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Phytochemical and biological investigations of *Eichhornia crassipes* (Mart.) Solms

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ABSTRACT

Eichhornia crassipes (Mart.) Solms was chemically and biologically evaluated. From the phytochemical study were isolated the mix β-sitosterol and stigmasterol, 2-hydroxy-8-(4-hydroxyphenyl)-1H-phenalen-1-one and shikimic acid that were spectroscopically identified by NMR (1D and 2D). Tests on the antioxidant activities by 1,1-diphenyl-2-picryl-hydrazyl , total phenolic contents by Folin-Ciocalteau, antimicrobial activities against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa and Candida albicans using paper disc diffusion biossay and toxicity Brine Shrimp Lethality (BSL) were carried out for the HEX, CH_2Cl_2, EtOAc and n-BuOH extract. The extract EtOAc demonstrated better antioxidant capacity (EC_{50} 66.46 ± 0.32 µg/mL) followed by extract CH_2Cl_2 (EC_{50} 107.28 ± 1.43 µg/mL), and the total phenolic contents was 64.12 and 66.97 (mg EAT/mL), respectively. All extracts were inactive against the tested microorganisms. In the Brine Shrimp Lethality test, the active extract was the CH_2Cl_2 with DL_{50} 49.40 ppm.

Keywords: Antimicrobial, Antioxidant, *Eichhornia crassipes*, Phenolic content, Phytochemical study

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INTRODUCTION

The *Eichhornia crassipes* (Mart.) Solms is a vascular aquatic plant member of the Pontederiaceae family. In Brazil popularly known as 'aguapé', orelha-de-jegue', 'jacinto d´água' and 'miriru', is native to South America. Because of its attractive purple flower, *E. crassipes* is a favorite amongst ornamental pond and garden enthusiasts, as a result, humans have spread it widely and due to its fast growth rate it now flourishes in all continents. *E. crassipes* is considered an aquatic invasive species due its rapid growth and proliferation [1]

E. crassipes is a bioindicator of water quality in rivers, lakes, etc. since the speed of growth and reproduction is directly related to the availability of nutrients, temperature and brightness of water [1,2]. The phytoremediator action from *E. crassipes* is investigated because is well known as an efficient absorber of nutrients, heavy metals and toxic compounds from eutrophic water bodies [3,4,5]. Researchers have discovered its allelopathic effects as algaecide [6,7].
E. crassipes is traditionally used as sedative, aphrodisiac, cooling, febrifuge and diuretic [8]. Phytochemical studies have reported compound as steroids [9], phenalene [10, 11, 12, 13] and humic acid, commonly in plant that absorber metals from the environmental [14]. From flowers have been reported pigments as antocianins [15].

Although this plant is native from South America [16], the most of reports about of chemical composition and biological activity from *E. crassipes* are about the plant cultured in other regions of the world [17, 18]. Our aim was the evaluation the biologic activity and chemical composition of *E. crassipes* collected in Parana state–Brazil.

EXPERIMENTAL SECTION

Chemicals

Folin-Ciocalteu reagent, DPPH (1,1-diphenyl-2-picryl-hydrazyl), solvents, silica gel, Sephaex® LH-20, vanillin and Liebermann-Burchard reagents. All other chemicals used in this study were of analytical grade.

Instrumentation

UV-Vis PG spectrophotometer, Column chromatography, TLC, UV light 254 and 365 nm, ^{1}H NMR, ^{13}C NMR, HMBC and HMQC spectra were recovered on VARIAN-300.

Plant Material

Eichhornia crassipes (Mart.) Solms was collected at Centro de Pesquisa em Aquicultura Ambiental – CPAA/UNIOESTE – Toledo City, Parana State – Brazil, in August 2010. A voucher specimen (number 6389) is deposited in the UNOP herbarium of the Universidade Estadual do Oeste do Parana (UNIOESTE).

Extraction and Purification of the Compounds

The dry leaves of *Eichhornia crassipes* (Mart.) Solms (1.135 kg) were powdered and macerated with EtOH-H_2O (8/2, v/v) at room temperature during six days. Hydroethanol crude extract was fractionated in increasing polarity successively to yield n-hexane fraction (HEX 14.70 g), dichloromethane fraction (DCM 13.97 g), ethyl acetate fraction (EtOAc 23.65 g), and n-butanol fraction (n-BuOH 186.55 g).

Part of the HEX fraction (6.33 g) was submitted to silica gel column chromatography (CC) (HEX/DCM/MeOH from 1/0/0 to 0/0/1) to produce nine fractions (FH1-9). Fraction FH6 (3.06 g) was fractionated in silica gel column chromatography eluting with a stepwise gradient of HEX−DCM−EtOAc-MeOH (from 1:1:0:0 to 0:0:0:1, v/v/v) to afford the sub-fraction 1-11 (250 mL). The sub-fraction 3 (360.0 mg) was purified by silica gel column chromatography eluting with DCM, to afford the mix **1** + **2** (20.0 mg).

Part of the DCM fraction (1.50 g) was subjected to reversed-phase silica gel column chromatography eluting with a stepwise gradient of MeOH/H_2O/EtOAc (from 1:1:0 to 0:0:1, v/v/v) to affortd eight fraction (DCA1-8). The fraction DCA1 (660.0 mg) was further purified by Sephadex LH-20 CC eluting with MeOH to give the compound **3** (9.0 mg).

Part of the EtOAc fraction (800.0 mg) was further fractionated on a Sephadex LH-20 column eluting with EtOAc/MeOH (1/1, v/v) to yield seven fractions (1−7). Fraction 4 (60 mg) was subjected to silica gel CC eluting with a stepwise gradient of EtOAc/MeOH (from 1:0 to 0:1) to afford **4** (20.0 mg).

β-sitosterol (**1**) + stigmasterol (**2**): white, amorphous powder. ^{1}H NMR data [300 MHz, $CDCl_3$, δ (ppm), multiplicity and *J* (Hz)]: δ 3.52 (*m*, H-3, **1** + **2**), 2.00 (*m*, H-4, **1** + **2**), 5.35 (*sl*, H-6, **1** + **2**), 2.30 (*m*, H-8, **1** + **2**), 1.50 (*m*, H-15, **1** + **2**), 1.85 (*m*, H-16, **1** + **2**), 0.68 (*s*, CH_3-18, **1**), 0.69 (*s*, CH_3-18, **2**), 1.01 (*s*, CH_3-19, **1** + **2**), 0.92 (*d*, *J*=6.5 Hz, CH_3-21, **1**), 1.02 (*d*, *J*= 6.5 Hz CH_3-21, **2**), 5.15 (*dd*, *J*=15.9 Hz, H-22, **2**), 5.01 (*dd*, *J*=15.9 Hz, H-23, **2**), 0.83 (*d*, *J*=6,5 Hz H-26, **1**), 0.84 (*d*, *J*=6.5 Hz, H-26, **2**), 0.81 (*d*, *J*=6.5 Hz, H-27, **1**), 0.79 (*d*, *J*=6.5 Hz, H-27, **2**), 0.84 (*t*, *J*=7.5 Hz, H-29, **1**), 0.80 (*t*, *J*=7.5 Hz, H-29, **2**). ^{13}C NMR data [75 MHz, $CDCl_3$, , δ (ppm)]: δ 37.3 (C-1, **1** + **2**), 31.7 (C-2, **1** + **2**), 72.6 (C-3, **1** + **2**), 42.3 (C-4, **1** + **2**), 140.7 (C-5, **1** + **2**), 121.9 (C-6, **1** + **2**), 31.9 (C-7, **1** + **2**), 31.6 (C-8, **1** + **2**), 50.2 (C-9, **1** + **2**), 36.5 (C-10, **1** + **2**), 21.2 (C-11, **1** + **2**), 39.8 (C-12, **1**), 38.7 (C-12, **2**), 42.2 (C-13, **1** + **2**), 56.8 (C-14, **1**), 56.9 (C-14, **2**), 24.3 (C-15, **1**), 24.3 (C-15, **2**), 28.2 (C-16, **1** + **2**), 55.9 (C-17, **1** + **2**), 11.8 (C-18, **1** + **2**), 19.4 (C-19, **1** + **2**), 36.1 (C-20, **1**), 40.4 (C-20, **2**), 18.9 (C-21, **1**), 21.1 (C-21, **2**), 33.1 (C-22, **1**), 138.5 (C-22, **2**), 39.1 (C-23, **1**), 129.5 (C-23, **2**), 45.8 (C-24, **1**), 51.2 (C-24, **2**), 26.1 (C-25, **1**), 31.9 (C-25, **2**), 18.8 (C-26, **1**), 19.4 (C-26, **2**), 19.8 (C-27, **1**), 18.9 (C-27, **2**), 23.1 (C-28, **1**), 25.4 (C-28, **2**), 11.9 (C-29, **1**), 12.2 (C-29, **2**).

2-hydroxy-8-(4-hydroxyphenyl)-1H-phenalen-1-one (**3**): Orange solid, UV (EtOH) (λ: 196, 274 and 416 nm). ^{1}H NMR data [300 MHz, acetone-d_6, δ (ppm), multiplicity and *J* (Hz)]: δ 7.40 (*d*, *J*=2.1 Hz, H-2'), 7.21 (*s*, H-3), 7.82 (*d*, *J*=6.6 Hz, H-4), 7.68 (*dd*, *J*=8.1 and 8.4 Hz, H-5), 7.04 (*d*, *J*=8.1 Hz, H-5'), 8.13 (*d*, *J*=7.8 Hz, H-6), 7.29 (*d*, *J*=8.4 Hz, H-6'), 8.60 (*d*, *J*=1.8 Hz, H-7), 8.83 (*d*, *J*=1.8 Hz, H-9). ^{13}C NMR data [75 MHz, acetone-d_6, δ (ppm)]: δ 181.1 (C-1), 151.4 (C-2), 114.6 (C-3), 129.3 (C-3a), 130.5 (C-4), 128.6 (C-5), 130.5 (C-6), 133.7 (C-6a), 133.6 (C-7), 129.5 (C-9), 129.6 (C-9a), 124.0 (C-9b), 132.3 (C-1'), 115.2 (C-2'), 146.8 (C-3'), 146.8 (C-4'), 117.0 (C-5'), 119.9 (C-6'), .

Shikimic acid (**4**): ^{1}H NMR data [300 MHz, acetone-d_6, δ(ppm), multiplicity and *J* (Hz)]: δ6.78 (*sl*, H-2), 4.36 (*sl*, H-3), 3.63 (*m*, H-4), 3.97 (*m*, H-5), 2.73 (*dd*, *J*=5.1 e 18 Hz, H-6a), 2.18 (*dd*, *J*=5.7 and 18.0 Hz, H-6b). ^{13}C NMR data [75 MHz, acetone-d_6, δ (ppm)]: δ 131.1 (C-1), 138.4 (C-2), 67.3 (C-3), 72.8 (C-4), 68.4 (C-5), 31.8 (C-6), 170.4 (COOH).

Toxicity test on Artemia salina Leach

The brine shrimp lethality assay was performed following the reported procedure [19]. The hexane, dichloromethane, ethyl acetate and n-butanol fraction were dissolved in artificial sea water (38 g/L, and 1% of DMSO) and diluted to different concentration (1, 10, 100 and 1000 ppm). 3 mL of the samples together with 10 shrimp were added in appropriate recipient and the final volume was adjusted to 5 mL, in triplicate. After 24 h incubation under light, the number of dead and survivor brine shrimps in each tube was counted. The DL_{50} values were calculated by graphics from concentration vs. lethality percentage using a Probit scale adjust. The extract with $DL_{50} \leq 200$ ppm were considered active.

Antioxidant activity

The antioxidant potential of the extracts was determined by DPPH (1,1-diphenyl-2-picryl-hydrazyl) method[20]. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH radical, through electron spin resonance (EPR) detection or by measuring the decrease of its absorbance monitored by a spectrophotometer. The result is normally expressed using the EC50 value, defined as the concentration of antioxidant that causes a 50% decrease in the DPPH absorbance.

The extracts solution were used in concentration 5.0, 10.0, 25.0, 50.0, 125.0 and 250.0 µg/mL. 0.3 mL of the extract solution were added to a 2.7 mL of the 40 µg/mL DPPH methanolic solution, the mixtures were shaken vigorously and left to stand in the dark for 30 min at room temperature, then absorbance was read at 517 nm. The experiments were run in quadruplicate. Radical scavenging capacity was expressed as percentage effect (AA%) and calculated using the following equation: AA% = [(Abs control – Abs sample)/Abs control] x 100. Negative control was used a DPPH methanolic solution, and positive control ascorbic acid and BHT (butylated hydroxytoluene).

The EC50 values were submitted to analyses of variance (ANOVA) following by Turkey`s test. Statistical program Minitab 14 and Excel® 2010 were used. A probability of $P < 0.05$ was considered as significant.

Total phenolic content

Total phenolic constituents (TPC) of plant extracts was determined by Folin-Ciocalteau (FC) reagent [21]. Aqueous solution of the extract in the concentration of 0.15 mg/mL was used in the analysis. The reaction mixture was prepared by mixing 1.0 mL of aqueous solution of extract, 0.5 mL of 10% Folin-Ciocalteu's reagent. After 5 minutes, 2.0 mL of a 20% Na_2CO_3 solution was added to the mixture. The mix was kept for 60 min., after the absorbance was read at 760 nm. The TPC was determined from extrapolation of calibration curve which was made by preparing tannic acid solution (concentration of 10, 20, 30, 40 e 50 µg/mL). The TPC was expressed as milligrams of tannic acid equivalents per mL of extract (mg EAT/mL).

Antimicrobial activity

The antimicrobial activity *in vitro* was tested against five microorganisms: *Staphylococcus aureus* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 3686) and *Candida albicans* (ATCC 10231) from General Microbiology Laboratory collection of the UNIOESTE.

The bioassay was performed by M2-A8 method from Clinical and Laboratory Standards Institute- CLSI [22]. The bioassay was performed in petri dishes with Agar Muller Hinton. It was added 50µL of each extract tested in concentration of the 20 mg/mL in hole in the plates. The plates were incubed at 35 ºC during 24 h to bacteria and 48 h to fungus, in duplicate.

RESULTS AND DISCUSSION

3.1 Identification of the Compounds

The chemical structure of the mix β-sitosterol (**1**) and stigmasterol (**2**) (figure 1) were determined by NMR data compared with the previous data reported [23, 24, 25].

^{1}H NMR spectrum showed the signal at δ5.35 (H-6, *sl*, **1** and **2**) suggesting an olefinic proton, two double doublet at δ5.15 and 5.01 (H-22 and H-23, respectively) from stigmasterol, and the multiplet at δ3.52 assigned to a proton attached to a oxygenated carbon (H-3, **1** and **2**). The signal at δ0.69 and 2.30 characteristic from methine and methylene protons were observed. In the ^{13}C NMR spectrum, the signals at δ 140.7 and 121.9 were assigned to

double bond between C-5 and C-6 for both β-sitosterol and stigmasterol, and the signals at δ 138.5 and 129.5 are characteristic for double bond between C-22 and C-23 from stigmasterol. The ratio of β-sitosterol (51.25%) and stigmasterol (48.75%) in the mix were estimated due the integration of the H-6 (relative intensity: 0.80, **1** + **2**), H-22 and H-23 (relative intensity: 0.39 of **2**). Greca and co-workers isolated, from *E. crassipes* collected in Italy, three steroids with allelochemical activity, were effective in inhibiting the growth of radish root (*Raphanus sativus* L.) [9].

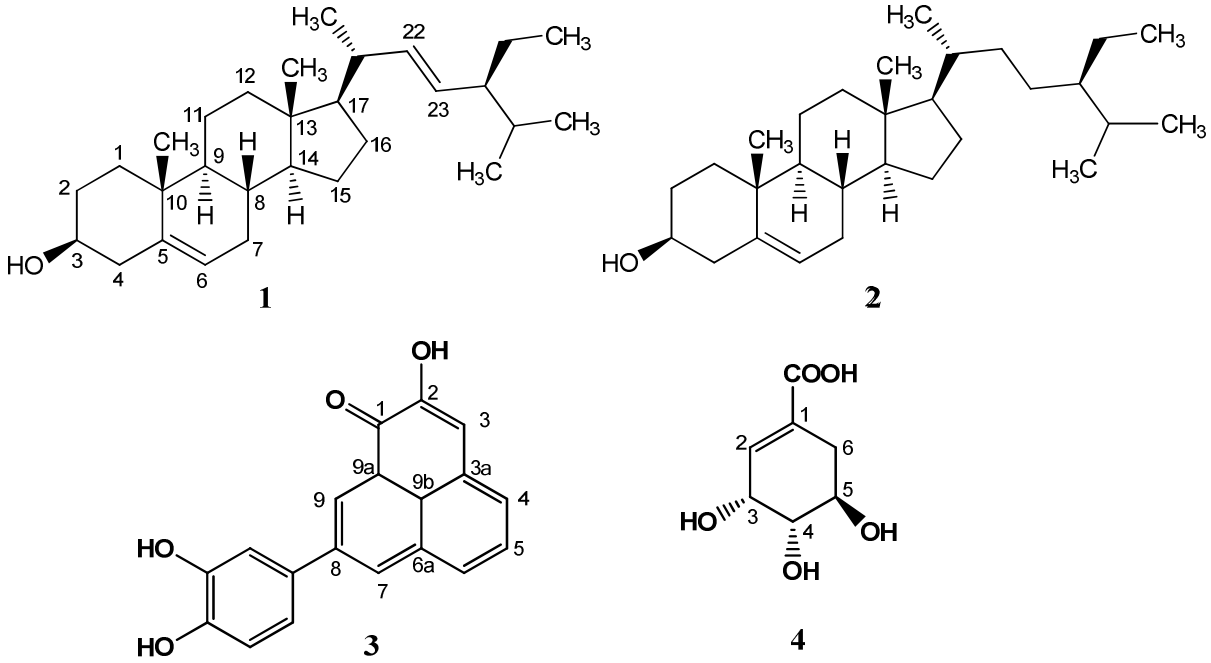

Figure 1: Compounds isolated from by *E. crassipes*

The chemical structure of the compound **3** (2-hydroxy-8-(4-hydroxyphenyl)-1H-phenalen-1-one) was elucidated using NMR data (1 and 2D) and comparison with the literature data reported [26]. The ^{1}H NMR spectrum showed nine signals at δ7.00 to 8.84 ppm. The tricyclic phenalenone system was confirmed by the presence of six aromatic protons, the doublet at δ7.29 (*J*=2.0 Hz), the double doublet at δ7.28 (*J*=8.3 and 2.0 Hz) and the doublet at δ7.03 (*J*=8.3 Hz) suggesting the presence of trissubstituted aromatic ring at 1, 3 and 4 position. The ^{13}C NMR spectrum showed characteristic signs of aromatic carbons. HMBC spectrum showed the correlation from the signals at δ7.21 (H-3), 7.82 (H-4), 8.13 (H-6), 8.60 (H-7) and 8.84 (H-9) to δ124.0, confirmed the C9b position and the phenalenone system. Phenylphenalenones compounds have been reported from *E. crassipes* [10, 11, 12, 13, 26, 27].

The shikimic acid (**4**) obtained from EtOAc extract was identified by NMR data 1 and 2D and comparison with the literature data reported [28]. The ^{1}H NMR spectrum showed six signals at δ2.18 to 6.78 ppm. The double doublets at δ2.73 and 2.18 (*J*=5.1 and 18.0) was assigned to methylene protons of C-6. In the COSY spectrum was observed the ^{1}H-^{1}H correlation for H-6 with H-5 (δ 3.97), and the system –CH_2–CH(OH)–CH(OH)–CH(OH)–CH– was confirmed. In the ^{13}C NMR spectrum, the signals at δ131.1 and 138.4 were assigned to olefinic carbons C-1 and C-2, and the signals at δ67.3, 72.8 and 68.4 were assigned to methine carbons C-2, C-3 and C-4 respectively. The HMBC spectrum showed the correlation from H-5 to C-1 (δ131.0) and C-3 (δ 67.0), H-2 to C-4 (δ72,8) and C-6 (δ 31,8).

The compounds isolated from plant species containing one or more aromatic ring, are biosynthesized by shikimate pathway, which the central intermediate in the pathway is shikimic acid. The shikimate pathway provides a route to aromatic amino acid as tyrosine, tryptophan and phenylalanine, they are precursors of a wide range of aromatic metabolites secondary [29], as well the phenylphenalenones [30].

3.2 Biologic assays

The brine shrimp (*A. salina*) assay is simple and useful bioassays for toxicity of crude extract from plants [31].The crude extracts obtained from *E. crassipes* were submitted to brine shrimp assay, the active one was the dichloromethane extract with DL_{50} 49.40 ppm. This is the first report about toxicity from crude extract of *E. crassipes*, this results established the presence of bioactive compound in the dichloromethane extract.

The crude extracts tested against the bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*) and the fungus (*Candida albicans*) were inactive. Shanab and co-workers found antimicrobial activity of the methanol extract and the hexane and ethyl acetate fractions against *Candida albicans*, *Strptococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis* [6]. Fareed and co-workers tested crude extracts from leaves and roots of the *E. crassipes* against seventeen microorganisms and all extracts were active, the best result was found to extracts from leaves against *Aspergillus niger* and *Fusarium oxysporum*, and for extracts from roots against *Bacillus subtilis* and *Bacillus cereus* [32].

The antioxidant activities and total phenolic content of all crude extracts obtained from *E. crassipes* are shown in Table 1. The total phenolic content to extracts was in range of 10.93 to 66.97 mg EAT/mL. The highest concentration of phenols was measured in dichloromethane and ethyl acetate extracts.

Table 1: Total phenolic content (TFC), antioxidant activity (EC_{50}) and yield (%) of the extracts

extract	Yield (%)	TFC (mg EAT/mL)	EC_{50} ± SD (µg/mL)
Hexane	1.30	13.79	322.52 ± 1.03
Dichloromethane	1.23	66.97	107.28 ± 1.43
Ethyl acetate	2.08	64.12	66.46 ± 0.32
n-Butanol	3.11	10.93	745.09 ± 45.96
BHT	-	-	10.33 ± 2.25
Ascorbic acid	-	-	16.80 ± 0.71

Some studies have demonstrated a correlation between phenolic content and antioxidant capacity. The highest concentration of phenols is related with good antioxidant capacity, our result is in agreement with other findings. The dichloromethane and EtOAc extracts showed highest concentration of phenols and highest antioxidant activity. The red color observed to both DCM and EtOAc extracts was assigned to phenylphenalenone compounds, which are phenolic pigments with antioxidant activity.

The quantitative antioxidant evaluation (AA%) for the extracts are shown in Figure 2. The DCM and EtOAc showed similar profile, antioxidant activity between 76 and 84% to the concentration 125 and 250 µg/mL.

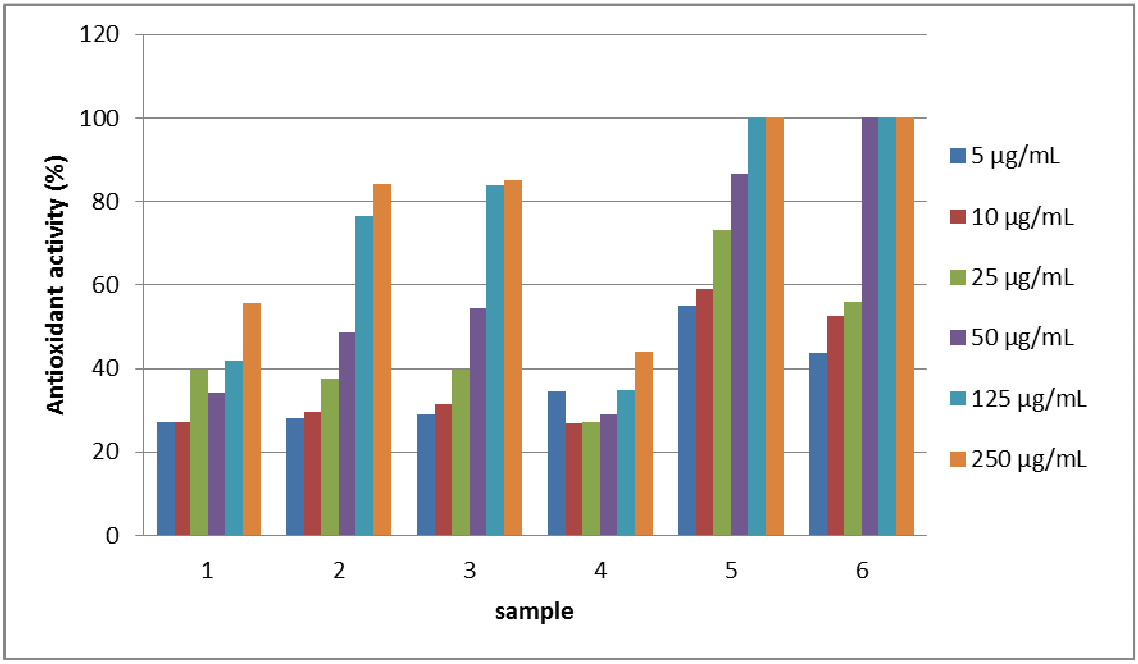

Figure 2: Porcentage of the antioxidant activity of extracts and controls: 1-HEX; 2- DCM; 3-EtOAc; 4- n-BuOH; 5- BHT; 6- ascorbic acid

Sduties developed by Ho Yu-Ling and co-workers with methanol and aquoseus extract from *E. crassipes* collected in Taiwan, were not significant, showed low antioxidant capacity [33]. However, the study using acetone/methanol extract from leaves and petiole, from plant collected in Tailand, showed a good antioxidant capacity (IC_{50} 145.33µg/mL to leaves and IC_{50} 179.18 µg/mL to petiole), the naringenine flavonoid was determinated as the principal phenolic compound found in the plant [18]. Studies performed with the plant collected in Canada, showed that the antioxidant effect from *E. crassipes* is as effective as soy and garllic [34].

Phytochemical investigation have been demonstrated that a species, depending of the region collected, may show a genetic variability resulting in plants with different chemical composition. Which explains for example, a species having a therapeutic application in one location and another region have sometimes different uses [35]. This is related to the production of secondary metabolites that can be influenced by biological and environmental factors [36].

This was observed for *E. crassipes* that depending on the region that was collected showed different biological activity and chemical composition. *E. crassipes* is one of the largest aquatic plants proliferation in lake of the Itaipu electric power, located in western Parana state, and their uncontrolled growth can affect the aquatic ecosystem. Thus, the results obtained in this study open the possibility of exploring this plant as a supplier of antioxidants.

CONCLUSION

The results obtained in this work showed a good antioxidant activity of *Eichhornia crassipes* extracts, particularly for dichloromethane and ethyl acetate, thus stimulating the investigation of the use of this plant as an agent supplier of antioxidants. Moreover, it can be an alternative to help the control of *E. crassipes*, which can affect the aquatic ecosystem and cause problems in the turbines of the Itaipu electric power.

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