely used in the traditional medicine of Mexico and Central America for the treatment of illnesses associated with inflammatory processes. In this study, antioxidant and hepatoprotective activity were assayed on the methanolic crude (ME), hexane (HE), ethyl acetate (AE), and butanol (BE) extracts of *H. patens*. The total phenolic content (TPC) as mg of gallic acid equivalents per g of dry extract was determined by Folin-Ciocalteu's method (ME=141.58±11.99, HE=33.96±1.13, AE=375.18±13.09, BE=132.08±3.62), and antioxidant activity by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical-scavenging method (EC_{50} ME=77.87±5.67, HE=236.64±26.32, AE=45.87±2.24, BE=50.97±0.85µg/mL). Hepatoprotective activity was evaluated through AST activity on HepG2 cells subjected to damage with CCl_4 (ME=62.5±3.41, HE=72.25±2.87, AE=63.50±4.20, BE=43.74±4.03). BE showed the greater hepatoprotective activity and a good antioxidant capacity, while HE did not show hepatoprotective or antioxidant activity. Cytotoxicity was evaluated on Vero cells cultures; none showed significant toxicity.

Keywords: *Hamelia patens*, hepatoprotective, antioxidant, HepG2, cytotoxicity.

INTRODUCTION

Liver diseases of viral, alcoholic, and recently the non-alcoholic steatohepatitis (NASH) origins present a serious health problem in the world. Their main complications are liver cirrhosis, which has high morbidity and mortality. In some Latin American countries, such as Chile, Mexico and Peru, liver cirrhosis is among the top six causes of death overall (Alonso, 2010; Campollo, 1997; Narro, 1992; Varvasovszky *et al*., 2000). Treatment options for cirrhosis, fatty liver, and chronic hepatitis are often limited in their effectiveness, present various adverse effects, and are too expensive, especially for developing countries. The effectiveness of treatments such as interferon, penicillamine, statins and corticosteroids are inconsistent and are associated with several adverse effects; more effective drugs with a low incidence of side effects are required.

The discovery of new pharmacologically active agents obtained from natural sources has led to the development of many bioactive principles of clinical use that play an important role in the treatment of various diseases (Potterat, 2006). Around 40% of the drugs currently in use are directly or indirectly related to compounds of natural origin (Newman, 2003).

Over the years, the effects of plants traditionally used to improve liver function have been examined, and then used for the treatment of liver diseases. Some of these plants are *Silybum marianum*, *Tilia argentea*, *Mucuna pruriens*, *Phyllanthus amarus*, *Picrorhiza kurroa*, *Glycyrrhiza glabra*, *Peumus boldus*, *Taraxacum officinale*, *Centaurium erythraea*, *Centaurea aspera*, *Fumaria officinalis*, *Cynara scolymus*, *Pinellia ternata*, *Magnolia officinalis*, *Pimpinella anisum*, *Salvia officinalis*, *Melissa officinalis*, *Ilex paraguariensis*, *Eclipta prostrata*, *Osbeckia chinensis*, and *Apium graveolens* (Adame, 2000; Gosh, 2011; Hegedus, 2011; Kianbakht, 2011; Xia, 2011). The traditional uses and effects of most of these plants have been determined, and the mechanisms and mode of action for some have been documented (Gosh, 2011).

Some of these extracts have molecules related to flavonoids, which provide antioxidant, antifibrotic, antiviral, and/or anticarcinogenic properties, including glycyrrhizin, phyllantin, silibinin, picroside, and baicalein (Ashfaq, 2011; Hodek, 2002; Xia, 2011;). In general, the protective effects of flavonoids have been attributed to their ability to capture free radicals.

Recently, it has been described that the main mechanism that produces liver damage is the oxidative stress in a proinflammatory environment, so the use of antioxidant agents in the different stages of liver diseases would be of great therapeutic interest (Ben Saad, 2015; Pinzani, 2011; Sánchez-Valle, 2012).

The majority of the plants with hepatoprotective activity present high antioxidant activity. Among the compounds extracted from plants with hepatoprotective activity, the most studied are silibinin and glycyrrhizin. The silymarin obtained from *Silybum marianum*, is a complex mixture

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Table 1: Total phenolic content (TPC), free radical-scavenging activity (EC_{50}), AST activity (AST), and cytotoxicity activity of crude extract (ME), hexane (HE), ethyl acetate (AE), and butanol (BE)

Extract	TPC	EC_{50}	AST	CC_{50}
ME	141.58±11.99	77.87±5.67	62.50±3.41	> 500
HE	33.96±1.13	236.64±26.32	72.25±2.87	132.06±12.85
AE	375.18±13.09	45.87±2.24	63.50±4.20	466.06±35.37
BE	132.08±3.62	50.97±0.85	43.74±4.03	>500
Quercetin	_	4.33±0.03	_	_
Silibinin	_	_	53.00±4.96	_

TPC values are expressed as mg of gallic acid equivalents per g of dry extract. EC_{50} antioxidant activity values in μg/mL All values of AST levels are expressed as IU; AST damage control =75.50±3.78 IU. All values of CC_{50} are expressed in μg/mL: positive cytotoxicity control of doxorubicin CC_{50}=3.13±0.23μg/mL. All values are mean ± SD (for TPC, EC_{50} and AST n=3; for CC_{50} n=5).

of four flavonolignans isomers (silibinin, isosibilinin, silydianin and silychristin) that have anti-inflammatory, cytoprotective, anticarcinogenic, antioxidant properties, and capture free radicals (Abenavoli, 2010; Chien, 2011). Using *in vitro* studies, Torres-Gozález *et al.* (2011) demonstrated that the hydroalcoholic extracts of some plants of the northeastern region of Mexico, including *Centaurea americana*, *Juglans mollis*, and *Turnera diffusa*, presented hepatoprotective activity and important antioxidant capacity; in addition, the extracts did not show cytotoxic activity on the Huh7 cells.

Hamelia patens (also known as chacloco, plant of coral, firebush) is a species of large evergreen shrub from subtropical and tropical America. It is perennial and belongs to the Rubiaceae family. It is found from Florida in the south of the USA to Argentina, principally in central and southern Mexico, and extending up to Costa Rica (Argueta, 1994; Reyes-Chilpa, 2004). This plant is used to treat superficial wounds through the application of cataplasms and to decrease the inflammation caused by blows. The infusion of this plant is taken to lessen the discomfort of abdominal swelling and menstrual cramps, and the few studies that have been undertaken of this species have determined anti-inflammatory and antioxidant activity in its crude extracts (Ahmad, 2012; Ruiz-Terán, 2008). Several compounds of flavonoids, as well as terpenoids, coumarins, sterols, and anthocyanins, have been isolated from this plant (Gomez, 2001).

The aim of this study was to evaluate the hepatoprotective and antioxidant activities of crude and differential extracts from *Hamelia patens* to validate its use in Mexican traditional medicine.

MATERIALS AND METHODS

Reagents

Special reagents: 2,2-diphenyl-1-picryl-hydrazyl (DPPH), trypan blue 0.4%. For cell culture: thiazolyl blue tetrazolium bromide (MTT), phosphate-buffered saline solution (PBS), dimethyl sulfoxide, doxorubicin hydrochloride, carbon tetrachloride (CCl_4) were purchased from Sigma (St. Louis, MO, USA). Penicillin-Streptomycin (Sigma-Aldrich) (as an antibiotic), L-glutamine 200 mM (100X), Dulbecco's Modified Eagle Medium (DMEM Advanced), bovine fetal serum, 0.25% trypsin 1X (Gibco), and aspartate aminotransferase (Instrumentation Laboratory) were used. Biological material: HepG2 and Vero (ATCC) cell lines were used.

Plant material

Leaves and stems of *Hamelia patens* were collected from the northern area of the state of Veracruz, Mexico. A specimen was deposited in the Institutional Herbarium of the Biology Faculty at the Universidad Autónoma of Nuevo Leon (Monterrey, Mexico) for authentication (Voucher UNL-01458).

Extraction

Leaves and stems were dried at room temperature for seven days. Two hundred grams of pulverized material was extracted with methanol at room temperature on a shaker for 60 min. The procedure was repeated three times. The solvent of the extract obtained was evaporated to dryness under reduced pressure. The ME was maintained at -4°C until use.

Twenty grams of ME was resuspended in 250mL of water and subjected to differential extraction with hexane (100mL × 3), ethyl acetate (100mL × 3) and butanol (100mL × 3). The hexane (HE), ethyl acetate (AE), and butanol (BE) extracts were evaporated at reduced pressure to dryness and stored frozen at -°C until use.

Total polyphenol content

To determine the total phenolic content (TPC) by the Folin-Ciocalteu method, 1 mg of each extract or fraction in 1mL of methanol, was prepared. Briefly, the extract solution (100μL) was diluted to 3mL with distilled water and then oxidized with the Folin-Ciocalteu reagent (500 μL); after 3 min, the reaction was neutralized with Na_2CO_3 20% solution (2mL) and then allowed to react for 60 min in darkness. The absorbance of the resulting blue-colored reaction was measured at 650 nm in a Beckman DU7500 spectrophotometer. The calibration curve was performed with gallic acid (concentration range 0.30-

9.00µg/mL) and the results were expressed as mg of gallic acid equivalents per g of dry extract. Data are reported as mean ± SD for three replicates (fig. 1).

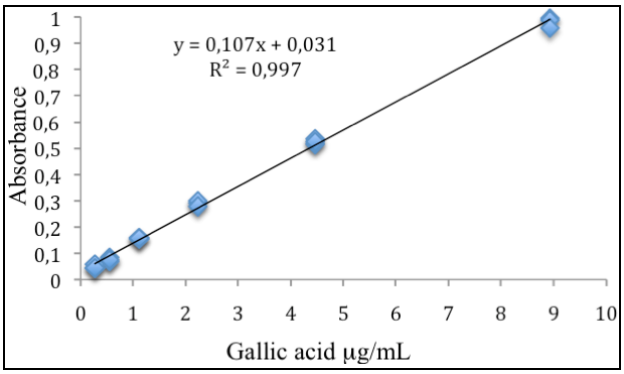

Fig. 1: Gallic acid linear regression to determine TPC. Each point was performed in triplicate.

Antioxidant activity

The free radical-scavenging activity of crude extract, hexane, AcOEt and BuOH extracts was determined using the DPPH free radical method in micro plate. All extracts from *H. patens* were evaluated in a concentration range of 5.0 to 230.0µg/mL. Briefly, serial dilutions in micro plates were performed with a total volume of 100µL methanol and the extract of each dilution. One hundred micro liters of a 280µM DPPH solution was added and allowed to react for 15 min in the dark. The plate was read in a Thermo Scientific Multiskan FC micro plate reader at a wavelength of 540 nm. Controls of 100µL methanol and 100 µL DPPH were included to determine the 100% of signal. Two hundred micro liters of methanol was used as a blank, and a serial dilution of the extract without DPPH was used as a sample blank. Quercetin was used for positive antioxidant activity control. To calculate the percentage reduction of DPPH, the following formula was used.

% Reduction = (Absorbance 100% signal control- Absorbance of sample) × 100
Absorbance of 100% signal control
where Absorbance 100% signal control = Average measure of 100% signal control DPPH minus the average of the solvent blank.

Absorbance of sample = Average measure of sample absorbance minus the dilution sample blank absorbance.

Each sample was assayed in triplicate. A linear regression curve was constructed using the percentage inhibition of DPPH versus concentration, and the effective concentration that reduced DPPH by 50% (EC_{50}) was interpolated. EC_{50} results were expressed as mean ± SD.

Cell culture

Human hepatocarcinoma cells (HepG2) and kidney epithelial cells from green monkey (Vero) were used.

Maintenance of cell lines. Cells were propagated in DMEM Advanced and supplemented with 10% fetal bovine serum (FBS), 1% antibiotic (penicillin and streptomycin), and 1% L-glutamine, and allowed to incubate at 37°C in a humidified atmosphere with 5% CO_2. Monolayer cells within 70-80% confluence were washed with saline solution (PBS) and detached with trypsin.

Hepatoprotective activity

Evaluation of hepatoprotective activity was performed as reported by Torres-González *et al*. (2011), with small changes. Briefly, one million HepG2 cells per well were cultivated in 6-well plates at 37°C, in a humidified atmosphere with 5% CO_2. After 12 h, the medium was removed and the cells were washed with PBS. The ME and differential extracts of HE, AE, and BE were dissolved in PBS to a concentration of 100µg/mL, and added to the culture, then incubated for 1 h under the same conditions. After this step, supernatants were removed and CCl_4 was added. Cultures were maintained for 2 h under the same incubation conditions, and then the supernatant was obtained and the AST activity was determined.

Aspartate aminotransferase activity (AST)

The AST activity was determined following the protocol recommended by the kit supplier (Instrumentation Laboratory) in an Ilab 300 Plus instrument. Determination was performed as recommended by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The decrease in absorbance due to the oxidation of NADH to NAD+ is directly proportional to AST activity in the sample. Each result was obtained in triplicate and expressed as mean ± SD.

Determination of cytotoxicity

This assay was performed by reducing 3-(4,5-dimethylthiazol-2-yl)-2,3- diphenyltetrazolium bromide (MTT) to formazan crystals, as described by Mossman (1983), with minor modifications; cellular reduction reaction involves the action of pyridine nucleotide cofactors NAD+/NADPH and mitochondrial dehydrogenase enzyme, which are functional in living cells. The cell membrane is impermeable to the formazan product, causing it to accumulate within the cell. The crystals are released when a cell lysing agent is added, allowing the quantification of the formed product. Product intensity is proportional to the number of living cells. Briefly, in 96-well plates, 1000 Vero cells were placed per well and incubated for 24 h at 37°C in a humidified atmosphere with 5% CO_2. Different concentrations of extracts (ME, HE, AE, and BE) were added to each well (0.5-00µg/mL) and the plates were incubated for 48 h under the same conditions. Subsequently, each well was washed twice with PBS and 200 µL of MTT (0.5mg/mL in culture medium) was added and allowed to incubate for

3 h. The supernatant was decanted and 200µL of dimethyl sulfoxide (DMSO) was added to each well; plates were shaken manually for 8 min and the absorbance at 540 nm was measured in a Thermo Scientific Multiskan FC plate reader. In every plate, some wells with only cells in culture medium (without test samples) were included; these were considered to have 100% viability. As a positive control, doxorubicin was used at different concentrations (0.025-25µg/mL). Each concentration was evaluated five times in three different plates.

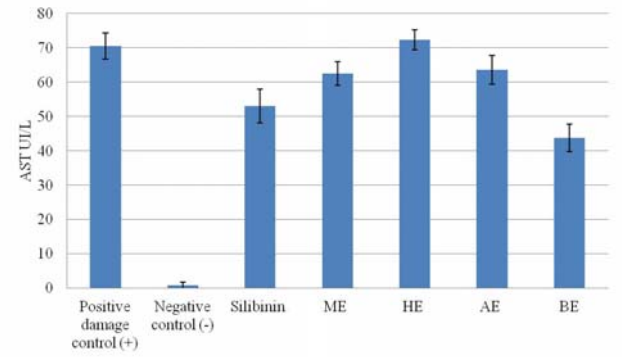

Fig. 2: AST activity for *H. patens* extracts on HepG2 cells. Silibinin was used as damage protection control, and CCl_4 was used as positive damage control.

Percent inhibition for each concentration was calculated by relating the absorbance value of each well with the absorbance value at 100% of control cell viability. A linear regression curve was constructed for each fraction using percentage inhibition versus concentration, and the concentration that reduces cell growth by 50% (CC_{50}) was interpolated. Results are expressed as mean ± SD.

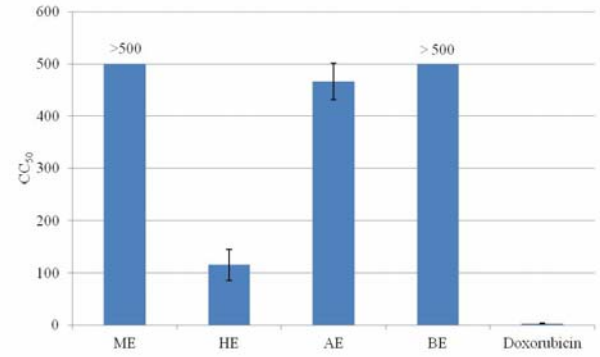

Fig. 3: Citotoxicity activity of *H. patens* extracts on Vero cells. Doxorrubicin was used as positive cytotoxicity control.

STATISTICAL ANALYSIS

All variables were tested three times for *in vitro* assays. Results are expressed as means ± SD. Data obtained were analyzed using Student's *t* test for independent samples using SPSS software (v15.0; SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered significant.

RESULTS

The total phenolic content TPC indicated that ethyl acetate extract (AE), followed by butanol extract (BE) showed the highest amount of these compounds, and this is also observed in the DPPH free radical-scavenging assay (table 1). The AST enzyme activity showed that ME, AE and BE extracts have a considerable hepatoprotective activity, even BE has greater activity than the control silibinin (Fig. 2). Except HE, none of the tested extracts showed cytotoxicity against Vero cell line (fig. 3).

DISCUSSION

The extracts obtained from *Hamelia patens*, ME, HE, and BE were evaluated by the Folin-Ciocalteu method to determine the total phenolics content and by DPPH free radical-scavenging method to determine antioxidant activity. As shown in table 1, TPC indicates that the polyphenolic compounds present in ME, AE, and BE were greater than in HE. The AE presented the highest content of all (375.18±13.09mg gallic acid equivalent). The results of antioxidant activity in the DPPH test are related to the amounts of TPC because AE (EC_{50}=45.87± 2.24µg/mL) and BE (EC_{50}=50.97±0.85µg/mL) showed the highest antioxidant activity with respect to ME (EC_{50} =77.87±5.67µg/mL) and HE (EC_{50}=236.64±26.32 µg/mL). These findings suggest that the antioxidant activity of the extracts may be due mainly to the presence of polyphenolic compounds.

On the other hand, the obtained levels of AST activity showed that ME, AE and BE have hepatoprotection activity comparable to silibinin (fig. 2), the compound used as positive control activity (53.00±4.96 IU AST). In this assay, BE shows the greatest hepatoprotective activity, with even lower levels of AST enzyme than silibinin. HE did not show hepatoprotection activity (72.25±2.87 IU AST) in comparison with control damage (75.50±3.78 IU AST). In fact, HE was the extract with the lowest amount of polyphenolic compounds (TPC) and the lowest DPPH free radical-scavenging activity. In the *in vitro* hepatoprotective activity assay, liver HepG2 cells are subjected to damage caused by CCl_4. During this process, the damage by the inducing agent (CCl_4) promotes the generation of reactive oxygen species (ROS) in the medium, which results in a lysis effect on liver cells. It is known that no matter what induces the damage to the liver tissue (alcohol, drugs, viruses, autoantibodies, obesity) this will result in a process of generating ROS. Therefore, recent treatments applied in diseases involving chronic liver damage have included antioxidants such as silibinin (isolated from *Silybum marianum*). According to the test results with HepG2 cells, this could indicate a relationship between antioxidant activity and hepatoprotective activity of the tested extracts of *Hamelia*

patens. Clearly, when there are no polyphenols, the protection of HepG2 cells is low. However, the presence of phenols, by itself, does not mean protection because it may depend on the type of polyphenols; BE was the most protective but had fewer polyphenols than ME and AE, suggesting that AE polyphenols are not as active as those of BE.

Against Vero cells, all extracts showed a significant difference from the positive cytotoxicity control of doxorubicin (CC_{50}=3.13±0.23µg/mL). ME, AE, and BE showed CC_{50} near or above 500µg/mL (fig. 3). In contrast, previous studies indicate that the ME of *H. patens* shows cytotoxic activity against such cancer cell lines as cervix adenocarcinoma (HeLa) and cervix squamous carcinoma (SiHa) (Mena-Rejón, 2009). In this study, the extracts were tested against normal cell lines and none were toxic. Therefore, it is important to continue with the bioassay-guided isolation of EA and BE extracts to find the compound or compounds responsible for the antioxidant and potential hepatoprotective activity of this plant.

CONCLUSIONS

Antioxidant activity was determined with a DPPH free radical-scavenging microplate method of crude and differential extracts from *Hamelia patens*. This is the first time that *in vitro* hepatoprotective activity was released on this plant. ME and AE have comparable hepatoprotective activity to the protection standard silibinin, while BE had greater hepatoprotective activity than the protection control. EA and BE are the extracts with the greater amount of polyphenolic compounds and better antioxidant activity from the three differential extracts. Antioxidant activity could be related to the hepatoprotective activity of these extracts. Except for HE, the extracts obtained from *H. patens* did not show cytotoxicity against Vero cells, so these are a potential source for isolating the compounds responsible for its activity.

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AUTHOR CONTRIBUTIONS

Participation of each author is as follows: Jonathan Pérez-Meseguer performed most of the experiments and wrote the final manuscript; Cecilia Delgado-Montemayor and Tania Ortíz_Torres helped with the *in vitro* assays and data analysis; Ricardo Salazar-Aranda helped with the cytotoxicity assays and with the redaction of the manuscript; Paula Cordero-Pérez was in charge of all the biological assays, conceived and designed the experiments and helped with the write up; and Noemí Waksman de Torres generated the idea and analyzed the data.

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