Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(8):1054-1062

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Bioactivities of extracts from *Eugenia uniflora* L. branches

Felipe Valente Fernandes, Luciana Segheto, Bruna Celeida Silva Santos, Glauciemar Del-Vechio-Vieira, Célia Hitomi Yamamoto, Ana Lúcia Santos Matos Araújo, Mírian Pereira Rodarte and Orlando Vieira de Sousa*

Department of Pharmaceutical Sciences, Faculty of Pharmacy, Federal University of Juiz de Fora, Rua José Lourenço Kelmer, s/n - Campus Universitário, Bairro São Pedro, Juiz de Fora, 36036-330, Minas Gerais, Brazil

---

ABSTRACT

Bioactivities of extracts from Eugenia uniflora L. branches were investigated using in vitro and vivo tests. Dried and powdered from E. uniflora branches were exhaustively extracted with hexane, ethyl acetate and ethanol by static maceration obtaining the hexane (HE), ethyl acetate (EAE) and ethanol (EE) extracts. Phytochemical screening and total phenolic and flavonoid contents were determined. The antioxidant activity was assessed by 2,2-diphenyl-1-pycrilhydrazin (DPPH) and iron reducing power tests. For evaluate the antinociceptive activity, acetic acid-induced writhing, formalin and hot plate assays were performed, while the anti-inflammatory activity by paw edema method. Flavonoids, tannins, coumarins, terpenes and sterols were revealed in the extracts. HE, EAE and EE presented expressive levels of total phenolic and flavonoids and a promising antioxidant effect. Doses of HE, EAE or EE (50, 100 or 200 mg/kg) decreased the number of writhes and the phases of paw licking time. The analgesic effect on central level was confirmed by the increase in reaction time on hot plate. EAE and EE (200 and 400 mg/kg) and HE (400 mg/kg) showed a reduction of paw edema after 4 h of carrageenan application. Therefore, E. uniflora presented important biological properties that may be associated with the chemical constituents and corroborate the medicinal uses of this species.

Keywords: *Eugenia uniflora*, Phytochemical screening, Phenolic content, Flavonoids, Antioxidant, Nociception, Inflammation.

INTRODUCTION

One of the major goals of scientific research in humans and animals is the search of new therapeutic agents for the treatment of a variety of pathophysiological conditions. The physiological changes cause disease in different organ systems, including cardiovascular and metabolic complications, diabetes, peptic ulcer, cancer, neurodegenerative diseases, among others, and can involve the generation of free radicals and oxidants associated with painful and inflammatory processes [1-2]. In these processes, acetylcholine, bradykinin, histamine, serotonin, leukotriene, substance P, platelet activating factor, prostaglandins, interleukins, tumor necrosis factor (TNF), nerve growth factor (NGF) and cyclic adenosine monophosphate (cAMP) have been highlighted as the major mediators [3-4]. On the other hand, the therapeutic agents (opioid analgesics and non-steroidal anti-inflammatory drugs, for example) used to inhibit the action of these mediators produce adverse effects, such as sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance, and respiratory depression, irritation of gastric mucosa and ulcer, water retention and nephrotoxicity, and compromise the treatment of patients [5-7]. In order to minimize the harmful effects of these drugs, one of the alternatives that have been employed over the generations is the use of medicinal plants and the development of antioxidant, analgesic and anti-inflammatory products [8].

Eugenia uniflora L. (Myrtaceae), known as “pitangueira” or Brazilian cherry tree, is found in South American, including Brazil, Southern Asia and Africa [9], and the fruits have mostly been used to produce juice and frozen

pulp [10-11]. In folk medicine, the leaves have been used to treat hypercholesterolemia, gout, hypertension, digestive disease, inflammation, rheumatism, rheumatic pain, cough, fever, hepatic disease, amygdalitis, sore throat and haemorrhoids, among other medicinal uses [11-12]. Pharmacological studies of *E. uniflora*, as antinociceptive and hypothermic [13-14], anti-inflammatory [14], antioxidant [15], [16], antihypertensive [9] and antitumor [17] and effects on the intestinal transit and prolongation of sleeping time [14] have provided sustainability for their medicinal applications. In addition, this species presented antibacterial, antiviral and antifungal [14-15, 17-19] and antiprotozoal [20-21] activities.

Chemically, compounds as flavonoids myricitrin, quercetin and 3-L-ramnoside quercitrin, steroids and terpenes, tannins, anthraquinones and phenols, cineol and essential oils [13, 15-17, 19, 21] were identified and related to biological properties. In particular, phenolic compounds, such as flavonoids, have been well-established for their antioxidant, antinociceptive and anti-inflammatory properties [22-23].

Chemical and pharmacologically, extracts from *E. uniflora* branches have not been previously studied. Based on this fact and in medicinal applications, this study assessed the antioxidant, antinociceptive an anti-inflammatory properties using *in vitro* and *in vivo* tests. In addition, phytochemical screening and phenolic constituents were determined.

EXPERIMENTAL SECTION

Collect and identification of plant material

Branches of *Eugenia uniflora* L. (Myrtaceae) were collected in January, 2010, at the city of Juiz de Fora, Minas Gerais State, Southeast region, Brazil (longitude 43º 21' 01" W and latitude 21º 45' 51" S). A botanic specialist, Dr. Fátima Regina Gonçalves Salimena, identified a voucher specimen that was deposited in the Herbarium Leopoldo Krieger (CESJ nº 49.187) of the Federal University of Juiz de Fora, Juiz de Fora, MG, Brazil.

After drying, all material was triturated by an electric grinder and pulverized using a tamise nº 18 for the extract preparation.

Extract preparation

480 g of dried and powdered branches were macerated in hexane (4.0 l) by static maceration (3 weeks) at room temperature until complete extraction. Then, using the same procedure, ethyl acetate was added to the resulting residue to obtain the ethyl acetate extract. At the end of this extraction, the resulting residue was extracted with ethanol to acquire the ethanol extract. After filtration, the extracts were evaporated at controlled temperature (50–55°C) by a rotary vacuum evaporator (Rotavapor RII, Büchi, Flawil, Switzerland). To complete this step, a desiccator containing silica removed the residual solvents and water of the hexane (HE), ethyl acetate (EAE) and ethanol (EE) extracts.

Drugs and reagents

For this research, the following chemicals were used: acetic acid (Vetec Química Farm. Ltda, Rio de Janeiro, RJ, Brazil); formaldehyde and acetylsalicylic acid (Reagen Quimibrás Ind. Química S. A., Rio de Janeiro, RJ, Brazil); aluminum chloride, potassium ferrocyanide P.A., metanol, etanol, pyridine and sodium carbonate (Labsynth, Diadema, SP, Brazil); Folin-Ciocalteu reagent P.A., trichloroacetic acid and ascorbic acid (Cromoline Química Fina, Diadema, SP, Brazil); morphine hydrochloride (Merck Inc., Whitehouse Station, NJ, USA); 1,1-Diphenyl-2-picrylhydrazyl (DPPH), gallic acid, rutin, naloxone hydrochloride, indomethacin and λ-carrageenan commercial grade Type II (Sigma Chemical Co, St Louis, MO, USA) and ketamine chloride and xylazine chloride (Syntec, Hortolândia, SP, Brazil). All chemicals used in the experiments presented purity certified by the suppliers.

Phytochemical screening

HE, EAE and EE were used to distinguish different chemical classes (tannins, flavonoids, terpenes and phytosterols, saponins, coumarins, anthraquinones, and alkaloids) of secondary metabolites according to the reactions described by Tiwari et al. [24]. These reactions were based on the visual observation of color change or formation of a precipitate after the addition of specific reagents.

Quantification of total phenolic

The Folin-Ciocalteu reagent was used to determine the total phenolic contents in HE, EAE and EE by spectrophotometric method [25]. The Folin-Ciocalteu reagent was neutralized with sodium carbonate to produce a blue coloration. Gallic acid solutions (40 to 120 µg/ml) in ethanol were used to acquire the calibration curve. The absorbance of the resulting solutions in triplicate was measured at 765 nm after 60 min with a double beam UV/Visible spectrophotometer. The total phenolic contents were expressed as gram of gallic acid equivalent (GAE) per 100g dry weight (g/100g).

Total flavonoids determination
Aluminum chloride colorimetric method was used for total flavonoid determination using rutin (reference standard) solutions in ethanol (5 to 40 µg/ml) to obtain the calibration curve [26]. HE, EAE or EE (0.4 ml), acetic acid (0.12 ml), pyridine:ethanol (2:8) (2 ml) and distilled water (1.98 ml) were reacted with 8% aluminum chloride (0.5 ml) at room temperature for 30 min. After reaction, the absorbance was measured at 420 nm with a double beam UV/Visible spectrophotometer. The flavonoid contents were expressed as gram of rutin equivalent (RE) per 100 g dry weight (g/100g).

DPPH radical scavenging activity
DPPH was used for determination of free radical-scavenging activity using the method described by Mensor et al. [27]. Different concentrations of HE (60 to 280 µg/ml), EAE (20 to 140 µg/ml) and EE (40 to 200 µg/ml) were added, at an equal volume, to methanol solution of DPPH (0.03 mM) and they remained under protection from light for 60 minutes at room temperature.. The absorbance was recorded at 518 nm using a double beam UV/Visible spectrophotometer. The experiment was performed in triplicate and rutin was used as standard control. EC_{50} values denote the concentration (µg/ml) of sample, which is required to scavenge 50% of DPPH free radicals.

Test of iron reducing power (FRAP)
The reducing power of iron was determined using a serial dilution of HE (300 to 80 µg/ml), EAE (120 to 20 µg/ml) and EE (220 to 40 µg/ml) with 0.2 mM phosphate buffer pH 6.6 (2.5 ml), and 1 % potassium ferrocyanide (2.5 ml) [28]. The mixture was incubated at 50ºC for 20 min. Then, five milliliters of this mixture received 10 % trichloroacetic acid (2.5 ml) followed by centrifugation at 3000 g for 10 minutes. The supernatant was separated and mixed with distilled water (2.5 ml) containing 1 % ferric chloride (0.5 ml). In triplicate, the absorbance of this mixture was measured at 700 nm. Ascorbic acid (12.5 to 0.5 µg/ml) was used as reference standard. The measurement was considered the possible antioxidant activity.

Animals
For this research, male Swiss albino mice (50-70 days with 25-30 g) and male Wistar rats (90-110 days with 180-220 g) were used in pharmacological tests. These animals were provided by the Central Animal Facility of the Federal University de Juiz de Fora (UFJF) and housed in clean plastic cages and maintained under standard laboratory conditions (temperature 24-2 °C and 12:12 light/dark cycles). They received water *ad libitum* and fed with the balanced feed (Nuvilab Rodents - Nuvital Nutrients, Colombo, Brazil) and the experiments were performed at the Natural Products Pharmacology Laboratory, Faculty of Pharmacy, UFJF. The procedures adopted in animal care and experimentation were in accordance with Guidelines for Care and Use of Laboratory Animals in Biomedical Research of the Brazilian College of Animal Experimentation (COBEA). The protocol was approved by the Experimentation Ethics Committee on Animal Use of the Federal University de Juiz de Fora (number 037/2010).

Writhing test in mice
According to Collier et al. protocol [29], male Swiss albino mice (n = 8) were pretreated with HE, EAE and EE (50, 100 and 200 mg/kg, v.o.). After one hour of application of the extracts, 0.1 ml/10 g of 0.6% v/v acetic acid was injected intraperitoneally. For test validation, reference drugs, acetylsalicylic acid (200 mg/kg, p.o.) and indomethacin (10 mg/kg, p.o.), were used in this experiment. Negative control was treated with 1% DMSO in 0.9% NaCl (10 ml/kg, v.o.). After injection of acetic acid, the number of abdominal constrictions (writhing) was counted between 10 and 30 min.

Formalin-induced pain test in mice
The method used in this assay was described by Hunskaar and Hole [30]. Groups of animals (n = 8) were pretreated with HE, EAE or EE (50, 100 and 200 mg/kg, p.o.), 1% DMSO in sterile saline (10 ml/kg, p.o., negative control), indomethacin (10 mg/kg, p.o., positive control) and morphine (5 mg/kg, s.c). After 1 hour of treatment, 20 µl of 2.5% formalin (37% formaldehyde) in sterile saline were injected in the subplantar right hind paw region. Immediately after application of formalin (0–5 min), the licking time the paw was recorded corresponding to the first phase (neurogenic), while the second phase (inflammatory) was evaluated between 15 and 30 min.

Hot-plate test in mice
To assess the central antinociceptive action, the hot plate test recommended by Eddy et al. [31] was performed. Fifteen groups of eight mice (n = 8) each were treated with HE, EAE or EE (50, 100 and 200 mg/kg, p.o.), morphine (5 mg/kg, s.c.), morphine (5 mg/kg, s.c., positive control) plus naloxone (2 mg/kg, s.c.), HE (200 mg/kg, p.o.) plus naloxone (2 mg/kg, subcutaneously), EAE (200 mg/kg, p.o.) plus naloxone (2 mg/kg, s.c.), EE (200 mg/kg, p.o.) plus naloxone (2 mg/kg, s.c.) or 1% DMSO in sterile saline (NaCl 0.9%) (10 ml/kg, p.o., negative control). Using a hot plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) at 55° ± 1°C, the latency time was measured in 0, 30, 60 and 90 min with a cut-off time of 30 s.

Carrageenan-induced paw edema in rats

To evaluate the anti-inflammatory effect, the carrageenan-induced paw edema test was used according to the method of Winter et al. [32] with slight modifications. One hour before the application of carrageenan, groups of animals were treated (n = 6) with HE, EAE or EE (50, 100 and 200 mg/kg, p.o.), indomethacin (10 mg/kg, p.o., positive control) and 1% DMSO in sterile saline (NaCl 0.9%) (10 ml/kg, p.o., negative control). A solution (0.1 ml) of 2% carrageenan was injected into the subplantar tissue of the right hind paw, while 0.1 ml of sterile saline (control) was injected in the left hind paw. To determine the volume displaced by the edema, a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain) was used in times of 1, 2, 3 and 4 h after the carrageenan injection.

Statistical analysis

The data were presented as the mean ± standard error of the mean (S.E.M.) and the statistical significance was determined using an analysis of variance (ANOVA) followed by Tukey or Student Newman-Keuls test. Values were considered significantly different at $p < 0.05$. All analysis was performed using by GraphPad Prism 5.0 program (Graph Pad Prism Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Phytochemical screening

480 g of dried and powdered branches yielded 15.79, 9.36 and 11.38 g of HE, EAE and EE, respectively, equivalent to 9.53, 7.45 and 8.34%.

Phytochemical screening showed that HE contains coumarins, terpenes and sterols, while tannins and flavonoids were detected in EAE and EE. Negative reactions to saponins, alkaloids and anthraquinones were observed in the samples.

Total phenolic and flavonoid contents

In *E. uniflora*, the total phenolic varied from 0.59 ± 0.07 to 5.56 ± 0.52 g/100 g and flavonoid ranged from 0.93 ± 0.05 to 1.82 ± 0.24 g/100 g (Table 1). HE showed no reactions to determine the total flavonoid content. In addition, as observed in Table 1, EAE exhibited the highest total phenolic and flavonoid contents.

Table 1. Total phenolic and flavonoid contents obtained of HE, EAE and EE

Plant extract	Total phenolic (g/100g)	Total flavonoid (g/100 g)
HE	0.59 ± 0.07	-
EAE	5.56 ± 0.52	1.82 ± 0.24
EE	2.96 ± 0.47	0.93 ± 0.05

The data represent the mean ± S.E.M. (n = 3).
There was significant difference between the means considering p < 0.05 after ANOVA followed of Tukey's test.
HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract.

DPPH radical scavenging and Fe^{+3} reducing power activities

Table 2 shows the scavenging effects obtained with samples on DPPH radical and Fe^{+3} reducing power in the following order: EAE > EE > HE. The EC_{50} values were statistically different ($p < 0.05$) that ranged from 79.97 ± 0.11 to 163.66 ± 0.88 µg/ml and 63.02 ± 0.36 to 182.36 ± 2.26 µg/ml using DPPH and Fe^{+3} reducing power, respectively (Table 2). EAE was more active to inhibit the DPPH radical and more potent in convert Fe (+3) to Fe (+2).

Table 2. Antioxidant activity of HE, EAE and EE by DPPH and Fe^{+3} reducing power methods

Plant extract/Chemical	EC_{50} (µg/ml)	
	DPPH	Fe^{+3} Reducing Power
HE	163.66 ± 0.88	182.36 ± 2.26
EAE	79.97 ± 0.11	63.02 ± 0.36
EE	111.00 ± 0.62	115.48 ± 0.62
Rutin	8.47 ± 0.08	104.37 ± 0.10
Ascorbic acid	-	5.36 ± 0.07

The data represent the mean ± S.E.M. (n = 3).
There was significant difference between the means considering p < 0.05 after ANOVA followed of Tukey´s test.
HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract.

Acetic acid-induced writhing in mice

When compared with the control group, HE reduced the number of abdominal writhes ($p < 0.001$) at doses of 50, 100 and 200 mg/kg (Figure 1). In Figure 1, EAE and EE also showed a decrease in writhes at doses of 100 (p <

0.05) and 200 mg/kg ($p < 0.01$ or $p < 0.001$). As drug references, ASA and indomethacin were effectives against acetic acid-induced nociception.

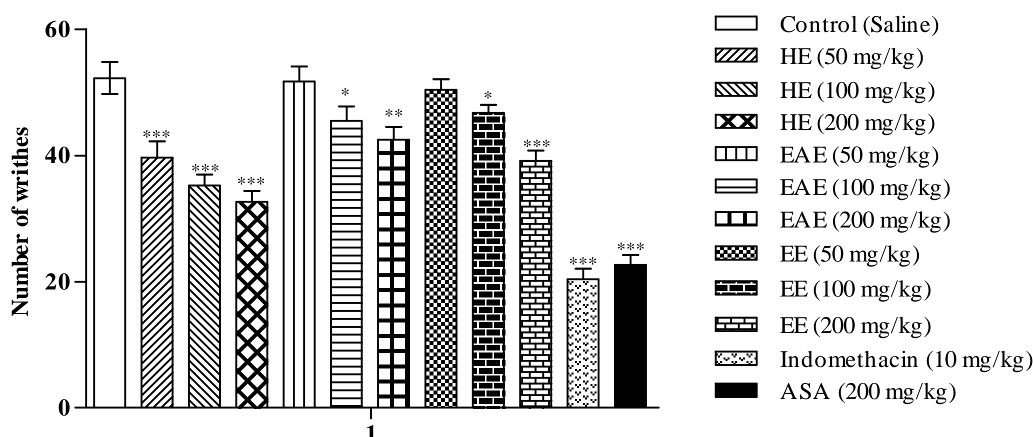


Figure 1. Effects of HE, EAE and EE on acetic acid induced writhing in mice

*The data represent the mean ± S.E.M. (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001 vs. control group after ANOVA followed of Student-Newman-Keuls' test. HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract; ASA: acetylsalicylic acid.*

Formalin test

Considering the first phase of the formalin test, doses of 50, 100 and 200 mg/kg de HE inhibited ($p < 0.001$) the licking time in 10.46, 20.76 and 58.77%, respectively (Table 3). This phase was also reduced after treatment with 100 and 200 mg/kg of EAE ($p < 0.05$ or $p < 0.01$) and 200 mg/kg of EE ($p < 0.001$). In Table 3, the second phase was reduced after treatment with HE (50, 100 and 200 mg/kg, $p < 0.001$), EAE and EE (100 and 200 mg/kg, $p < 0.01$ or $p < 0.001$). Indomethacin was not active in the first phase, but morphine inhibited both phases of the formalin test (Table 3).

Table 3. Effects of HE, EAE and EE on formalin-induced nociception in mice

Group	Dose (mg/kg)	Duration of paw licking (s)			
		First phase		Second phase	
		Time	Inhibition (%)	Time	Inhibition (%)
Control	Saline	77.62±1.77	-	84.12±2.67	-
	50	69.50±1.35***	10.46	71.25±2.55***	15.30
HE	100	61.50±1.28***	20.76	64.37±1.52***	23.48
	200	45.62±1.58***	58.77	54.37±1.27***	35.37
	50	74.12±1.98	4.51	81.87±2.47	2.67
EAE	100	71.50±2.34*	7.88	74.37±2.43**	11.59
	200	70.87±1.54**	8.70	73.25±2.30**	12.92
	50	75.37±1.18	2.90	82.50±1.73	1.92
EE	100	74.25±1.48	4.34	75.50±1.84**	10.25
	200	59.50±1.32***	23.34	67.25±2.05***	20.05
Indomethacin	10	74.75±2.43	3.70	25.75±1.14***	69.39
Morphine	1	18.12±11.86***	76.65	22.12±1.85***	73.70

*1st phase (0–5 min) and 2nd phase (15–30 min). The data represent the mean ± S.E.M. (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001 vs. control group after ANOVA followed of Student-Newman-Keuls' test. HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract.*

Effects on the hot plate test

In the hot plate assay, after 30 minutes of treatment, dose of 200 mg/kg HE increased the reaction time (Table 4). In this table, it is also showed that HE (50, 100 and 200 mg/kg), EAE (100 and 200 mg/kg) and EE (200 mg/kg) significantly ($p < 0.05$, $p < 0.01$ or $p < 0.001$) increased after 90 min of treatment. In the presence of naloxone, an opioid antagonist, antinociceptive response was inhibited to the morphine, EAE and EE, but it was not to HE.

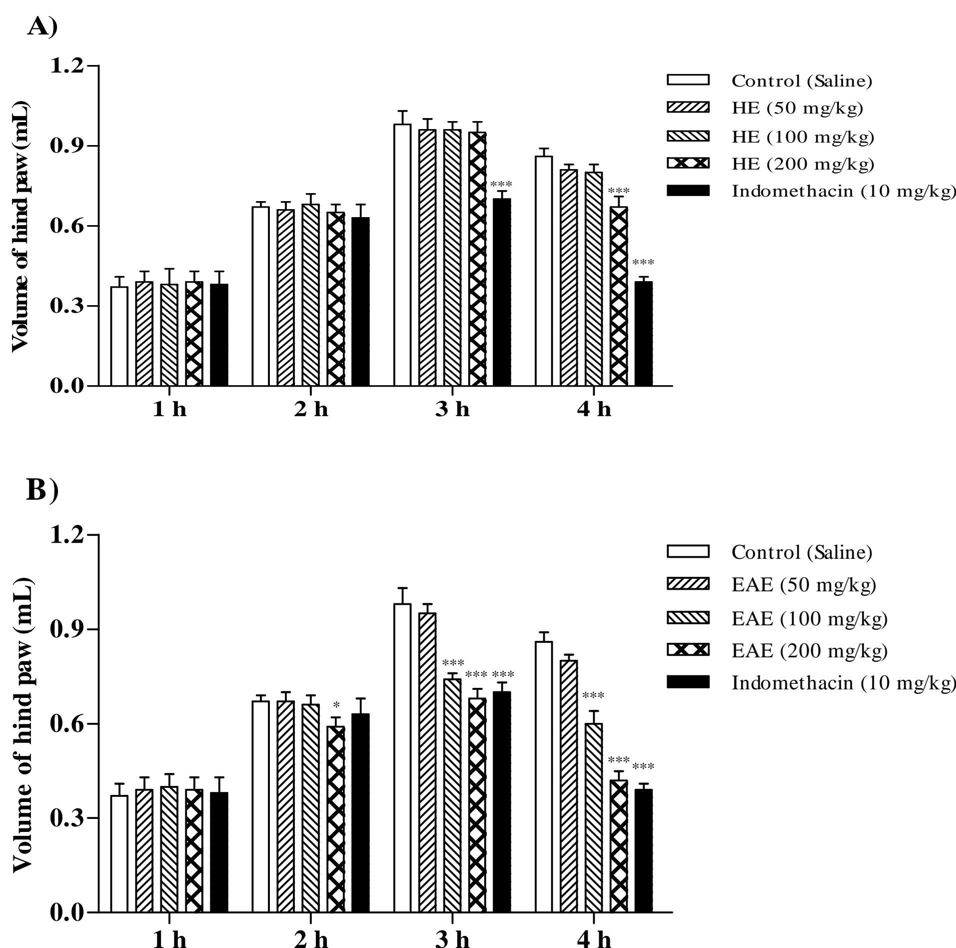
Table 4. Effects of HE, EAE and EE on the latency time of mice exposed to the hot plate test

Groups	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	6.23±0.33	6.65±0.18	6.88±0.18	7.10±0.24
HE	50	7.92±0.79	7.92±0.80	7.82±0.42	8.80±0.45*
	100	8.30±1.00	8.31±1.00	10.13±0.90***	10.29±0.48***
	200	10.67±0.83	10.67±0.83***	12.11±0.25***	12.61±0.67***
EAE	50	6.25±0.37	6.82±0.33	6.79±0.36	7.30±0.20
	100	6.34±0.38	6.94±0.35	6.97±0.38	7.89±0.22*
	200	6.42±0.40	7.13±0.35	7.48±0.26*	8.06±0.27**
EE	50	6.10±0.29	6.73±0.36	7.02±0.24	7.19±0.22
	100	6.15±0.36	6.86±0.35	7.07±0.35	7.57±0.26
	200	6.11±0.44	7.06±0.34	7.59±0.25*	8.36±0.22***
Morphine	1	6.47±0.17	11.05±0.34***	13.24±0.18***	14.58±0.27***
Naloxone+Morphine	1+1	6.54±0.19	9.17±0.39*	8.82±0.38*	7.68±0.30
Naloxone+HE	1+200	6.38±0.20	9.57±0.46*	11.58±0.45***	10.55±0.58***
Naloxone+EAE	1+200	6.40±0.34	7.05±0.36	7.27±0.29	7.47±0.19
Naloxone+EE	1+200	6.42±0.44	6.95±0.26	6.99±0.24	7.30±0.29

*The data represent the mean ± S.E.M. (n = 8). *p < 0.05; **p < 0.01; ***p < 0.001 vs. control group after ANOVA followed of Student-Newman-Keuls' test. HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract.*

Effects on edema induced by carrageenan

Dose of 200 mg/kg of EAE inhibited the paw edema in 11.94% ($p < 0.05$) after 2 hours of carrageenan application (Figure 2B). In Figure 2, 3 hours after carrageenan injection, doses of 100 and 200 mg/kg of EAE and EE significantly ($p < 0.01$ or $p < 0.001$) reduced the paw edema when compared with control group (Figures 2B and 2C). The anti-inflammatory effect was also observed at time of 4 hours after treatment with HE, EAE and EE. As expected, indomethacin, a nonsteroidal anti-inflammatory, was active against the paw edema (Figure 2).



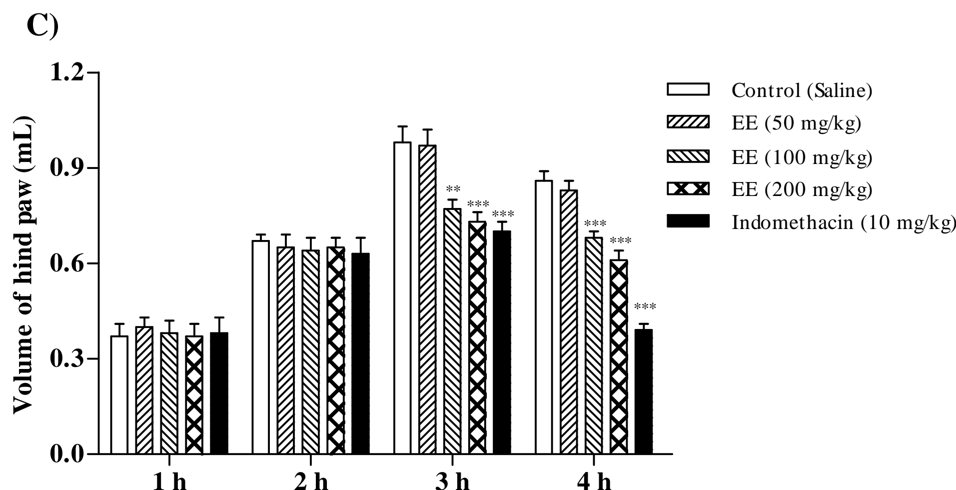


Figure 2. Effects of HE (A), EAE (B) and EE (C) on the rat paw edema induced by carrageenan

*The data represent the mean ±S.E.M. (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001 vs. control group after ANOVA followed by Student Newman-Keuls' test. HF: Hexane extract; EAE: Ethyl acetate extract; EE: Ethanol extract.*

The phytochemical screening of HF, EAE and EE indicated the presence of terpenes, sterols, coumarins, flavonoids and tannins, which is consistent with the chemical profile described for *E. uniflora* [13,15-17, 19, 21]. In addition, Table 1 shows that the variation of the total phenolic and flavonoid contents in the extracts was influenced by the polarity of solvent, since hexane is able to remove terpenes, steroids and coumarins, while free flavonoids, tannins and phenolic compounds are extracted with ethyl acetate [33]. Glycosylated flavonoids and tannins are separated by the ethanol action [33]. Our results also revealed that the flavonoid content of EE was higher than that found in the leaves [16]. These constituents, as well as tannins, triterpenoids, essential oils and other secondary metabolites, have been reported with actions against oxidative, nociceptive and inflammatory mechanisms [22-23, 34], which can give explanation for the traditional use of *E. uniflora* [11-12].

The extracts from *E. Uniflora* branches showed a promising antioxidant effect, which were able to inhibit the stable radical DPPH and chelate iron (Table 2). This effect can be related to the presence of phenolic compounds, since that EAE presented a higher total phenolic flavonoid contents that correlate with EC_{50}, confirming literature findings [16]. In addition, phenolic compounds exhibit mechanism against these radicals [22-23], and this action can also justify the inhibition of signaling pathways that modulate pain and inflammation [35]. The antioxidant activity can be associated with other components as essential oil from leaves, which it was active against the DPPH and FRAP assays [15].

One aspect to be considered on *E. uniflora* is the use in popular medicine for the treatment of inflammation and rheumatic pain [11]. According to our findings (Figure 1), HE, EAE and EE reduced the writhing induced by acetic acid proving to be important products for use as antinociceptive agent. This effect may be due to the presence of different constituents that decreased the response characterized by twisting of the trunk and extension of the hind limbs [29]. A similar result was shown by Amorim et al. [13] using essential oil (sesquiterpenes as the major compounds) and isolated terpenoids from *E. uniflora* leaves. Considering the nociceptive mechanisms, the nociceptive response can be produced by activation of acetic acid-sensitive ion channels and transient receptor potential vanilloid 1 (TRPV1) located at primary afferent pathways [36] and, the acetic acid induces a release of TNF-α, interleukin 1β and interleukin 8 [37], prostanoids and bradykinin [38]. Under these aspects, our results revealed a dose-dependent effect after treatment with HE, EAE and EE (Figure 1) that may include central and/or peripherical actions.

Based on data in Table 3, HE, EAE and EE produced significant inhibition in both phases of formalin-induced pain. However, HE caused a dose-dependent promising effect after oral application. The formalin promotes a local tissue injury to the paw producing tonic and inflammation pain characterized by a biphasic response called first (neurogenic) and second (inflammatory) phases [30]. In the first phase, immediately after intraplantar application, the formalin produces an intense pain by direct stimulation of nociceptors for 5 minutes with release of substance P and bradykinin [30]. The second phase occurs between 15 and 30 minutes after formalin injection and involves the formation of inflammatory mediators (histamine, serotonin, prostaglandin, bradykinin, TNF-α and IL-1β) [39]. Opioid drugs (as morphine) inhibit both phases, while steroidal and nonsteroidal anti-inflammatory agents (aspirin, oxyphenbutazone, hydrocortisone and dexamethasone) have actions against the second phase [30], [39]. Our

findings showed that HE, as well as EAE and EE, decreased both phases confirming the results of the writhing test. In this sense, the action of these extracts justifies the traditional use of *E. uniflora* and the mechanism may involve the inhibition of central pain and inflammation pathways.

As shown in the first phase of the formalin test (Table 3), HE, EAE and EE promoted a central antinociceptive effect that could be confirmed by hot plate assay, which is a selective method able to screen centrally acting opiate analgesic drugs [40]. Our results showed a pronounced activity from 2 hours after administration of HE corroborating the study described by Amorim et al. [13], which attributed to essential oil (sesquiterpenes) and isolated terpenoids this central action. In hot plate test, the thermal stimulus induces paw licking and jumping and is associated with the activation of TRPV and propagation of the action potential by nociceptive pathways [41]. According to the presented data (Table 4), the oral treatment with HE, EAE and EE exerts a central action, confirms the results of the first phase of formalin test. Our results also showed that the naloxone did not revert the antinociceptive effect of HE (Table 4). Probably, other inhibitory pathways are contributing for this effect.

Our results showed a possible anti-inflammatory effect of HE, EAE and EE using formalin test, which is consistent with data described by Schapoval et al. [14] and with the traditional use for inflammation and rheumatism [11]. To confirm this hypothesis, the anti-inflammatory effect of HE, EAE and EE was evaluated using the carrageenan-induced paw edema model. The carrageenan produces three phases involving different mediators [32]: 1st phase (1–2 h, serotonin and histamine), 2nd phase (kinins) and 3rd phase (3–5 h, prostaglandins) [42]. Among the tested extracts, EAE provided a better effect, which was observed from 2 hours after carrageenan application. Probably, EAE contains compounds (flavonoids, for example) that are able to inhibit the generation of mediators such as histamine, serotonin and prostaglandins, demonstrating a spectrum of action against different inflammation pathways. In addition, the lower expression of anti-inflammatory activity of HE may be due to its potent antinociceptive action that decreased both phases of the formalin test with similar action to opioids. Therefore, our results indicate that HE, EAE and EE present actions against inflammatory mediators and are an important implement for the development of anti-inflammatory products.

CONCLUSION

The results showed that the extracts from *E. uniflora* branches have antioxidant, analgesic and anti-inflammatory effects, which can justify the traditional uses of this species. In addition, *E. uniflora* can be an active source of bioactive substances, representing promising targets for future medicines.

Acknowledgments
This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and Pró-Reitoria de Pós-Graduação, Pesquisa e Inovação of the Federal University of Juiz de Fora. We are grateful to Éder Luis Tostes and Jésus de Paula Sarmento, for the technical support.

REFERENCES

[1] BE Sansbury; M Spite, *Circ. Res.*, **2016**, 119(1), 113-130.
[2] V Afonso; R Champy; D Mitrovic; P Collin; A Lomri, *Joint Bone Spine*, **2007**, 74(4), 324-329.
[3] V Verma; Z Sheikh; AS Ahmed, *Acta Neurol. Belg.* **2015**, 115(3), 213-220.
[4] F Amaya; Y Izumi; M Matsuda; M Sasaki, *Curr. Neuropharmacol.*, **2013**, 11(6), 592-597.
[5] Bacchi S; P Palumbo; A Sponta; Coppolino MF, *Anti-Inflamm. Anti-Allergy Agents Med. Chem.*, **2012**, 11(1), 52-64.
[6] R Benyamin; AM Trescot; S Datta; R Buenaventura; R Adlaka; N Sehgal; SE Glaser; R Vallejo, *Pain Physician*, **2008**, 11(2 Suppl), S105–S120.
[7] Z-Y Ren; X-Q Xu; Y-P Bao; J He; L Shi; J-H Deng; X-J Gao; H-L Tang; Y-M Wang; L Lu, *Pain Physician*, **2015**, 18(2), 131-152.
[8] E Christodoulou; NP Kadoglou; N Kostomitsopoulos; G Valsami, *J. Pharm. Pharmacol.*, **2015**, 67(12), 1634-1649.
[9] AE Consolini; MG Sarubbio, *J. Ethnopharmacol.*, **2002**, 81(1), 57-63.
[10] AL Oliveira; RB Lopes; FA Cabral; MN Eberlin, *Food Chem.*, **2006**, 99(1), 1-5.
[11] G Daniel; S Krishnakumari, *J. Pharmacogn. Phytochem.*, **2015**, 4(2), 40-43.
[12] I Arai; S Amagaya; Y Komatsu; M Okada; T Hayashi; M Kasai; M Arisawa; Y Momose, *J. Ethnopharmacol.*, **1999**, 68(1-3), 307-314
[13] ACL Amorim; CKF Lima; AMC Hovell; ALP Miranda; CM Rezende, *Phytomedicine*, **2009**, 16(10), 923-928.
[14] EES Schapoval; SM Silveira; ML Miranda; CB Alice; AT Henriques, *J. Ethnopharmacol.*, **1994**, 44(3), 137-142.

[15] FN Victoria; EJ Lenardão; L Savegnago; G Perin; RG Jacob; D Alves; WP Silva; AS Motta; OS Nascente, *Food Chem. Toxicol*., **2012**, 50(8), 2668-2674.
[16] HA Martinez-Correa; PM Magalhães; CL Queiroga; CA Peixoto; AL Oliveira; FA Cabral, *J. Supercrit. Fluids*, **2011**, 55(3), 998-1006.
[17] IA Ogunwande; NO Olawore; O Ekundayo; TM Walker; JM Schmidt; WN Setzer, *Int. J. Aromather*., **2005**, 15(3), 147-152.
[18] AC Adebajo; KJ Oloke; AJ Aladesanmi, *Fitoterapia*, **1989**, 60(5), 451-455.
[19] DP Costa; EG Alves Filho; LMA Silva; SC Santos; XS Passos; MRR Silva; JC Seraphin; PH Ferri, *J. Braz. Chem. Soc*., **2010**, 21(5), 851-858.
[20] KKA Santos; EFF Matias; SR Tintino; CES Souza; MFBM Braga; GMM Guedes; M Rolón; C Veja; AR Arias; JGM Costa; IRA Menezes; HDM Coutinho, *Exp. Parasitol*., **2012**, 131(1), 130-132.
[21] KAF Rodrigues; LV Amorim; JMG Oliveira; CN Dias; DFC Moraes; EHA Andrade; JGS Maia; SMP Carneiro; FAA Carvalho, *Evid. Based Complement. Alternat Med*., **2013**, 2013, 1-10.
[22] S Kumar; AK Pandey, *Sci. World J*., **2013**, 2013, 1-16.
[23] RJ Nijveldt; E Nood; DEC Hoorn; PG Boelens; K Norren; PAM Leeuwen, *Am. J. Clin. Nut*., **2001**, 74(4), 418-425.
[24] P Tiwari; B Kumar; M Kaur; G Kaur; H Kaur, *Int. Pharm. Sci*., **2011**, 1(1), 98-106.
[25] CMM Sousa; HR Silva; GM Vieira-Junior; CLSC Ayres; DS Araújo; LCD Cavalcante; EDS Barros; PBM Araújo; MS Brandão; MH Chaves, *Quím. Nova*, **2007**, 30(2), 351-355.
[26] TJSP Sobrinho; CHTP Silva; JE Nascimento; JM Monteiro; UP Albuquerque; ELC Amorim, *Braz. J. Pharm. Sci*., **2008**, 44, 683-689.
[27] LL Mensor; FS Menezes; GG Leitão; AS Reis; TC Santos; CS Coube; SG Leitão, *Phytother. Res*., **2001**, 15(2), 127-130.
[28] M Oyaizu, *Jpn J. Nutr. Diet*., **1986**, 44(6), 307-315.
[29] HO Collier; LC Dinneen; CA Johnson; C Schneider, *Br. J. Pharmacol. Chemother*., **1968**, 32(2), 295-310.
[30] S Hunskaar; K Hole, *Pain*, **1987**, 30(1), 103-114.
[31] NB Eddy; D Leimbach, *J. Pharmacol. Exp. Ther*., **1953**, 107(3), 385-393.
[32] CA Winter; EA Risley; GW Nuss, *Proc. Soc. Exp. Biol.* Med., **1962**, 111(3), 544-547.
[33] V Cechinel Filho; RA Yunes, *Quím. Nova*, **1998**, 21(1), 99-105.
[34] MT Souza; JR Almeida; AA Araujo; MC Duarte; DP Gelain; JC Moreira; MR Santos; LJ Quintans-Júnior, *Basic Clin. Pharmacol. Toxicol*., **2014**, 115(3), 244-256.
[35] AA Geronikaki; AM Gavalas, *Comb. Chem. High Throughput Screen*., **2006**, 9, 425-442.
[36] D Julius; AI Basbaum, *Nature*, **2001**, 413(6852), 203-210.
[37] RA Ribeiro; ML Vale; SM Thomazzi; AB Paschoalato; S Poole; SH Ferreira; FQ Cunha, *Eur. J. Pharmacol*., **2000**, 387(1), 111-118.
[38] ID Duarte; M Nakamura; SH Ferreira, *Braz. J. Med. Biol. Res*., **1988**, 21(2), 341–343.
[39] M Shibata; T Ohkubo; H Takahashi; R Inoki, *Pain*, **1989**, 38(3), 347-352.
[40] FV Abbott; R Melzack, *Brain Res*., **1982**, 251(1), 149-155.
[41] D Le Bars; M Gozariu; SW Cadden, *Pharmacol. Rev*., **2001**, 53(4), 597-652.
[42] M Di Rosa; JP Giroud; DA Willoughby, *J. Pathol*., **1971**, 104(1), 15-29.