

Phenolic Composition and Evaluation of Antioxidant and Cytoprotective Activity of *Chiliadenus montanus*

Tarek F. Eissa, Elena González-Burgos, M. Emilia Carretero and
M. Pilar Gómez-Serranillos *

Department of Pharmacology, Faculty of Pharmacy, University Complutense of Madrid, Spain

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Abstract: The antioxidant and cytoprotective activities of the hydroalcoholic extract of *Chiliadenus montanus*, widely used in Egyptian traditional medicine, were investigated. The antioxidant potential, determined using ORAC assay, revealed that *Chiliadenus montanus* extracts are active radical scavengers (ORAC value 1.720 $\mu\text{mol TE/mg}$ sample). Total phenolic content, measured by Folin-Ciocalteu, was 107.4 mg galic acid/g sample. HPLC and HPLC-MS analysis allowed individual polyphenolic compounds to be identified. Furthermore, the cytoprotective effect of *Chiliadenus montanus* hydroalcoholic extracts was analyzed in an *in vitro* oxidative stress model employing H_2O_2 as an oxidant inductor and the human astrocytoma U373-MG cell line as cell model. The results obtained showed that *Chiliadenus montanus* hydroalcoholic extracts exert a protective action by decreasing cell death and by inhibiting intracellular ROS production, suggesting that these polyphenol-enriched extracts may be useful for those oxidative stress-related neurodegenerative diseases.

Keywords: *Chiliadenus montanus*; phenolic compounds; antioxidant; oxidative stress.

1. Introduction

Egypt's Sinai deserts have hundreds of medicinal plants, whose traditional uses were passed down from the ancient Egyptians through the generations and till now some people in the desert of Sinai have great experiences of treating different diseases by using them.

Chiliadenus montanus (Vahl.) Brullo (Family Asteraceae), popularly known as Haneida, is common in the Sinai Peninsula. This medicinal plant is traditionally used for diarrhea, renal troubles, stomachache and chest diseases [1]. Moreover, evidences for their hypoglycemic, anticholestatic and antioxidant activities have been recently investigated [2,3]. Previous phytochemical researchers have identified the presence of active constituents in the aerial parts, including phenolic compounds that give *Chiliadenus montanus* their medicinal values [1,4].

Oxidative stress, mediated by reactive oxygen species (ROS), has been implicated in the pathophysiology of many diseases including CNS disorders. The excess ROS can damage lipids, proteins and DNA, leading to cytotoxic and genotoxic effects and consequently serious human diseases such as the just mentioned CNS disorders [5]. Antioxidants remain as the medical choice strategy for protection against this unbalanced oxidant-antioxidant status. Among natural antioxidants, phenolic compounds such as flavonols, coumarins, cinnamic acids and caffeic acids are well known

* Corresponding author: E-Mail: pserra@farm.ucm.es; Tel.: +34-91-394-17-67; Fax: +34-91-394-22-76.

for its proven and demonstrated chemopreventive potential against ROS overproduction through direct and indirect antioxidant mechanisms [6,7].

The above mentioned medicinal activities of *Chiliadenus montanus* encouraged the authors to investigate for the first time the antioxidant and cytoprotective properties of its hydroalcoholic extracts under oxidative stress conditions H₂O₂-induced in astrocytes.

2. Materials and Methods

2.1. Materials

All chemicals were from Sigma–Aldrich (St. Louis, MO) and cell culture products from Gibco (Grand Island, NY).

2.2. Plant material

Chiliadenus montanus plants were collected in May 2008 from mountains of Saint Katherine, Sinai, Egypt and were authenticated in the Department of Plant Biology (MAF) of Faculty of Pharmacy of the University Complutense of Madrid, where voucher specimens were deposited with the number identification MAF 169946.

2.3. Extracts preparation

Chiliadenus montanus plants were dried at room temperature for 3–4 days. Small cut portion of the plant material (500g) were successively extracted with 70% ethanol by using a Soxhlet extractor for 72 h at room temperature. The combined ethanol extracts were filtered using Whatman filter paper (N°: 1) and then concentrated on Rotary Evaporator at 55 °C. The concentrate extract allowed drying under suction, being the yield concentrates 19g/100g. That concentrates were kept at 4 °C prior to use.

2.4. Total phenolic content

Total phenolic compounds content was determined according to Folin-Ciocalteu method [8]. Results are expressed as gallic acid equivalents in mg/mL hydroalcoholic extract.

2.5. HPLC-MS analysis

The analysis was carried out on a HPLC-PAD Waters system (Milford, Mass, USA), comprising an autoinjector, a quaternary pump, a photodiode-array detector 2001 and a column Nova-Pak C18 (300 x 3.9 mm, 4 μm). The analytical conditions were based on those described by [9] with some modifications. Two mobile phases were employed for elution, A: water/acetic acid (98:2 v/v) and B: water/acetonitrile/acetic acid (78:20:2 v/v/v). The gradient profile was 0–55 min, 100%–20% A; 55–70 min, 20%–10% A; 70–80 min, 10%–5% A; 80–100 min, 100% B. The flow rate was 1 ml/min from the beginning to 55 min and 1.2 ml/min from this point to the end. Detection was performed by scanning from 210 to 400 nm with an acquisition speed of 1s. A volume of 25 μL was injected. The samples were analyzed in duplicate.

Mass spectra were obtained using a Hewlett Packard 1100MS (Palo Alto, CA) chromatograph equipped with an API source, equipped with an electrospray ionisation (ESI) interface. The solvent gradient and column employed were identical to those for HPLC-PAD analyses. The ESI conditions were as follows: negative-ion mode of analysis; N₂ as the nebulising gas at 275 kPa, drying gas flow rate and temperature of 10 L/min and 340°C, respectively; voltage at the capillary entrance was set at 4000 V; and variable fragmentation voltage at 100 V (*m/z* 200–1000) and 250 V (*m/z* 1000–2500). Mass spectra were recorded from an *m/z* of 100–2500.

2.6. ORAC assay

Antioxidant activity was measured using oxygen radical absorbance capacity (ORAC) assay [10]. Dilutions of hydroalcoholic extracts samples and of Trolox (antioxidant reference compound) were made in phosphate buffer (pH 7.4, 10 mM). Then, the reaction mixture containing samples or Trolox and fluorescein (70 nM final concentration) were incubated for 10 min at 37°C. Following, 2,2'-azobis(2-amidino-propane) dihydrochloride (12 mM final concentration) was added to the mixture. Fluorescence was read every 56 s for 98 min using FLUOstar Optima (BMG Labtech) fluorometer. Area under curve (AUC) was calculated for each sample and compared with AUC corresponding to Trolox. Results were expressed as μM of Trolox Equivalents (TE)/mg of sample.

2.7. Cell culture

The human astrocytoma U373 MG cell line was cultivated in DMEM containing 10% FBS and gentamicine (0.5%) at 37 °C in a humidified atmosphere of 5% CO₂ - 95% air.

2.8. Cytotoxicity and cytoprotective assays (MTT assay)

MTT assay was performed as [11]. After treatments, cells were incubated with MTT (2 mg/mL PBS, 1 h, 37 °C). The formed dark blue formazan crystals were dissolved with DMSO. Absorbance was measured at 550 nm. Results are expressed as percentage of cell viability (%), assuming control cells as 100%.

2.9. DCFH-DA assay

Intracellular reactive oxygen species (ROS) generation was determined by using 2,7-dichlorofluorescein diacetate (DCFH-DA) assay [12]. DCFH-DA (0.01 M) in PBS-glucose was added to cells for 30 min at 37 °C. Then, cells were washed with PBS-glucose and treated with two different concentrations of each hydroalcoholic extract sample and with H₂O₂ (0.1 mM). Fluorescence was determined at 485 nm (excitation) and 530 nm (emission).

2.10. Statistical analysis

Results are expressed as mean \pm standard deviation (S.D.) of at least three independent experiments. The statistical evaluation was determined by one-way analysis of variance followed by Tukey's test. p value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Antioxidant capacity and total phenolic content

The antioxidant capacity of the *Chiliadenus montanus* hydroalcoholic plant extracts was investigated using ORAC assay. This method is widely used to assess the scavenging activity of antioxidants and it is based in the peroxy radical scavenging activity. Peroxy radicals are one of the most common ROS found in the body [11]. The antioxidant activity of plant extracts was compared to Trolox, a water-soluble vitamin E analogue used as the standard, and the results were plotted against Trolox equivalence in mg sample. The ORAC value was 1.720 $\mu\text{mol TE}/\text{mg sample}$. This value reflects that the hydroalcoholic extract investigated has potent active radical scavenging properties by hydrogen atom transfer pathway (Table 1). The ORAC radical-scavenging activity demonstrated by *Chiliadenus montanus* hydroalcoholic plant extracts may protect against the harmful action of oxidative species, and therefore from their ability to induce lipid peroxidation in cell membranes, DNA breakage and protein oxidation. These events on biological systems eventually trigger apoptotic and necrotic cell death and consequently they are involved in the pathophysiological development of many different oxidative stress-related diseases [13].

Next, the total content in phenol compounds of *Chiliadenus montanus* hydroalcoholic extracts was investigated using Folin-Ciocalteu assay. The results obtained from the Folin-Ciocalteu assay, expressed in milligram of galic acid per gram of sample, revealed that *Chiliadenus montanus* contain high quantities of phenolic compounds (107.4 mg galic acid/g sample) as shown in Table 1. Phenolic-type compounds have been reported to be the main contributors of antioxidant activity and this activity has been structurally-related with the hydroxyl groups attached to benzene ring [14]. The high content in substances with phenols group explains, at least in part, its radical scavenging activity in ORAC assay.

Table 1. Antioxidant capacity and total phenolic content of *Chiliadenus montanus* hydroalcoholic extracts ^a.

Hydroalcoholic extracts	ORAC value ($\mu\text{mol TE} / \text{mg sample}$)	Phenolic content (mg galic acid / g sample)
<i>Chiliadenus montanus</i>	1.720 ± 0.33	107.4 ± 0.032

^aResults are expressed as (medium \pm S.D) and are representative of at least three independent experiments.

3.2. Analysis of phenolic compounds

The HPLC-MS was employed to analyze the polyphenolic compounds profile of the hydroalcoholic plant of *Chiliadenus montanus* and thus identifying the natural components responsible for the antioxidant activities. As shown in Figure 1, this extract is a rich source of cinnamic acids [chlorogenic acid derivatives (1, 2, 3), caffeoil quinic derivates (4, 5, 6, 7, 8, 9, 12, 13, 14)]. Cinnamic acids have been previously reported to be efficient antioxidant compounds; the double bond presented in its structure is the main responsible for its antioxidant action through radical resonance-stabilization mechanisms [14]. Moreover, flavonols [quercetin glucuronide (10), syringetin galactoside or glucoside (11), kaempferol 3-O-acetyl-glucoside (15)], with beneficial antioxidant properties through its H-donating capacity, are also displayed.

3.3. Cytoprotective effects

3.3.1. Effect of hydroalcoholic extracts on U373 MG cell viability

None of concentrations assayed (0.025; 0.25; 2.5; 25 and 250 $\mu\text{g/mL}$; 24 h) of the *Chiliadenus montanus* hydroalcoholic plant extracts were cytotoxic for U373-MG (Figure 2).

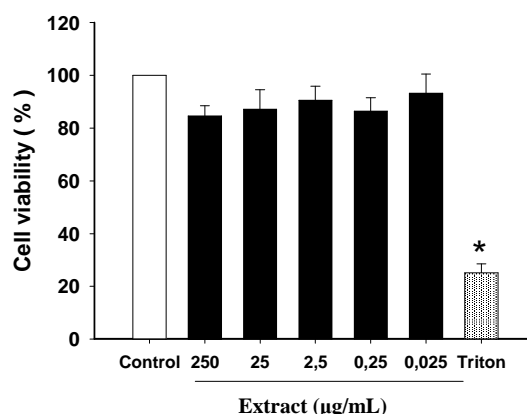


Figure 2. Effect of *Chiliadenus montanus* hydroalcoholic extracts on U373 MG cell viability. U373 MG cells were treated for 24 h with different concentrations of plant extracts (range of concentrations from 0.025 to 250 $\mu\text{g/mL}$). Cell viability was assayed using MTT assay. Results are expressed as cell viability (%) (medium \pm S.D) relative to control cells and are representative of at least three independent experiments. * $p < 0.05$ versus control cells.

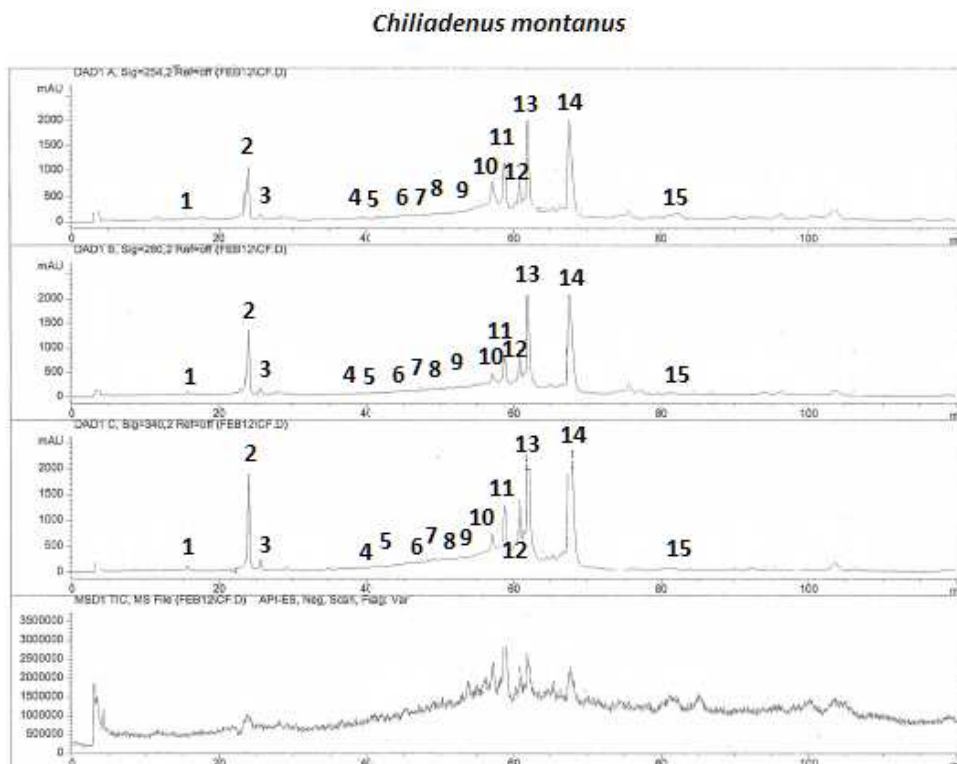


Figure 1. HPLC–MS chromatograms for *Chiliadenus montanus* hydroalcoholic extracts. Mass spectrometric profile at 254 nm, 280 nm and 340 nm. The compounds: Chlorogenic acid derivatives (**1-3**), caffeoyl quinic derivatives (**4-9, 12-14**), quercetin glucuronide (**10**), syringetin galactoside or glucoside (**11**), kaempferol 3-O-acetyl-glucoside (**15**).

3.3.2. Effect of hydroalcoholic extracts on U373 MG cells under oxidative stress conditions

Oxidative stress plays a very important role in the pathogenesis of several CNS disorders. Antioxidants can protect the human body from oxidative stress derived damage, resulting from ROS overproduction [5]. *Chiliadenus montanus* hydroalcoholic extracts contain high amounts of phenolic compounds and possess good antioxidant properties as we have demonstrated in the above assays. Then, we evaluated the possible cytoprotective role of these extracts in an *in vitro* oxidative stress model caused by an application of exogenous H₂O₂ in an astrocytoma cell line.

Figure 3 shows that H₂O₂ (1 mM, 30 min) caused a significant cell viability reduction of 71.5% compared to control cells. However, a protection against H₂O₂-induced oxidative stress was observed when U373 MG cells were pretreated (24 h) with the hydroalcoholic extracts of *Chiliadenus montanus* (range of concentrations from 0.025 to 250 µg/mL). The maximum cellular protective effect was observed for 0.25 µg/mL and 0.025 µg/mL concentrations by increasing cell viability 22.7% and 25.6%, respectively, respect to cells treated only with H₂O₂. As 0.25 and 0.025 µg/mL concentrations exerted a significant protective effect, they were selected for the study of the effect of hydroalcoholic plant extracts on ROS formation H₂O₂-induced.

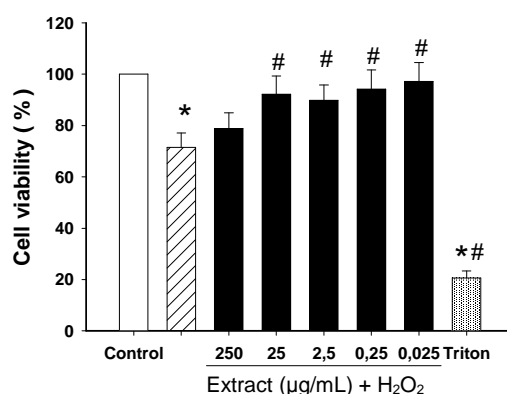


Figure 3. Protective effect of *Chiliadenus montanus* hydroalcoholic extracts under oxidative stress conditions. U373 MG cells were treated for 24 h with different concentrations of plant extracts (range of concentrations from 0.025 to 250 $\mu\text{g/mL}$), previous to an exposition of 1 mM of H_2O_2 . Cell viability was assayed using MTT assay. Results are expressed as cell viability (%) (medium \pm S.D) relative to control cells and are representative of at least three independent experiments. * $p < 0.05$ versus control cells, # $p < 0.05$ versus H_2O_2 .

3.3.3. Effect of hydroalcoholic extracts on ROS production H_2O_2 -induced

Hydrogen peroxide is the major ROS in the human body and a source of other potent free radicals such as hydroxyl radical [15]. We explored the potential ROS scavenging activities of *Chiliadenus montanus* hydroalcoholic extracts in a cellular model. H_2O_2 caused a significant ROS production time-dependently as shown DCFH-DA assay (Figure 4). A significant inhibition of ROS production H_2O_2 -induced was observed when U373 MG cells were pretreated with both 0.25 and 0.025 $\mu\text{g/mL}$ concentrations of hydroalcoholic extracts.

These findings support those reported previously about the effectiveness of different *Chiliadenus montanus* extracts on different oxidative stress situations and experimental models (animals, cells) and provide evidences about their mechanism of action [2, 3].

4. Conclusions

The hydroalcoholic extracts of *Chiliadenus montanus* have been found to possess good antioxidant properties by scavenging oxygen derived radicals commonly found in the human body. The developed analytical method for identifying phenolic compounds demonstrated the presence of a high variety and quantity of cinnamic acids. The beneficial effects of the hydroalcoholic extracts of *Chiliadenus montanus*, based on their antioxidant actions, have a potential chemopreventive role for those diseases associated with oxidative stress including neurodegenerative disorders such as Parkinson's and Alzheimer's diseases. Further studies for isolation of phenolic compounds to identify which are chiefly particularly responsible for this activity and to further investigate the mechanistic basis of this protection are in prospect.

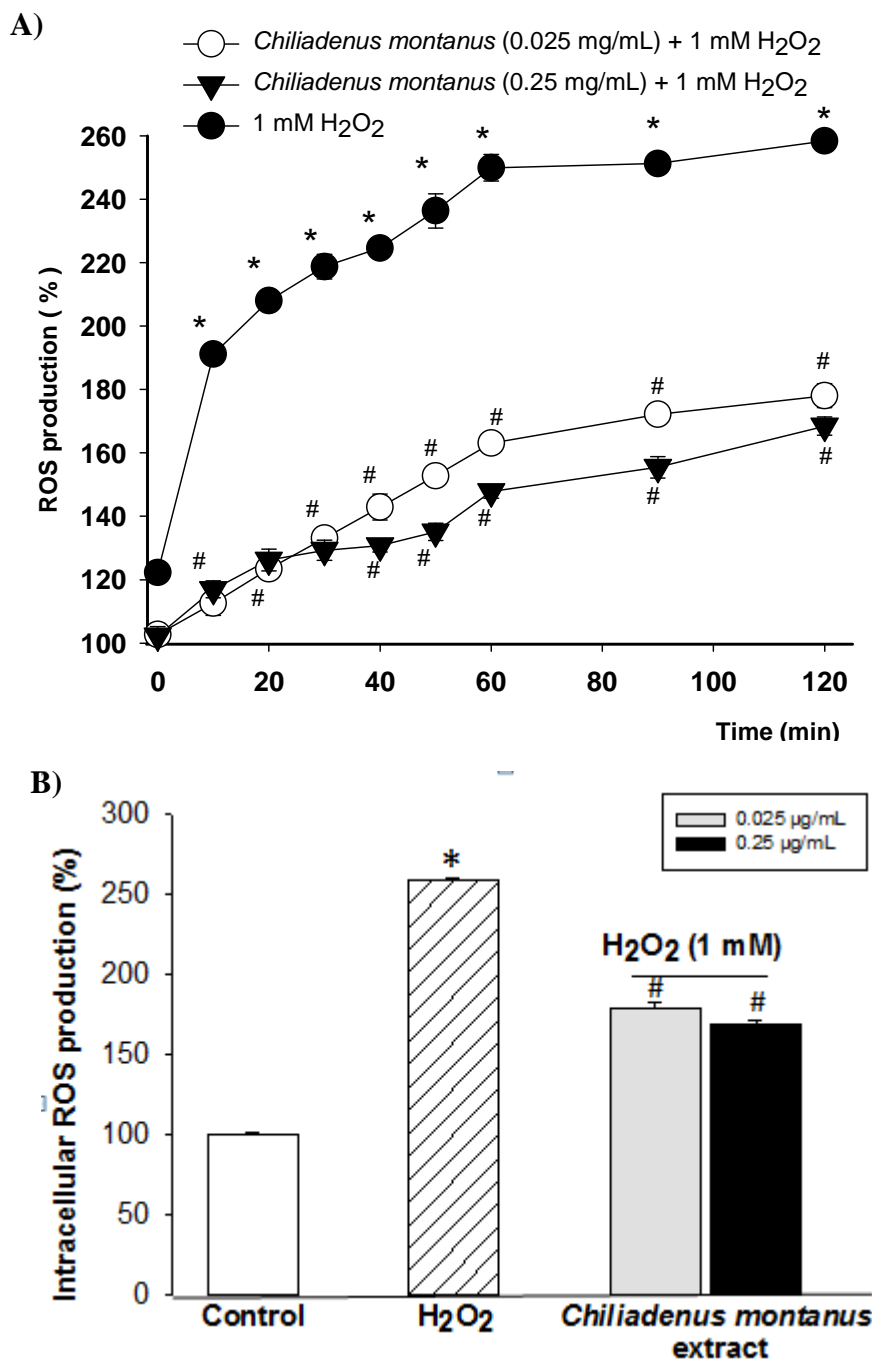


Figure 4. Effect of *Chiliadenus montanus* hydroalcoholic extracts on ROS production H₂O₂-induced. U373 MG cells were treated with plant extracts (0.025 and 0.25 µg/mL) and 1 mM of H₂O₂. ROS production was assayed using DCFHA assay. A) Curves of intracellular ROS production (%) versus time. B) Percentage of ROS production after 120 min of the assay. Results are expressed as free radical production (%) (medium ± S.D) and are representative of at least three independent experiments. *p < 0.05 versus control cells, #p < 0.05 versus H₂O₂.

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