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ORIGINAL ARTICLE

Pharmacognostical Analysis and Protective Effect of Standardized Extract and Rizonic Acid from *Erythrina velutina* against 6-Hydroxydopamine-Induced Neurotoxicity in SH-SY5Y Cells

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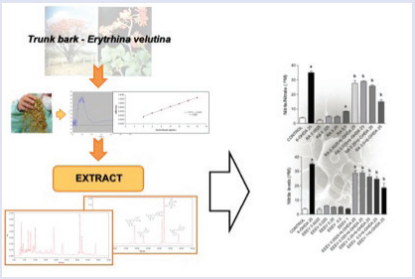
ABSTRACT

Background: *Erythrina velutina* is a tree common in the northeast of Brazil extensively used by traditional medicine for the treatment of central nervous system disorders. **Objective:** To develop a standardized ethanol extract of *E. velutina* (EEEV) and to investigate the neuroprotective potential of the extract and rizonic acid (RA) from *E. velutina* on neuronal cells. **Materials and methods:** The plant drug of *E. velutina* previously characterized was used for the production of EEEV. Three methods were evaluated in order to obtain an extract with higher content of phenols. The neuroprotective effect of standardized EEEV (HPLC-PDA) and RA was investigated on SH-SY5Y cell exposure to the neurotoxin 6-hydroxydopamine (6-OHDA). **Results:** The powder of the plant drug was classified as moderately coarse and several quality control parameters were determined. EEEV produced by percolation gave the highest phenol content when related to others extractive methods, and its HPLC-PDA analysis allowed to identify four flavonoids and RA, some reported for the first time for the species. EEEV and RA reduced significantly the neurotoxicity induced by 6-OHDA in SH-SY5Y cells determined by the MTT assay and the nitrite concentration. EEEV also showed a free radical scavenging activity. **Conclusion:** This is the first pharmacological study about *E. velutina* which used a controlled standardized extract since the preparation of the herbal drug. This extract and RA, acting as an antioxidant, presents a neuroprotective effect suggesting that they have potential for future development as a therapeutic agent in neurodegenerative disease as Parkinson.

Key words: Antioxidant effect, *Erythrina velutina*, neuroprotective activity, quality control, rizonic acid

SUMMARY

- The powder of *Erythrina velutina* was classified as moderately coarse and several quality-control parameters were determined.
- Ethanolic extract from *E. velutina* (EEEV) produced by percolation gave the highest phenol content when related to others extractive methods and its HPLC–PDA analysis of EEEV allowed to identify four flavonoids and rizonic acid (RA), some reported for the first time for the species.
- The EEEV and RA reduced significantly the neurotoxicity induced by 6-OHDA in SH-SY5Y cells determined by the MTT assay and the nitrite concentration.
- The EEEV also showed a free radical scavenging activity.



Abbreviations used: ±: More or less, %: Percentage, °C: Degree Celsius, <: Less than, µg: Microgram, µL: Microliter, µM: Micromol, [1D] MNR: One-dimensional nuclear magnetic resonance spectroscopy, [2D] MNR:Two-dimensional nuclear magnetic resonance spectroscopy, 6-OHDA: [6-] Hydroxydopamine. Abs: Absorbance, CFU: Colony forming units, CH_2Cl_2: Dichloromethane, $CHCl_3$: Chloroform cmCentimeter, DMEM/F12: *Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12*. DMSO: Dimethyl sulfoxide, DPPH: 1,1-Diphenyl-2-picrylhydrazyl, EAG: Gallic acid equivalents, EEEV: Ethanolic extract of Erythrina velutina, EtOAc: Ethyl acetate, g: Gram, h: Hour, $H2_O$: Water, HPLC: High-performance liquid chromatography, H REIMS: Hydrogen rapid evaporative ionization mass spectrometry, Kg: Kilogram M: Molar, m: Metro, MeOH: Methanol, mg: Milligram, min: Minute, mL: Milliliter, mm: Millimeter, MTT: Bromide 3 [4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium, N: Normal, NBT: Nitroblue tetrazolium, nm: Nanometer, PDA: Photodiode array detector, TPC: Total polyphenol content, RA: Rizonic acid, RP: Reverse phase, SOD: Superoxide dismutase, v/v: Volume per volume, Vs: Versus W: Watts

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INTRODUCTION

In Brazil, the wide traditional use of medicinal plants and their pharmacological potential have increasingly attracted the interest of pharmaceutical industries and government.[1] Since the use of medicinal plants is growing steadily, studies to establish the toxicological and pharmacological profile as well as the quality control of the whole production process for herbal medicines are urgently required. The quality control of herbal products includes pharmacognostic methods,

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chemical finger print, analysis of chemical markers, and microbiological parameters.[2]

E. velutina Willd. (Fabaceae) a tree popularly known as "mulungu," is traditionally used in folk medicine for the treatment of anxiety, insomnia, and nervous system excitation. Chemical studies of its trunk bark allowed the isolation of alkaloids, terpenes, and various bioactive flavonoids.[3] Phenols compounds including flavonoids and phenolic acids have been attracting more attention of researchers because their wide range of pharmacological effects acting as powerful antioxidants and anti-inflammatory.[4,5] Previous study we demonstrated the neuroprotective effect of amburoside A, phenol glucoside from *Amburana cearensis*, against 6-hydroxydopamine-induced neurotoxicity an experimental model of Parkinson disease.[4]

Then clinical toxicological assessment of *E. velutina* showed low toxicity in rats whereas pharmacological studies demonstrated anxiolytic, anticonvulsant, antinociceptive, and antiedematogenic activities of the nonstandardized hydroalcoholic extract from it trunk bark in rodents.[3] Then, the present study aimed to prepare and characterize the herbal drug and extract, and to investigate the neuroprotective activity of this standardized extract and rizonic acid from *E. velutina* on SH-SY5Y cells exposure to the neurotoxin 6-OHDA.

MATERIAL AND METHODS

Plant material

The trunk bark of *E. velutina* was collected at the city of Mulungu (Ceará, Brazil) in July 2010.Voucher specimen was deposited at the Herbarium Prisco Bezerra of the Federal University of Ceará under the number 44802.

Characterization of herbal drug

The trunk bark of *E. velutina* was dried in a drying chamber with (Tecnal, Brazil) and without (Olidefcz, Linea, Brazil) circulation of air at 80°C ± 2°C for different periods (24, 48, 72 h) monitored for moisture and total polyphenols contents. The humidity was determined using infrared balance, whereas mean size of the particles, density, total ash content, acid insoluble ash, microbiological analysis, and water or ethanol extractable content were determined as previously described.[6,7] Total polyphenol content (TPC) was determinate by the Folin–Ciocalteu colorimetric method.[8] The amount of TPC was calculated from regression equation of calibration curve ($y = 0.077x + 0.0468$; $r = 0.9985$) of gallic acid (4–16 μg/mL). All samples were analyzed in three replications.

Preparation and characterization of the ethanol extract: total phenols content

Three extractives methods were investigated: maceration (24 h), percolation preceded by maceration (24 h), and soxhlet extractor (6 h). The percentage efficiency of the extraction process was obtained by the ratio between the masses of the extract and plant material. TPC in extracts was determinate by the Folin–Ciocalteu colorimetric method.[8]

Extraction and isolation of phenols compounds from *E. velutina*

The EtOH extract the trunk bark of *E. velutina* (75.1 g) was dissolved in a mixture of MeOH: H_2O (1:1) and submitted to partition with hexane, CH_2Cl_2, EtOAc, and MeOH. The CH_2Cl_2 fraction (2.78 g) was further purified over Sephadex LH-20 by elution with MeOH to afford five fractions. The subfraction 4 (390.4 mg) was chromatographed by HPLC on a semipreparative column, utilizing a mixture of hexane: EtOAc (3:7) to yield the RA (39.0 mg) and abssinine (4.6 mg). Then, the chromatography of the subfraction 5(935.0 mg) on a silica gel column was performed using hexane, $CHCl_3$, EtOAc, and MeOH as binary mixtures with increasing polarity yielding four fractions. The subfraction 5 and 2 (310.2 mg) was further submitted to semipreparative HPLC with a Si gel column using hexane: EtOAc (46:54) system as the eluent to yield sigmoidin C (5.0 mg), hesperid in (8.1 mg), and homoesperidin (29.7 mg).

Chromatographic finger printing analysis of *E. velutina* extract by HPLC-PDA

HPLC–PDA analysis was performed in order to identify chemical markers in the ethanol extract. Hesperidin, abssinine, homoesperidin, RA, and sigmoidin C were isolated and their identities were established on the basis of spectroscopic methods, mainly 1D and 2DNMR, and HREIMS. Solutions of each compound at appropriate concentrations were prepared by dissolving them with acetonitrile and purified water 1:1 (v/v). Samples were subjected to preliminary stage of purification by solid phase extraction.[9] HPLC analysis was carried out on an Alliance HPLC-PDA system (Waters, USA) using a 5 μm RP-8 column (4.6 mm × 250 mm). The solvent A was ammonium acetate buffer (5 mM; pH = 3.46) and the solvent B was acetonitrile. The gradient elution at 30°C was performed, whereas the detection was from 260 nm to 286 nm, varying according to the retention time of each component.

Neurotoxicity induced by 6-OHDA in SH-SY5Y cells

The effect of tested drugs on neurotoxicity induced by 6-OHDA (25 μM) was investigated through the cell viability assay and the measurement of nitrite concentration in SHSY5Ycell line (0.7×10^5 cells/mL; medium: DMEM/F12 [1:1], 10% fetal bovine serum, and 1% antibiotics).

MTT assay

EEEV and RA (0.0025; 0.025; 0.25; 0.5; 1 μg/mL) were added to the culture after 1 h of cells exposure to 6-OHDA (25 μM) and then after 24 h incubation, the MTT assay was performed, with the absorbance measured at 595 nm.[10]

Nitrite determination

For the assessment of nitrite/nitrate, 100 μL of the Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1% *N*-(1-naphthyl)-ethylene diamine dihydrochloride/1% H_3PO_4/distilled water, 1:1:1:1) were added to 100 μL of cell-culture supernatant. EEEV or RA (0.0025; 0.025; 0.25; 0.5; 1 μg/mL) were added to the culture after exposure of cells to 6-OHDA or to 100 μL $NaNO_2$ at concentrations ranging from 0.75 M to 100 M (standard curve). The absorbance was measured in a reader plate (Synergy HT, Bio-systems, Brazil) at 560 nm.[11]

Free radical scavenging activity

DPPH assay

The antioxidant potential was assessed on the basis of scavenging activity of EEEV by DPPH free radical.[12] Briefly, na aliquot (0.1 mL) of EEEV(10, 25, 50, 100, 200 μg/mL) or α-tocopherol (50 μg/mL) was mixed with 3.9 mL of DPPH (0.3 mM in a 1:1 MeOH/EtOH solution). The absorbance (517 nm) of the mixture was determined, and the percentage of neutralized free radicals was calculated.

NBT assay

Aliquot of EEEV (10, 25, 50, 100, 200 μg/mL) or ascorbic acid (50 μg/mL) was mixed with phosphate buffer (0.1M, pH7.0), methionine (11.6 μg/mL), nitro-blue tetrazolium (NBT; 367 μg/mL), riboflavin and was exposed to fluorescent light (80 W) for 30 min.[13] Absorbance (ʃ= 560 nm) was measured in both illuminated and not illuminated extracts and

the differences between the absorbances were used for determining the SOD activity.

Statistics

All the procedures were repeated three times with three distinct samples. The data were analyzed with the program Graph Pad Prism 5.0 (USA). Data were analyzed by a one-way ANOVA using Student's t-test or Turkey's test as *post-hoc* test to assess differences between means. A significant difference was considered when $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the effects of the drying time (24, 48, and 72 h) and type of oven (with or without air circulation) in the preparation of the herbal drug from *E. velutina* using as response moisture content and concentration of total polyphenols. Fresh plant (78.4 ± 0.13%) after dried in the oven with circulation of the air had reduced its moisture content significantly (3.34 ± 0.07%) since at 24 h of drying and in this time the concentration of total phenol was 161.28 ± 4.16 µg EAG/mg. The moisture content of the herbal drug prepared in the oven without circulation of the air was from 12.0 ± 0.22 to 4.41 ± 0.13% (24–72 h) and the maximal TPC was 162.20 ± 8.85 µg EAG/mg at 72 h of drying. Excessive water in herbal drug may encourage growth microorganisms like fungi, yeasts, and bacteria and the action of enzymes that can degrade chemical constituents by hydrolysis.[14] In the present study, both drying conditions of the trunk bark from *E. velutina* resulted in a herbal drug having a moisture content below the maximum recommended limit of 8–14%.[7] However, as the drying process performed in the oven without circulation of air showed some technical drawbacks and the drying process performed in oven with air circulation resulted in a concentration of total polyphenol higher in the first 24 h when related to the other one, the drying process of choice to produce herbal drugs from *E. velutina* was the oven with air circulation.

After drying, trituration of the herbal drug enabled the material to be reduced to particles of a small size characterized as a moderately coarse powder[8,14] with a mean diameter of 0.296 mm [Table 2]. Previous studies[15,16] have shown that the efficiency of solid/liquid extract processes can be influenced by several parameters, including temperature, nature of solvent, time of extraction and structure of solid matrix, mainly particle size. In this context, the moderately course powder of *E. velutina* is advantageous, being the use of powders of this nature recommended for the majority of herbal drugs. Other parameters as total and acid-insoluble ash, contents of substances extractable were also determined [Table 2].

Microbiological control is essential to ensure the quality of botanical raw material[16] and the control of *E. velutina* dried showed that there was no microbial growth of *Salmonella* spp. or *Eshcherichia coli*, both considered pathogenic microorganisms. In the total count of bacteria and fungi/yeasts, the contamination level found was 2.25×10^3 CFU/g and 1.75×10^3 CFU/g, respectively [Table 2]. The growth of bacteria detected was below the maximum recommended limit to herbal drug. On the other hand, the total number of fungi and yeasts in *E. velutina* drug was below or slightly above the pharmacopoeia specifications,[8] depending on the extraction method (hot extraction process: 10^5 CFU/g; cold extraction process: 10^3 CFU/g).

Table 2: Physical and chemical analyses of the trunk bark of *Erythrina velutina*.

Parameters	Results
Mean diameter of particles	0.296 ± 0.007mm (2.56)
Apparenty density	0.264 ± 0.021g/mL (1.75)
Total ashes	7.54 ± 0.04% (0.54)
Ashes insoluble acid	0.22 ± 0.01% (1.26)
Contents extractable in water	10.69 ± 0.31% (2.87)
Contents extractable in ethanol	33.42 ± 0.24% (0.71)
Microorganism	
Aerobic bacteria	2.25×10^3 CFU/g
Yeats and moulds	1.75×10^3 CFU/g
Escherichia coli	Absent
Salmonella spp	Absent
Total phenols	
Percolation	155.14 ± 3.31 (2.13)
Maceration	150.08 ± 2.78 (1.85)
Soxhlet	147.96 ± 6.65 (4.49)
Extraction yield	
Percolation	5.63 ± 0.23 (4.01)[b,c]
Maceration	2.66 ± 0.08 (3.01)[a,c]
Soxhlet	8.83 ± 0.49 (5.51)[a,b]

Analyses were carried out in triplicate. The results were expressed as mean ± standard deviation (coefficient of variation). a *vs* percolation; b *vs* maceration; c *vs* soxhlet ($p < 0,05$ – ANOVA and Tukey's*post hoc* test).

The determination of extractable matter is important to assess the ability of the solvent to extract active compounds under predetermined conditions.[17] The content of substances extractable in ethanol from the trunk bark of *E. velutina* was about three times greater than that of extractable substances in water [Table 2]. These data corroborated with previous phytochemical studies of genus *Erythrina* known for bioproduction of several organic molecules including bioactive phenols.[3] A comparison of extraction methods (percolation, maceration, and soxhlet) using ethanol as solvent observed that percolation was slightly a more efficient at extracting the total phenols when related to other extraction methods, while the soxhlet produced an extract with the best yield. Based on obtained results and considering the lower cost of extraction process, the percolation method was used for all subsequent experiments performed, such as chromatographic analysis and pharmacological evaluation.

The HPLC-PDA analysis of ethanol extract allowed detect simultaneously four flavonoids (hesperidin, abssinin, homoesperidin, and sigmoidin C) and one phenol acid (rizonic acid) [Fig. 1]. This data corroborates several previous studies[18,19] which report the presence of a wide variety of these chemical constituents in *E. velutina*; however, only homoesperidin was previously described in the plant[19] and sigmoidin C was previously described in *Erythrina eriotricha*.[20] The other substances are being reported for the first time for the species.

Table 1: Moisture content of plant drug (%) from *Erythrina velutina* determined by method of drying on an infrared balance and prepared in an oven with or without circulation and renewal of air.

Time (h)	Moisture content (%)		Total polyphenol content (µg EAG/mg)	
	Oven without circulation and continuous renewal of air	Oven with circulation and continuous renewal of air	Oven without circulation and continuous renewal of air	Oven with circulation and continuous renewal of air
24	12.00 ± 0.22 (2.11)	3.34 ± 0.07 (1.82)	156.28±10.50 (6.72)	161.28±4.16 (2.57)
48	3.49 ± 0.06 (1.76)	4.30 ± 0.08 (1.73)	151.38±2.95 (1.95)	153.69±4.05 (2.63)
72	4.41 ± 0.13 (1.84)	5.18 ± 0.10 (2.84)	162,20±8,85 (5.46)	157.34±14.96 (9.51)

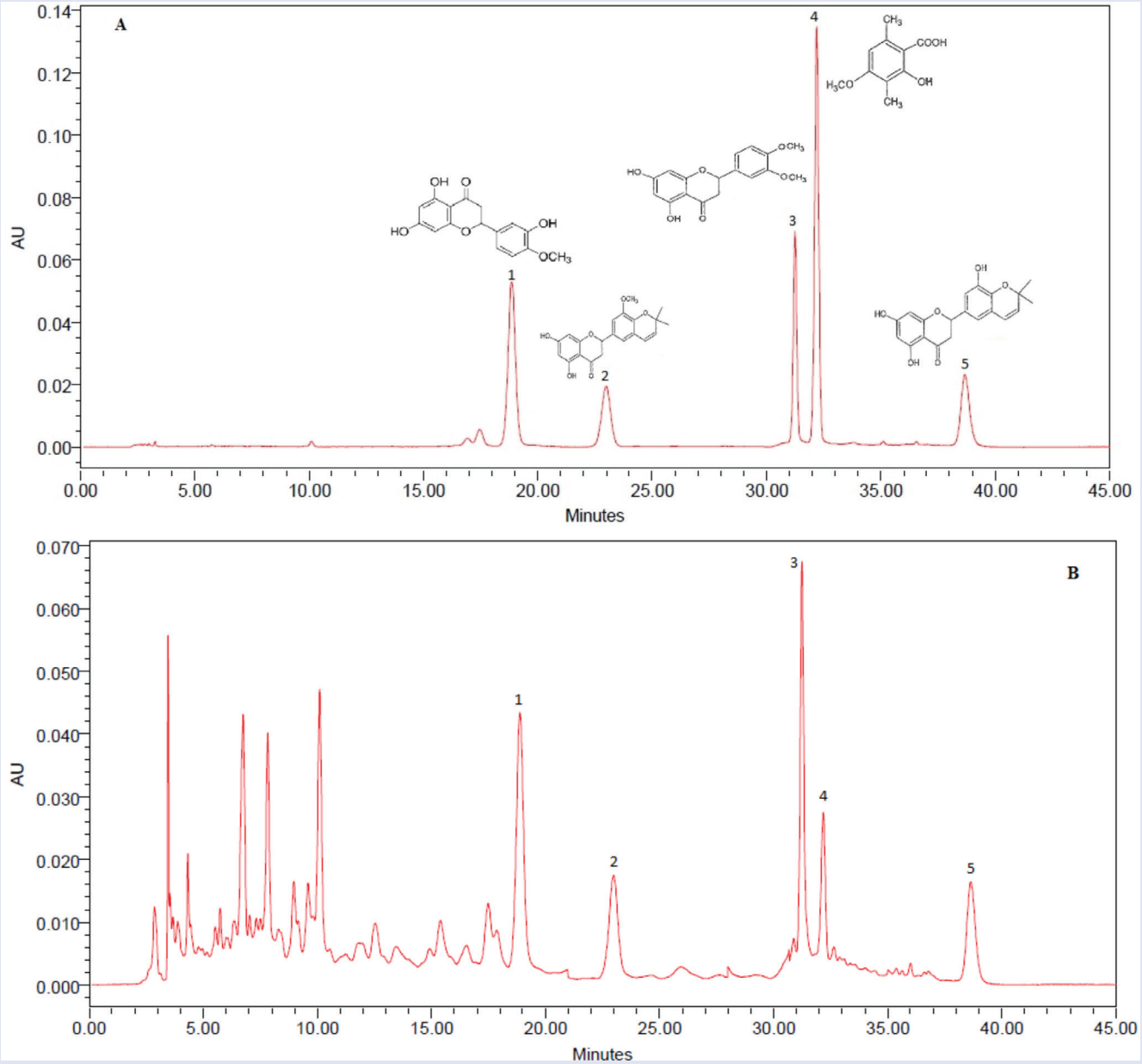

Figure 1: Chromatograms (HPLC-PDA) of (A) isolated compounds from stem bark of *E. velutina* – 1, hesperetin; 2, abissinin; 3, homohesperetin; 4, rizonic acid; 5, sigmoidin C – and (B) ethanolic extract of *E. velutina* (EEEV). Conditions: C8 column, mobile phase [(a) ammonium acetate buffer 5 mM, pH = 3.46; (b), acetonitrile], elution gradient, injection volume of 20 µL (microliter), flow rate of 1.0 mL/min and detection: 0–21 min at 286 nm, 21–28 min at 260 nm, 28–30; 7 min at 286 nm, 30; 7–35 min at 267 nm; and 35–45 min at 282 nm.

Considering the effects of *E. velutina* in the central nervous system demonstrated previously by our research group[21,22] and the lack of pharmacological data about standardized EEEV, it was investigated the neuroprotective activity of the extract and RA from *E. velutina* on rat SH-SY5Y cell-culture exposure to the neurotoxin 6-OHDA, an experimental model of Parkinson disease (PD).

Currently, one of the most commonly used cell lines for the study of events related to PD is the SH-SY5Y which has characteristics of dopaminergic neurons.[23] The neurotoxin 6-OHDA is formed endogenously in patients with PD and induces common features of this disease including oxidative stress and release of several inflammatory mediators.[24]

The SH-SY5Y cells exposure to 6-OHDA reduced in 47.6 % the cell viability (MTT absorbance: 0.226 ± 0.016) as related to group not treated (control) (MTT absorbance: 0.4227 ± 0.0213). On the other hand, the addition of EEEV (0.0025–1 µg/mL) or RA (0.0025–0.5 µg/mL) alone to the cell culture produced no effect on cell viability. Both tested drugs with concentration as low as 0.5 µg/mL protected significantly the neuronal cells against the neurotoxic effect of 6-OHDA (MTT absorbance: EEEV 0.5 + 6-OHDA = 0.339 ± 0.024; RA 0.5 + 6-OHDA = 0.357 ± 0.040) when related to treated 6-OHDAcells [Fig. 2].

The cellular damage caused by ROS and ERNs (reactive oxygen and nitrogen species, respectively) has been linked to the pathology of several central nervous system disorders such as Parkinson and Alzheimer disease, dementia, brain edema, and others.[23] In the present study, the addition of EEEV or RA on the culture of SH-SY5Y cells did not change the concentration of nitrite/nitrate as related to controls group, except

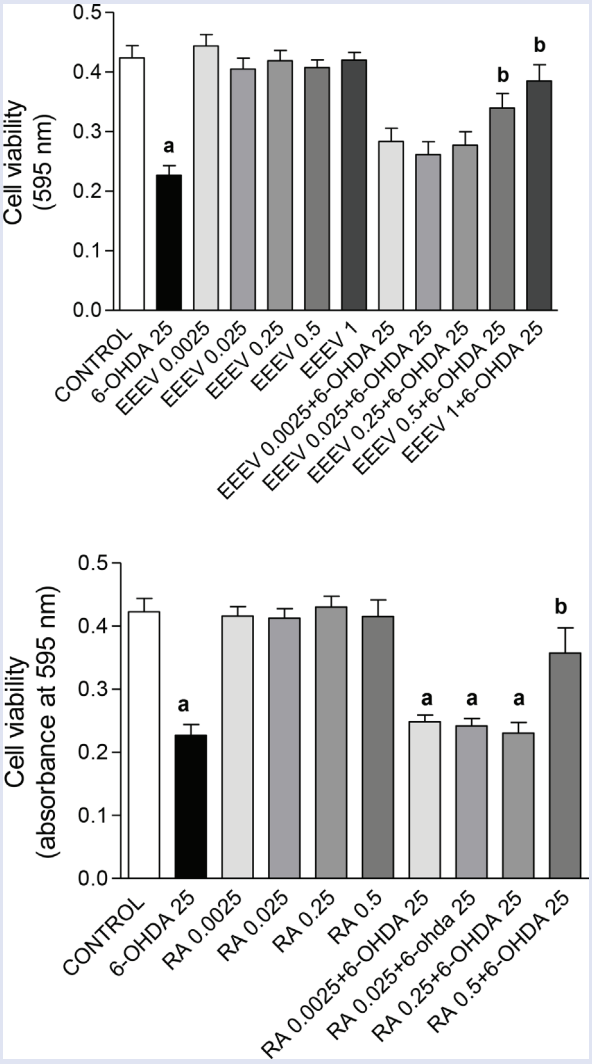

Figure 2: Effect of EEEV and RA on cell viability before 6-OHDA exposure. EEEV or RA (0.0025 – 1 µg/mL) was added to SHSY5Y cell culture 1 h before 6-OHDA (25 µg/mL). After 24 h, cell viability was evaluated by the MTT assay. A versus control, B versus 6-OHDA ($P < 0.05$, ANOVA and Tukey as the post-hoc test). 6-OHDA, 6-hydroxydopamine; EEEV, ethanolic extract of *E. velutina*; RA, rizonic acid.

for the highest concentration of RA (0.5 µg/mL). After cells exposure to 6-OHDA, a great increase in nitrite concentration was detected, relatively to control group and this effect was reduced in 45.42% and 42.86% by EEEV (1 µg/mL) and RA (0.5 µg/mL), respectively, as related to treated 6-OHDA cells [Fig. 3].

EEEV at concentrations ranging from 10 to 200 µg/mL reduced the DPPH radical up to 48%, but did not show scavenging activity on superoxide anion investigated through NBT assay (data not shown) and α-tocopherol used as the reference drug showed radical scavenging activity [Table 3]. These results suggest that EEEV has potential to scavenge free radicals, but it does not seem to be correlated with the removal of superoxide anion. Previous study also showed the potential to scavenge free radicals of the tincture of *E. velutina*.[25]

The reduction of DPPH radical by phenol compounds, such as flavonoids and phenol acids have been extensively demonstrated[+]and for that the number and position of hydroxyl or methoxyl groups on molecules seem to be one of the main factors. In this sense, it is possible that the DPPH

Table 3: Antioxidant activity of ethanolic extract of *Erythrina velutina* (EEEV): DPPH test.

Groups (µg/mL)	% Neutralised free radicals
EEEV 10	3.73 ± 0.48
25	9.89 ± 1.55
50	18.10 ± 1.70
100	30.22 ± 1.72
200	48.36 ± 0.99
α-toc 50	94.02 ± 1.10

Data are reported as mean± S.E.M. of three experiments.

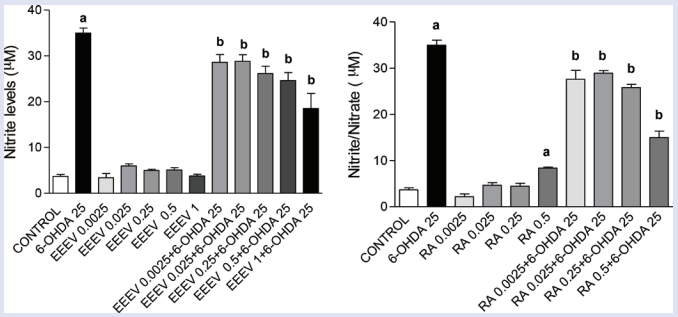

Figure 3: Effect of EEEV and RA on nitrite formation in SH-SY5Y cell culture before 6-OHDA exposure. EEEV or RA (0.0025 –1 µg/mL) was added to cell culture 1 h before 6-OHDA (25 µg/mL). After 24 h, nitrite formation was evaluated by the Griess reaction. A versus control, B versus 6-OHDA ($P < 0.05$, ANOVA and Tukey as the post-hoc test). 6-OHDA, 6-hydroxydopamine; EEEV, ethanolic extract of *E. velutina*; RA, rizonic acid.

scavenging activity of the EEEV is due at least in part to the presence of flavonoid in the extract, such as hesperetin, abissinin, homohesperetin, and sigmoidin C, which have hydroxyl and/or methoxyl groups in the A and B rings. Indeed, previous study[28] has shown that hesperetin, has a powerful radical scavenger activity. However, the role of the rizonic acid, a phenol acid, also found in *E. velutina* extract, cannot be ruled out. Taken together, the present results suggest that EEEV has neuroprotective effect that is at least in part due to the antioxidant activity of phenols compounds, such as flavonoids and rizonic acid present in *E. velutina*. Corroborating this hypothesis, previous study of our laboratory[5] also showed the neuroprotective potential of phenols compounds, such as the amburoside A from *A. cearensis*.

CONCLUSION

Pharmacognostical investigation of *E. velutina* permitted the preparation and characterization of the herbal drug and extract, including isolation and simultaneous identification of five bioactive phenols by HPLC–PDA and determination of content of polyphenols. This standardized extract and rizonic acid from *E. velutina*, acting as an antioxidant, presents a neuroprotective activity, suggesting that they could provide benefits as a therapeutic agent in neurodegenerative disease as Parkinson. This finding corroborates the popular use of *E. velutina* for the treatment of central nervous system disorders.

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Nil

Conflicts of interest

There are no conflicts of interest

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