

# Preliminary studies on antioxidant and anti-cataract activities of *Cheilanthes glauca* (Cav.) Mett. through various in vitro models

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Aqueous extracts of *Cheilanthes glauca* (Cav.) Mett., (Adiantaceae), commonly named Doradilla, are often used in folk medicine as anti-inflammatory and for diabetes treatment. In the present study the antioxidant capacity of freeze-dried extract of *Ch. glauca* was investigated through different tests, i.e. i) 1,1-diphenyl-2-picrylhydrazyl (DPPH), ii) 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activities iii) ferric reducing antioxidant power (FRAP) iv) Oxygen Radical Absorbance Capacity (ORAC) v) lipid peroxidation in rat liver microsomes and vi) anti-cataract effect on cultured bovine lens. The total phenolic content of the plant was also determined to estimate the antioxidant activity as gallic acid equivalent (GAE). Doradilla extracts exhibited a strong antioxidant capacity, which was concentration-dependent in all assays. To ascertain the possible explanation for this potent activity, the percentages of rutin and total flavonoids were estimated by planar chromatography, found values of 3.50% ± 0.05% and 11.70% ± 0.08% respectively. Only rutin was able to partially inhibit cataract formation in high glucose conditions. The results suggest that rutin could be related to some of pharmacological effects of *Ch. glauca*. In vitro models clearly established the antioxidant properties of this plant and its use in pathological conditions where oxidative stress was involved.

## Introduction

Diabetes mellitus represents the final consequence of a chronic and progressive syndrome. The costs of diabetes treatment and its associated complications have great implications, and therefore prevention measures are very important. A recent study of the World Health Organization estimated that the worldwide prevalence of diabetes in 2002 was 170 million, which has a predicted growth to 366 million by 2030 [1, 2]. Type 1 and 2 diabetes, exhibit hyperglycemia as their hallmark. Type 1 diabetes, accounting for 5-10% of diabetes diagnoses, is caused by a failure of the pancreatic  $\beta$ -islet cells producing an insulin deficiency. Type 2 diabetes encompasses 90% of diabetes and it is characterized by insulin resistance often accompanied by obesity and dyslipidemia. The vascular complications of diabetes are divided into macrovascular and microvascular categories. Microvascular complications such as retinopathy, neuropathy, and nephropathy are important causes of morbidity and mortality in diabetic patients and have been involved in oxidative stress. There are several studies demonstrating that patients with diabetes not only have increased levels of circulating markers of free radical-induced damage, but also have reduced antioxidant defenses [3]. Hyperglycemia can induce oxidative stress via several mechanisms. These include glucose autooxidation, formation of advanced glycation end-products (AGE), and activation of the polyol pathway. The latter induce intracellular overload of sorbitol and have been proposed as initial event of various types of ocular lesions. Additionally, this polyol was involved in generation of peripheral neuropathy [4]. The biochemical reactions known as "polyol pathway" represent only a 3% of glucose metabolism. During hyperglycemia or osmotic stress

the pathway activity increases several times. Association of this condition with chronic complications in diabetes has been observed in experimental models of hyperglycemia, where significant alterations affected the lens. These changes are characterized by high sorbitol levels, alterations on the membrane permeability, loss of glutathione (GSH) and a diminution of the protein synthesis [5]. Additionally, diabetic individuals present a polymorphism in the promoter region of ARL2 gene, generating major susceptibility to develop cataracts, neuropathy and retinopathy [6]. By the other side, oxidative stress is associated with cataracts formation by production of hydrogen peroxide through glucose auto-oxidation [7]. Levels of hydrogen peroxide in normal lens and aqueous humor are between 20 and 30 mol L<sup>-1</sup>. This value is 10 or 20 times higher in lens with cataracts. This finding strongly suggests that hydroxide peroxide is the most important reactive oxygen intermediary with deleterious effect upon lens [8]. Moreover, as diabetic lens has lower GSH levels than normal ones, the probability of protein damage is increased [9]. Therefore, now is established that in diabetes, oxidation events (lipids and proteins), AGE, sorbitol accumulation and other events contribute to cataracts formation and retinopathy [10, 11].

From biological point of view an antioxidant is defined as any molecule that in relatively low concentrations prevents free radical generation and neutralizes oxidation of biological substrates [12]. Antioxidant substances could act as reducing agents, free radical scavengers, singlet oxygen quenchers or by complex formation with metallic ions like Fe<sup>2+</sup> and Cu<sup>2+</sup>.

There is a plethora of information about foodstuff and medicinal plants with antioxidant capacity evaluated by different methods. *Vaccinium myrtillus* (billberry), *Ginkgo biloba*, *Vitis vinifera* and *Rosmarinus officinalis* among others, have been investigated for some complications associated with diabetes like retinopathy and atherosclerosis. The contribution of these plants in lowering glucose levels was also investigated elsewhere [13, 14]. In our previous work rutin and kaempferitrin were isolated as predominating flavonoids from *Cheilanthes glauca* (Cav.) Mett. Only rutin displayed notorious scavenging activity on DPPH assay [15]. In the same study the contents of rutin and total flavonoids were not determined, hence is not clear if these compounds could be responsible of relevant biological effects. Therefore, presence of high levels of kaempferitrin could be important due to its significant hypoglycemic effect proved in animal models. Hypoglycemic activity of kaempferitrin and other flavonoids has been demonstrated for some *Bauhinia* species. Recently, a quercetin glycoside isolated from *B. megalandra* caused inhibition of

hepatic neoglucogenesis and glucose-6-phosphatase [16]. Also, it has been reported that *Bauhinia candicans* shown hypoglycaemic effect stimulating glucose uptake in isolated gastric glands of normal and diabetic rabbits [17, 18]. Additionally, administration of pure flavonoids such as quercetin, rutin and kaempferitrin induced antihyperglycaemic and antioxidant effects in streptozotocin-induced diabetic rats [19, 20]. Consumption of polyphenols containing foods and herbal teas has been proposed as a useful practice to decrease the oxidative damage in the body. Because of this, the objectives of the study were to assess the antioxidant activity of *Cheilanthes glauca* extracts using some general assays, i.e. DPPH, ABTS, ORAC and FRAP and to evaluate its anti-cataracts effect through an in vitro model with bovine lens. Furthermore the effect of the extracts on the consumed oxygen and on lipid peroxidation in the hepatic microsomes, was measured. For quantitative evaluation of rutin and total flavonoids levels an HPTLC method was developed.

## Materials and Methods

### Chemicals and reagents

2,4,6-tripyridyl-s-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH),  $\beta$ -phycoerythrin ( $\beta$ -PE), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Triton X-100, bovine serum albumin (BSA) and Folin-Ciocalteu reagents were purchased from Sigma (St. Louis, MO, USA). Thiobarbituric acid (TBA), trichloroacetic acid (TCA) and all the others chemical and solvents were obtained from Merck (Darmstadt, Germany).

### Plants material

*Cheilanthes glauca* (Cav.) Mett. (CONC 148497) samples were collected in January of 2005 in Peña Negra, located 80 Km from Chillan, 600 m s.m. (36° 58' S – 71° 48' W) in the VIII Region, Chile.

### Extraction

100 g of dried *Ch. glauca* stems were extracted twice with 1 L of methanol-water (8:2 v/v). Pooled extracts were rotary evaporated under vacuum (40° C) and the aqueous extract was freeze-dried to yield 9.24 g of material (EChg). Polyphenols-free extract of *Ch. glauca* was prepared dispersing 100 mg of freeze-dried EChg in 5 mL of ethanol. This solution was centrifuged (2500 g, 15 min) and the supernatant was loaded onto a column packaged with 5 g of

diethylaminoethyl cellulose (DEAE cellulose). Column was eluted with 50 mL of ethanol and the resulting extract was dried under nitrogen current stream (EChg-DEAE yield 2.5 mg).

### Total polyphenol analysis (TP)

Total phenolic content in *Ch. glauca* extracts were determined with Folin-Ciocalteu reagent according to the method of Singleton and Rossi [21], using gallic acid as standard. Briefly, 100  $\mu\text{L}$  of 1  $\text{mg mL}^{-1}$  extract was mixed with 0.2 mL of Folin-Ciocalteu reagent, 2 mL of distilled water, and 1 mL of 200  $\text{g L}^{-1}$   $\text{Na}_2\text{CO}_3$ , and the mixture was measured at 765 nm after 1 hour at room temperature using a V-500 Jasco spectrophotometer (Easton, USA). A Gallic acid calibration plot was established from 5 to 20  $\text{mg L}^{-1}$  (final concentration in cuvette). The results were expressed as  $\text{mg g}^{-1}$  gallic acid equivalents (GAE). All measurements were performed in triplicate.

### Rutin content by planar chromatography (HPTLC):

For quantitative evaluation of rutin, we adapted a previously published preparative TLC separation [15]. The solvent system used was ethyl acetate, formic acid and water with a volume ratio of 9:1:1 v/v/v for flavonoids analysis. The chromatography was performed on silica gel 60 F<sub>254</sub>, 10 cm x 10 cm HPTLC plates from Merck. The plates were previously washed with methanol by pre-chromatography for 30 minutes and dried at room temperature in a fume hood. Before analyses, plates were activated at 120 °C for 30 min. Sample and standard zones were applied to the layer as bands by means of the Automatic TLC Sampler (ATS) 3 and Linomat III automated spray-on applicator equipped with a 100  $\mu\text{L}$  syringe, both from CAMAG (Muttens, Switzerland), operated with the following settings: band length 6 mm, application rate 4  $\text{s } \mu\text{L}^{-1}$ , table speed 10  $\text{mm s}^{-1}$ , distance between bands 4 mm, distance from the plate side edge 6.5 mm, and distance from the bottom of the plate 10 mm. Plates were developed up to a migration distance of 50 mm in a CAMAG HPTLC twin-trough chamber equilibrated with the mobile phase for 15 min. Approximately 15 mL of mobile phase were used for each development, which required approximately 18 min. For multiple developments, the plates were run three times as maximum. Freeze-dried extract (100 mg) was dissolved in methanol, filtered through cotton and diluted to 50 mL with methanol, no further clean up was necessary. This operation was performed in triplicate, and developed three times. For rutin analysis, different volumes were applied in the plate (2–8  $\mu\text{L}$ ). For total flavonoids analysis, 1  $\mu\text{L}$  of samples was applied on the plate and the detection for both was performed in UV absorption

mode using the TLC Scanner 3 from CAMAG at  $\lambda = 355 \text{ nm}$ .

### DPPH assay

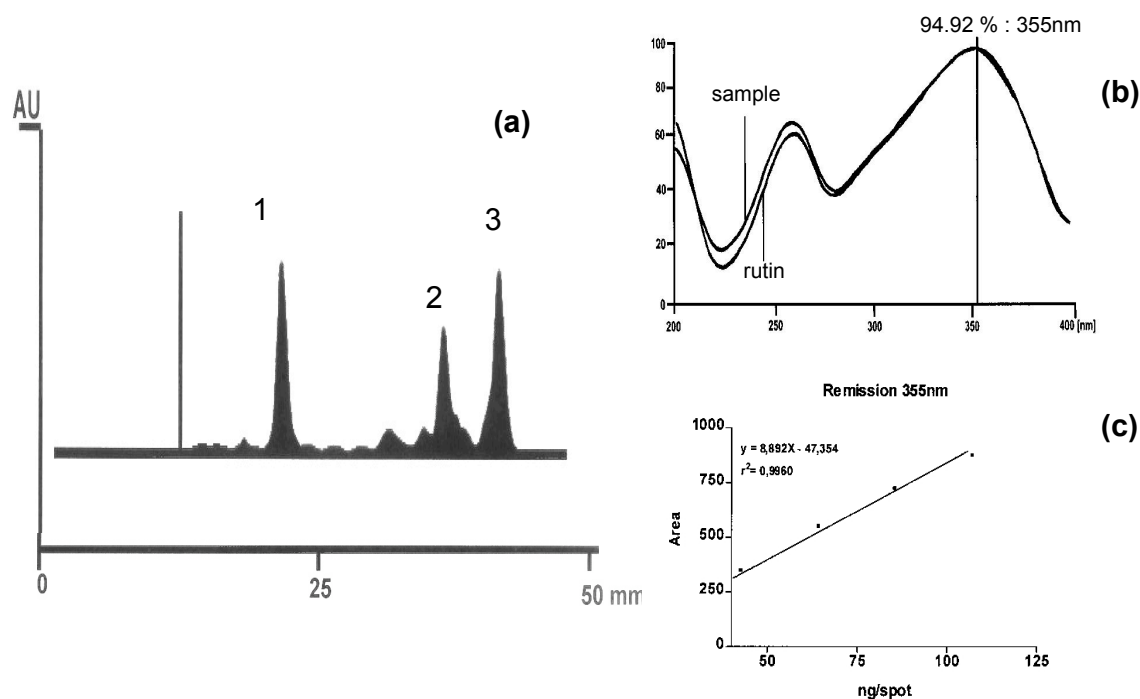
For radical scavenging capacity DPPH assay was carried out according to Bonoli *et al.* with slight modifications [22]. Briefly, 100  $\mu\text{L}$  sample of each extract were added to 2.9 mL of 0.1  $\text{mol L}^{-1}$  DPPH solution in methanol-water (8:2 v/v). A decrease in absorbance was determined at 517 nm every 0.5 seconds intervals in a 0–30 min range at 25 °C. The blank reference cuvette contained only methanol-water (8:2 v/v). Delta absorbancies were evaluated according to the Trolox calibration plot established from 20 to 200  $\mu\text{mol L}^{-1}$ , the results were expressed as  $\mu\text{mol L}^{-1}$  of Trolox equivalent per gram of extract. All measurements were performed in triplicate.

### ABTS assay

Trolox Equivalent Antioxidant Capacity (TEAC) assay using ABTS<sup>•+</sup> radical cation, was done according to Pellegrini *et al.* [23]. ABTS<sup>•+</sup> (7  $\text{mmol L}^{-1}$ ) was mixed with potassium persulfate (final concentration: 2.42  $\text{mmol L}^{-1}$ ) and kept for 12–16 h at room temperature in the dark. For the assay, this stock solution was diluted with ethanol to an absorbance of 0.70 at 734 nm. After the addition of 1.0 mL of ABTS<sup>•+</sup> solution to 10  $\mu\text{L}$  of each extract (1  $\text{mg mL}^{-1}$ ), the mixture was stirred for 10 s and the readings were taken every 0.5 seconds until 15 min (25 °C). Delta absorbancies were evaluated regarding the Trolox calibration plot established from 20–100  $\mu\text{mol L}^{-1}$  and the results were expressed as  $\mu\text{mol L}^{-1}$  of Trolox equivalent per gram of extract. All measurements were performed in triplicate.

### Oxygen Radical Absorbance Capacity Assay (ORAC)

This procedure was based on a previously reported method [24]. The assay was done in a final volume of 500  $\mu\text{L}$  solution constituted by:  $\beta$ -PE (16.7  $\text{nmol L}^{-1}$ ), AAPH 2.2  $\text{mg mL}^{-1}$  and 2.5–10.0  $\mu\text{g mL}^{-1}$  of *Ch. glauca* extract. After addition of the AAPH, fluorescence was recorded every 1 min for 70 min with emission and excitation wavelengths of  $\lambda = 535$  and 485 nm, respectively, using a blank composed by phosphate buffer (pH 7.4),  $\beta$ -PE and AAPH. All readings were carried out at 37 °C using a RF 5301-PC fluorescence spectrophotometer from Shimadzu (Kyoto, Japan) and the ORAC values were calculated as area under the curve (AUC) and expressed as  $\mu\text{mol Trolox equivalent (TE) per gram}$ . All measurements were performed in triplicate.



**Figure 1.** Chromatographic profile of EChg. Samples were applied on HPTLC silica gel 60 plates and developed as indicated in material and methods section. (a) HPTLC separation of EChg: rutin (1), unknown (2), kaempferitrin (3). (b) Comparison of UV spectra of sample and rutin standard. Additionally, peaks assignment was assessed by comparison with spectroscopic data from previous work[15]. (c) Amounts of flavonoids were calculated from calibration curve (area versus rutin concentration).

### FRAP assay

For FRAP assay [25], a mixture of  $0.1 \text{ mol L}^{-1}$  acetate buffer (pH 3.6),  $0.01 \text{ mol L}^{-1}$  TPTZ, and  $0.02 \text{ mol L}^{-1}$  ferric chloride (10:1:1 v/v/v) was prepared (FRAP reagent). To  $900 \mu\text{L}$  of FRAP reagent,  $90 \mu\text{L}$  of water and  $30 \mu\text{L}$  of sample were added. The absorbance readings started immediately after the addition of sample, and they were performed at  $593 \text{ nm}$  with readings every  $0.5$  seconds for  $10$  min. The blank consisted in  $120 \mu\text{L}$  of water and  $900 \mu\text{L}$  of reagent. The final absorbance of each sample was evaluated regarding the Trolox calibration plot established from  $10$  to  $100 \mu\text{mol L}^{-1}$ . The results were expressed as  $\mu\text{mol L}^{-1}$  of Trolox equivalent per gram of extract. All measurements were performed in triplicate.

### Animals

Adult male Sprague Dawley rats ( $200$ – $250 \text{ g}$ ) from a stock maintained at the University of Chile, were used. Animals had free access to pelleted food, maintained with controlled temperature ( $22 \text{ }^\circ\text{C}$ ) and photo-period (lights on from  $07:00$  a.m. to  $7$  p.m. hr). All animal procedures were performed using protocols approved by the Ethical Committee of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile.

### Liver microsomal preparations

Animals were fasted for  $15$  hr with water *ad libitum* and sacrificed by decapitation. Livers were perfused *in situ* 4 times with  $25 \text{ mL}$  of  $9 \text{ mg mL}^{-1}$  NaCl, excised, and placed on ice. All homogenization and fractionation procedures were performed at  $4 \text{ }^\circ\text{C}$  (ice bath). The centrifugation were performed using either a Suprafuge 22 Heraeus centrifuge (Osterode, Germany) or an XL-90 Beckman ultracentrifuge (Palo Alto, CA, USA). Liver tissue ( $9$ – $11 \text{ g}$  wet weight), devoid of connective and vascular tissue, was homogenized with five volumes of  $0.154 \text{ mol L}^{-1}$  KCl and eight strokes in a Dounce Wheaton B homogenizer (Millville, NJ, USA). Homogenates were centrifuged at  $9.000 \times g$  for  $15$  min at  $4 \text{ }^\circ\text{C}$ , and sediments were discarded. Supernatants were then centrifuged at  $105.000 \times g$  for  $60$  min at  $4 \text{ }^\circ\text{C}$ . Sediments (microsomes, enriched in endoplasmic reticulum) were stored at  $-80 \text{ }^\circ\text{C}$  until use. Protein determinations were performed according to Lowry *et al.* [26].

### Microsomal lipid peroxidation

Lipid peroxidation was estimated measuring the thiobarbituric acid reactive substances (TBARS) according to Buege and Aust [27] protocol.

**Table 1.** In vitro antioxidant activity of *Ch. glauca* extracts determined by ORAC, FRAP, ABTS and DPPH assays

Antioxidant activity	EChg-DEAE ( $\mu\text{mol Trolox /g}$ )	EChg ( $\mu\text{mol Trolox /g}$ )	equation $y = ax + b$
ORAC	3	1245 $\pm$ 11 (115.0)	$y = 2043x + 124$ ( $r^2 = 0.9840$ )
FRAP	n.d	486 $\pm$ 30 (44.9)	$y = 0.0449x + 0.07$ ( $r^2 = 0.9983$ )
ABTS	n.d	852 $\pm$ 130 (78.7)	$y = 0.0334x + 0.0148$ ( $r^2 = 0.9902$ )
DPPH	n.d	855 $\pm$ 41 (79.0)	$y = 0.022x + 0.0301$ ( $r^2 = 0.9958$ )

n.d. : non-detectable. Values are expressed as mean  $\pm$  standard deviation ( $n = 3$ ). Values in parentheses indicate  $\mu\text{mol Trolox}$  equivalents per gram of dried plant.

Oxidative damage was induced by ascorbate- $\text{Fe}^{2+}$  system. Incubations were carried out at 37°C for 30 min in a shaker-water bath. After the incubation, the samples were boiled with TBA reagent for 30 min. The pink color of TBARS formed was measured at 532 nm as malondialdehyde equivalents (MDA) after accounting for appropriated blanks. A MDA standard was prepared by acid hydrolysis of tetraethoxypropane. The extinction coefficient used was  $0.156 \text{ mol}^{-1} \times \text{cm}^{-1}$ . All measurements were performed in triplicate.

### Oxygen Consumption

Measures of oxygen consumption were investigated polarographically using a 2 mL chamber and a Clark electrode N° 5331 (Yellow Springs, instrument model 5300 Biological Oxygen Monitor, Ohio, USA). Reaction mix contained: plant extracts [ $\text{IC}_{50}$  from lipoperoxidation assay],  $10 \mu\text{mol L}^{-1} \text{ CuSO}_4$  and  $1 \text{ mmol L}^{-1}$  sodium ascorbate. All measurements were performed in triplicate.

### Bovine Lens assay

Extract anti-cataract effect was evaluated using a a previously published *in vitro* protocol [28]. Bovine lens were obtained from young and healthy animals, slaughtered in the same day of the sampling (Chiguayante abattoir in Chile). Lens were quickly removed and stored at -20°C. Krebs Ringer carbonate buffer ( $132 \text{ mmol L}^{-1} \text{ NaCl}$ ,  $4.8 \text{ mmol L}^{-1} \text{ KCl}$ ,  $1.2 \text{ mmol L}^{-1} \text{ Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and  $1.2 \text{ mmol L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$ , pH 7.5) was used, plus  $1.3 \text{ mmol L}^{-1} \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $25 \text{ mmol L}^{-1} \text{ NaHCO}_3$  and glucose. Buffer was sparged with a low flow of  $\text{CO}_2$  at 37°C. Extracts were dissolved in 25 mL of buffer and filtered through  $0.22 \mu\text{m}$  membrane. Lenses were incubated in this medium for 24 hours at 37 °C with 5%  $\text{CO}_2$  and 95% air. Positive and negative controls were prepared with and without glucose ( $0.03 \text{ mol L}^{-1}$ ), respectively. Hyperoside was used as aldose reductase inhibitor. Image of the lens were digitally acquired by Reprostar 3 system from CAMAG.

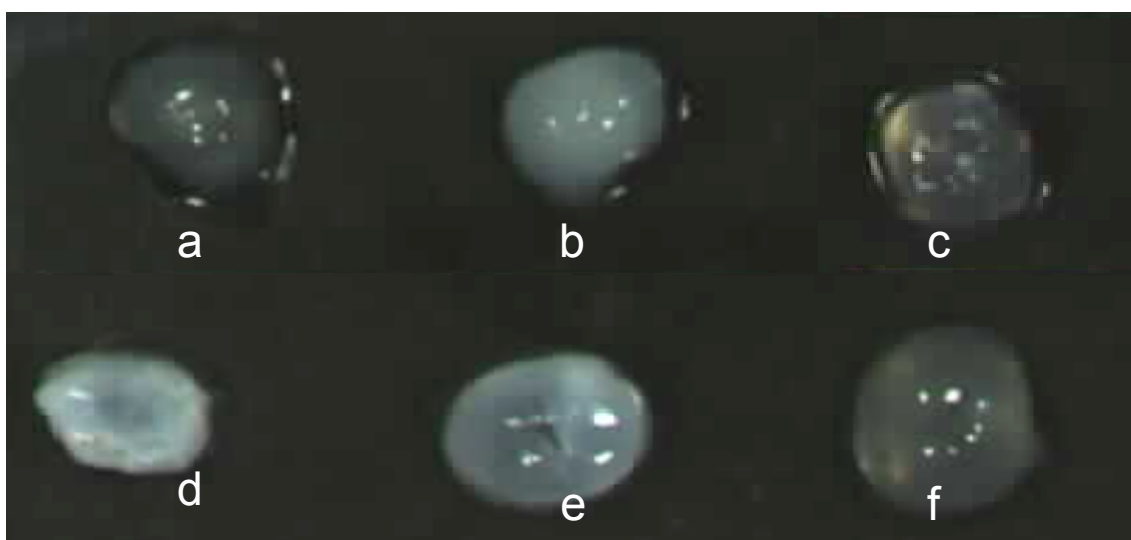
## Results and Discussion

### Total polyphenols (TP) and rutin content:

Using the equation of gallic acid calibration curve was  $y = 0.091x + 0.0229$  ( $r^2 = 0.9951$ ) the TP calculated for EChg was  $148 \pm 3 \text{ mg GAE}$  per gram of dried extract. The rutin and total flavonoid content in dried plant (expressed as rutin equivalents) were  $3.50\% \pm 0.05\%$  and  $11.70\% \pm 0.08\%$  respectively. As expected, TP in EChg-DEAE was  $< 1 \text{ mg GAE}$ . From HPTLC profiles (figure 1), it is possible to calculate a kaempferitrin (peak 3) content of  $3.80\% \pm 0.06\%$  in the plant, which is exceptionally higher than contents reported for some *Bauhinia* species. However, due to obvious differences in the response factors for rutin and kaempferitrin, in the future this content must be corrected using an adequate calibration curve with an authentic sample.

### Antioxidant Capacity (DPPH, ABTS, ORAC, FRAP)

Table 1 depicts results of antioxidant capacity of *Ch. glauca* extracts addressed by different methods. Antioxidant capacity assayed with DPPH and ABTS radicals showed similar results. Both radicals are stable in absence of antioxidant substance and became to decolorize mainly through a single electron transfer mechanism [29]. In spite of widely use of FRAP to estimate antioxidants in different products, results only reflex the presence of reducing substances, which not necessarily involve polyphenol compounds. It should be noted however, that the reaction of *Ch. glauca* polyphenols with FRAP reagent has not been finished within the 10 min period. Some polyphenols causes a continuous increasing of the FRAP reagent absorbance sometimes for hours. Therefore, if short recording times are used, antioxidant potential of these compounds cannot be determined accurately. Also, presence of non-phenolic substances (vitamin C and Cu (I)) must be considered in order to a correct interpretation of total polyphenol analysis by means of Folin-Ciocalteu reagent. Nevertheless, correlate total polyphenol content with antioxidant capacity is a routine practice in food and medicinal plants investigation [30].



**Figure 2.** *In vitro* anti-cataract effect of EChg. Bovine lenses were incubated as described in material and method section: (a) without glucose; (b) with 0.030 mol L<sup>-1</sup> glucose (c) 50 mg L<sup>-1</sup> hyperoside; (d), (e) and (f) *Ch. glauca* extract (1, 100 and 1000 mg L<sup>-1</sup>, respectively).

ORAC assay was chosen because it is becoming a very popular method, which is considered one of the most sensitive and reliable assays for antioxidant capacity evaluation in foodstuff and medicinal plants [31-33]. In the present work the original method published by Cao *et al.* [24] was used. For this reason, R-Phycoeritrin was employed as fluorescence probe instead of sodium fluorescein, which is considered more stable and inexpensive. The ORACPE value of 1245 Trolox equivalents per gram of extract, ranks *Ch. glauca* in the same category of some fruits extracts like bilberry, elderberry which published ORACPE values are 1283 and 1174, respectively [34].

In agreement with the chemical analysis, presence of rutin and kaempferitrin could explain the antioxidant capacity of *Ch. glauca*. However, in a previous study we found that kaempferitrin only produced a slight DPPH bleaching rate in comparison with rutin [15]. To our knowledge, there is not evidence of other antioxidant substances in this plant. In order to confirm if the antioxidant capacity of *Ch. glauca* extract is related to the presence of polyphenols we prepared a polyphenols-free extract using DEAE-cellulose which efficiently removed polyphenolic material. As expected, DEAE-EChg showed weaker antioxidant capacity than EChg in the entire battery of tests, suggesting that polyphenolic substances may be related to this property. In other approach, we used dot-blot over TLC with DPPH as visualizing reagent. Only one spot with antioxidant activity was observed 5 minute after spraying the layers with a DPPH solution. After scrapped and eluted the zone from preparative TLC plates, rutin was identified by spectra comparison UV, <sup>1</sup>H-NMR and <sup>13</sup>C-NMR (data not show) [15] and by co-chromatography with a

certified standard. Nevertheless, it is important to complete the phytochemical profile of *Ch. glauca* searching new substances with biological activity.

**Table 2.** Inhibition of microsomal lipid peroxidation induced by Fe<sup>2+</sup>/ascorbate ([FeCl<sub>3</sub>]: 600 μmol L<sup>-1</sup>; [ascorbate]: 1 mmol L<sup>-1</sup>). IC<sub>50</sub> values were calculated from dose-response curves obtained for each extract.

Extract	IC <sub>50</sub> (μg/mL)
EChg	50,8
EChg-DEAE	>1000

**Table 3.** Effect of the *Ch. glauca* extracts on oxygen consumption. Concentrations used were the IC<sub>50</sub> determined in the lipid peroxidation assay (Table 2).

Extract	Concentration (μg/mL)	% Inhibition
EChg	51	24.6 %
EChg-DEAE	1000	21.3%

### Microsomal Lipid peroxidation and oxygen consumption

From the dose-response curves obtained with the extracts, IC<sub>50</sub> values were calculated as is showed in Table 3. These values represent the extract concentration capable to inhibit in a 50% the microsome lipid peroxidation induced by the Fe<sup>2+</sup>/ascorbate pro-oxidant system. Because EChg-DEAE extract shown a very low anti-lipoperoxidative activity, IC<sub>50</sub> value was not determined.

Nevertheless, EChg and EChg-DEAE extracts display only a low antioxidant activity (IC<sub>50</sub>) upon consumed oxygen assays (Tables 2 and 3)

### Bovine lens assay

As is shown in figure 2, *in vitro* anti-cataract effect of *Ch. glauca* on bovine lenses incubated in a high glucose medium was dose-dependent. This effect was manifested as amelioration of glucose-induced bovine lens opacity. However, hyperoside was still more effective. As reported by other researchers there are some structural requirements that determine aldose reductase (AR) inhibitory effect of flavonoids [35-38]. For instance, the ortho orientation of the hydroxyl groups in positions 3' and 4' of ring B, number of hydroxyl groups and glycosylation pattern appears as structure-activity relevant requisites. In a recent report, using a Genetic Algorithm (GA) analysis and Artificial Neural Network (ANN) approach, the importance of the carbonyl group on the aromatic rings was remarked. Such groups can form hydrogen bonds with Tyr48, and His110 residues in the active site of AR. The same authors observed that the absence of the hydroxyl substituent at position 4' drastically decreases the AR inhibitory activity [41]. In spite of its powerful free radical scavenging properties, the potential mechanism of *Ch. glauca* anti-cataract effect also could be associated to a flavonoids inhibitory activity over the bovine AR. This could be proved in an *in vivo* model like the streptozotocin-induced diabetic rat [39]. This model allows investigate if *Ch. glauca* could inhibit cataract rating and lipid peroxidation or glycation of plasma and lens proteins [40]. Nevertheless, it must be kept in mind the potential anti-diabetic effects associated to the presence of kaempferitrin. This polyphenol, lowering the glucose levels of plasma and lens, could produce more relevant outcomes than those related to the antioxidant and AR inhibitory properties.

### Concluding remarks

This work supports the possibility that *Ch. glauca* polyphenols reduce the oxidative stress and could protect diabetes mellitus patients against hyperglycemia-mediated lens damage. Further research is necessary to determine if the ingestion or administration of this plant extracts could partially abrogate diabetes mellitus chronic complications via anti-diabetic and/or antioxidant effects

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