Full Length Research Paper

Comparative studies between the chemical constituents and biological properties of the extracts from the leaves and barks of *Myracrodruon urundeuva* Fr. All.

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The present study describes the comparison between the chemical constituents and biological properties of leaves and barks of *Myracrodruon urundeuva* for proposing the medicinal use of the plant from leaves, avoiding the degradation by remotion of the barks. The results of chromatographic profile of the hydroalcoholic extracts and fractions of leaves and barks showed similarities in the composition of the phytoconstituents, with variation for some of them that are in greater quantity of the bark extracts. The hydroalcoholics extracts from leaves (HELMu) and barks (HEBMu) exhibited promising 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} = 4.8 and 7.0 µg/ml, respectively), comparable to gallic acid (IC_{50} = 4.8 µg/ml). No significant cytotoxicity was observed in the concentrations evaluated (IC_{50} > 400 µg/ml). The HEBMu exhibited greater nociceptive activity than HELMu, however HELMu was more active against oral pathogens. This study provides support for the use of the leaves in traditional medicine instead of bark.

Key words: Anacardiaceae, phytochemical and chromatographic profile, biological activities, sustainable use.

INTRODUCTION

Myracrodruon urundeuva Fr. All. is commonly used in northeastern Brazil because of its high medicinal power (Goes et al., 2005). It is popularly known as aroeira-do-cerrado, aroeira-do-sertão, aroeira-preta, urundeuva, among others (Indian Prairie Educational Foundation (IPEF), 2012; Viana et al., 1995). It is used to treat ulcers

and lesions of the skin and mucous membranes, against infections of the respiratory, digestive and genitourinary systems (Berger et al., 2007; Santos et al., 2007; Viana et al., 1995). Furthermore, studies have observed antifungal, anti-inflammatory, antiulcerogenic, antihistamine, antibradicinina and analgesic effects coupled with the absence of toxic and teratogenic effects of bark extracts (Botelho et al., 2007; Carvalho, 1994; Moraes et al., 2005; Naruzawa and Papa, 2011). Despite such great importance, the extractive exploration, due to the excellent physical, chemical and biological properties, generated losses of genetic material and committed to conservation of existing population (Pacheco, 2006). With consolidated properties and uses, it necessary to provide alternative sustainable use of the remaining populations. Therefore, this study aims to determine similarities between the phytochemical profile, chromatographic and biological potential of leaves and bark of *M. urundeuva*, to propose the use of leaves as a more sustainable alternative to medicinal use of this species.

MATERIALS AND METHODS

Plant material and extraction procedure

M. urundeuva leaves and barks were collected in Glaucilândia (Minas Gerais, Brazil) and identified by Prof. Dr. Rubens Manoel dos Santos in Unimontes (Universidade Estadual de Montes Claros), Minas Gerais. A voucher specimen was deposited in the Unimontes herbarium under number 3534. The leaves and barks were dried naturally in the shade for 96 h, pulverized, stored in paper bags, and refrigerated at -10°C. The hydroalcoholic extracts of the leaves (HELMu) and bark (HEBMu) were prepared by maceration exhaustive method in which 100 g of powdered vegetable material were placed in 1000 ml of ethanol/water (7:3 v/v). The extracts were stored in the dark at room temperature for 7 days, with occasional shaking. The extracts were filtered, evaporated and refrigerated at -10°C. This extraction process was repeated with the residues for another three times (HELMu yield 24.3%. w/w and HEBMu yield 23.9% w/w). The solid extracts were partitioned with hexane, dichlorometane, ethyl acetate and isobutanol to yield hexane, dichlorometane, ethyl acetate and isobutanol fractions. The solvents were evaporated under reduced pressure below 40°C.

Phytochemical analysis

Plant materials were screened for the presence of alkaloids, saponins, tannins, total phenols, anthraquinones, flavonoids, cardiotonic glycosides and sterols using the methods previously described by Harborne (1999).

Chromatographic profiles

Chromatographic plates of silica gel G 60 F_{254} (TLC) were used to determine the chromatographic profiles of extracts and fractions from the leaves and barks of *M. urundeuva*. Mixtures of hexane/ethyl acetate in proportions of 8:2 to 2:8 were used as mobile phase. Plates were observed in UV light at a wavelength of 254 nm and then reveled with vanillin sulfuric solution. The retention factors (Rf) were used as a comparison measure. The

comparison between high performance liquid chromatography (HPLC) profiles was performed in waters liquid chromatography using a Phenomenex-C18 (250 × 4.6 mm, 10 µm) analytical column and a diode array detector. The partitioned extracts were solubilized at 500 µg/ml using a gradient system with water and methanol, starting with 0% of organic phase up to 100% (variation 10% every 2.0 min).

Antioxidant assay

To measure the antioxidant activity of the extracts (HELMu and HEBMu, 0.5 to 10 µg/ml) and standards (gallic acid and ethanol), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the procedure described previously (Alvarenga et al., 2013), with slight modifications. Briefly, the DPPH radical scavenging activity was measured in triplicate, in a reaction mixture containing 0.1 ml of 1 mM DPPH radical solution, 0.8 ml of ethanol 99%, and 0.1 ml of extract (in methanol). The same mixture was used for the standards. The solution was rapidly mixed, and the scavenging capacity was measured spectrophotometrically by monitoring the decrease in the absorbance at 517 nm. The antioxidant activity was expressed as IC_{50}, which was defined as the concentration of the extracts required to inhibit the formation of DPPH radicals by 50%.

Toxicity to mammalian cells

The LLC-MK2 fibroblast cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 5% of inactivated fetal calf serum, and maintained at 37°C in 5% CO_2. A cell suspension was seeded at a concentration of 1×10^6 cells/ml in a 96-well microplate containing RPMI 1640 medium. Thereafter, the cells were treated with HELMu and HEBMu at different concentrations (6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml). The plates were incubated at 37°C for 24 h, and the biological activity was evaluated by using the MTT colorimetric method [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in a microplate reader at 515 nm. RPMI 1640 medium plus dimethyl sulphoxide (DMSO) and RPMI 1640 medium were used as positive and negative controls, respectively (Twentyman and Luscombe, 1987). All the experiments were performed in triplicate. The percentage of cell viability was determined by the formula:

% cell viability = 1 - [(Y - N) / (N - P)] × 100

Where Y = absorbance of wells containing cells and HELMu or HEBMu at different concentrations; N = negative control; P = positive control.

Antimicrobial activity

Antibacterial sensitivity testing using disc diffusion method

The agar diffusion method using paper discs was performed according to procedures described by the National Committee for Clinical Laboratory Standards (NCCLS) (2003) against *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 15922) and *Shigella flexneri* (ATCC 12022). Sterile paper discs were impregnated with equal volume (50 µl) of each crude extracts (250 mg/ml). After drying, they were placed on Mueller Hinton Agar plates inoculated with suspensions of the test strains. The antibiotics chloramphenicol 30 µg and gentamicin 120 µg and solution of 5% Tween 80 in saline were used as controls. The

Table 1. TLC chromatographic profile of fractions obtained from HELMu and HEBMu.

Extracts/fractions		Retention factor							
A	Leaves	0.90	0.84	-	-	0.53	-	0.18	-
	Barks	-	-	0.71	0.63	-	0.32	-	-
B	Leaves	0.82	0.72	0.67	0.56	0.33	0.27	-	0.08
	Barks	0.82	0.72	0.67	0.56	-	0.27	0.15	0.08
C	Leaves	0.58	-	0.21	-	-	-	-	-
	Barks	-	0.26	-	-	-	-	-	-
D	Leaves	0.90	0.74	-	-	-	-	-	-
	Barks	0.90	0.74	-	-	-	-	-	-

A: hexane fraction; B: dichlorometane fraction; C: ethyl acetate faction; D: isobutanol fraction obtained from of HELMu and HEBMu

plates were incubated for 24 h at 35 ± 2°C. After this period, the inhibition zones were measured in millimeters. All procedures were performed in triplicate. Inhibition zones with diameter less than 12 mm were considered as having low antibacterial activity. Diameters between 12 and 16 mm were considered moderately active, and those with > 16 mm were considered highly active (Indu et al., 2006).

Antibacterial activity by microdilution minimum inhibitory concentration (MIC) assay methods

The antimicrobial activity of the HELMu and HEBMu was examined by the broth microdilution method against strains of oral bacteria *Streptococcus salivarius* (ATCC 7073), *Streptococcus oralis* (*Streptococcus sanguis*) (ATCC 10557), *Streptococcus mutans* (ATCC 25175) and *Lactobacillus rhamnosus* (ATCC 9595) and strains of disc diffusion test. Minimal inhibitory concentration (MIC) and minimal bactericidal concentrations (MBC) were determined according to the NCCLS (2005). All tests were performed in Mueller Hinton Broth (Valgas et al., 2007). Overnight broth cultures of each strain were prepared at a final concentration of 1.5×10^8 CFU/ml for bacteria in a 96-well microtiter plate (Oliveira et al., 2006). Extracts were investigated in the ranges 10 to 500 µg/ml to oral bacteria and 0.12 to 250 mg/ml for other microorganisms. Resazurin was added to the culture medium as a growth indicator (0.02%). Microbial growth was determined after incubation at 37°C for 24 h. The MIC is defined as the lowest concentration of the extract at which the microorganism does not demonstrate visible growth. MBC is defined as the lowest concentration of the extract at which inoculated microorganisms are completely killed. To determine the MBC, broth was taken from each well (100 µl) and inoculated in Mueller Hinton Agar for 24 h at 37°C (Palomino et al., 2002). All determinations were performed in triplicate: two positive growth controls were included (Dalirsani et al., 2011).

Preliminary analgesic activity: acetic acid-induced writhing in mice

The assay was performed as described by Koster et al. (1959) using albino mice Swiss. The nociceptive effect caused by injection of acetic acid was detected by observing abdominal writhing associated with stretching of the whole body. Animals were treated with different doses of HELMu and HEBMu (100, 300, and 500 mg/kg, *per or*), saline, or indomethacin (5 mg/kg), which was used as a reference compound. Thirty minutes after treatment, all the animals received 0.6% acetic acid i.p.; 10 min later, the number of abdominal constrictions was recorded for 20 min, by visual observation of the animals. The experiment was performed by protocol number 001/2012/CEUA according to Colégio Brasileiro de Experimentação Animal (COBEA) and approved by Comissão de Ética no Uso de Animais (CEUA) of Engineering College of UNESP/ Ilha Solteira.

Data analysis

All determinations were realized in triplicate. The results were statistically analyzed by analysis of variance (ANOVA) followed by the Dunnett or Tukey test, with significant differences being considered if $p < 0.05$. All values are presented as mean ± standard error of the mean (SEM). The level of significance used in analysis of the data was less than 0.05 ($p < 0.05$).

RESULTS

Phytochemical study

The phytochemical study not detected alkaloids or cardiotonic glycosides, but detected the presence of antraquinones in low concentrations in both HELMu and HEBMu. Flavonoids and steroids are present in moderate concentrations, while tannins and saponins are in high concentrations in both extracts.

Chromatographic profile

Were performed analysis by thin layer chromatography (TLC) and HPLC of the hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) fraction obtained from of HELMu and HEBMu. It was possible to verify by TLC (Table 1) similarities in relation to compounds mainly the majoritarian. The analysis of TLC (Table 1) and HPLC

Table 2. Antimicrobial activity of HELMu and HEBMu by diffusion disc method (inhibition zones and SD in mm).

Extract (250 mg/ml)	Inhibition zones ± SD				
	S. aureus	*E. faecalis*	*E. coli*	*P. mirabilis*	*S. flexneri*
HELMu	12.0 ± 1.00	9.3 ± 0.58	NI	9.5 ± 0.50	15.7 ± 0.58
HEBMu	NI	NI	NI	NI	NI
Chloramphenicol	21.1 ± 1.00	20.2 ± 0.30	NI	NI	NI
Gentamicin	NI	NI	26.3 ± 0.58	26.3 ± 4.0	24.0 ± 1.00

NI- no inhibition.

(Figures 1 and 2) showed good similarities of compounds; however the proportions in the fractions are different. In the fraction A of the leaves the broad peak in 0.66 min can be attributed to the volatile (essential oil) compounds that did not occur in the barks, the other peaks are present in both chromatogram but in lower proportions in the leaves. The chromatographic profile of the fractions B and D are very similar, varying only in the area of some peaks, which are higher in the fractions of the extracts from bark. However, the chromatograms of fraction C differ because peaks appear in the range of 4 to 6 min to HEBMu that are not present in HELMu.

Antioxidant assay

DPPH is a free radical compound that is widely used to test the free radical scavenging ability of various samples. Antioxidants interacting with DPPH, they can either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character (Ramirez-Mares and De Mejia, 2003). The color of the reaction changes from purple to yellow, which can be quantified by the change in the absorbance at 517 nm. The HELMu and HEBMu were capable of scavenging DPPH radicals in a concentration-dependent manner. The HELMu displayed IC_{50} = 4.8 µg/ml and HEBMu IC_{50} = 7.0 µg/ml whereas the value of IC_{50} found for gallic acid was 4.8 µg/ml. These results show that DPPH radical scavenging activity of the HELMu is comparable to the gallic acid, indicating that their compounds may contribute to neutralize the oxidant agents produced during pain and inflammatory states. The DPPH radical scavenging activity of the HEBMu, though lower than gallic acid, is very satisfactory.

Toxicity to mammalian cells

The cultures of LLC-MK2 mammalian fibroblast cells were treated with HELMu and HEBMu (separately) at concentrations of 6.2, 12.5, 25, 50, 100, 200 and 400 µg/ml for 24 h, in triplicate. The viability of the cultures was determined by establishing a relation between the absorbance values obtained in the treated and untreated

(control) groups, as shown in Figure 3. No significant cytotoxicity was observed in the concentrations evaluated after 24 h of treatment, only control 25% DMSO display significant toxicity with p < 0.05. These results showed that both extracts no present toxicity at concentration evaluated with IC_{50} > 400 µg/ml.

Antimicrobial activity

Using the diffusion method was possible to observe that HELMu showed moderate activity on *S. aureus* and *S. flexneri* with inhibition zone of 12.0 and 15.7 mm, respectively, and the HEBMu did not display activity against any of the strains tested (Table 2). Antibiotics used as control showed inhibition zones within the standards established by the NCCLS (2003). In the microdilution test (Table 3) HELMu showed for this same microorganisms MIC ≥ 62.5 and 125 mg/ml on *S. aureus* and *S. flexneri,* respectively. For HEBMu, MIC or MBC could not be determined for these microorganisms because antimicrobial activity in the agar diffusion test was not observed. In relation to oral bacteria, the HELMu presented results more significant (MIC ≤ 200 µg/ml and MBC ≤ 300 µg/ml against *S. oralis*) than HEBMu. MIC and MBC of the HEBMu were not determinate for *S. salivarius* and *S. mutans.*

Analgesic activity

Acetic acid-induced writhing in mice

HELMu showed inhibition percentage lower (42.3, 65.8 and 55.4 at doses of 100, 300 and 500 mg/kg, respectively) than HEBMu that presented significant peripheral analgesic activity at all the evaluated doses (Figure 4). The inhibition percentage of the number of writhing of acetic acid-induced writhing in mice for HEBMu was 84.0, 85.9 and 74.7% at doses of 100, 300 and 500 mg/kg, respectively. The HEBMu at doses 100 and 300 mg/kg presented significant analgesic activity with 84.0 and 85.9% (p < 0.0001) inhibition of acetic acid writhing compared to control, but the effect was lower than produced by indomethacin (99.0%). The inhibitory

Table 3. Minimal inhibitory concentration (MIC in µg/mL) and Minimum Bactericidal Concentration (MBC in µg/mL) of extracts and positive controls.

Microorganism	HELMu (µg/ml)		HEBMu (µg/ml)		Control MIC (µg/ml)
	MIC	MBC	MIC	MBC	
S. salivarius	≤ 300	≤ 400	ND	ND	0.5[Chlorh]
S. oralis	≤ 200	≤ 300	≤ 400	≤ 400	8[Chlorh]
S. mutans	≤ 400	≤ 500	ND	ND	0.5[Chlorh]
L. rhamnosus	≤ 400	≤ 400	≤ 400	≤ 400	10[Chlorh]
S. aureus	≤ 67.5	≤ 125	ND	ND	0.5[Chlorh]
E. faecalis	≤ 67.5	≤ 125	ND	ND	0.5[Chlor]
P. mirabilis	≤ 125	≤ 250	ND	ND	0.5[Gent]
S. flexneri	≤ 125	≤ 250	ND	ND	20[Gent]

ND – Not determined. The positive control: Chlorh: chlorhexidine, Gent: Gentamicin.

effect of the extract is not dose-dependent: increasing the extract dose from 300 to 500 mg/kg which reduces the inhibition percentage from 85.9 to 74.7% with HEBMu and 65.8 to 55.4 with HELMu.

DISCUSSION

Preliminary phytochemical screening and chromatographic profile showed the presence of several chemical compounds classes in the hydroalcoholic extracts suggesting great similarity in chemical composition between leaves and bark of *M. urundeuva*. Despite the similarity of the chromatographic profiles between HEBMu and HELMu, the fraction C of HEBMu showed peaks in the range of 4 to 6 min that did not appear in the fraction C of HELMu. The presence of this compounds in the HEBMu can have relation with the higher analgesic activity presented by bark. In chromatographic study of *Vernonia polifanthes*, for example, using a mobile phase containing 90% methanol was possible to observe the presence of several flavonoids such as protocatechuic acid and para-hydroxy-benzoic acid with retention times of 4.58 and 6.37 min, respectively (Pereira, 2010).

Moreover, the fraction chromatograms were very similar to the profiles obtained by Viana et al. (1995) for alcoholic and hydroalcoholic extracts of *M. urundeuva*. These presented peaks between 1 and 6 min, suggesting the presence of tannins and other phenolic compounds. The similarities as well as the chemical variations in the production of secondary metabolites have been the subject of several studies of this species (Da Silva et al., 2013). The variation in the secondary metabolites is associated at climate, water and nutrients availability, UV radiation and attack of pathogens which are factors to which the plant is daily exposed (Falkemberg et al., 2003; Gobbo-Neto and Lopes, 2007).

The phytochemical and chromatographic studies did not detect any alkaloids and cardiac glycosides but identified high concentrations of saponins, flavonoids and tannins. The presence of saponins and flavonoids can be directly related to peripheral analgesia exibited by HEBMu and HELMu (Mutalik et al., 2003; Zayachkivska et al., 2005; Owoyele et al., 2008). The classification of antinociceptive drugs is usually based on their mechanism of action; these drugs can act on either the peripheral nervous system or the central nervous system (Planas et al., 2000). The acetic acid writhing test is a standard sensitive test for both opioid and non-opioid analgesics (Steranka et al., 1987; Habib and Waheed, 2013; Wang et al., 2013). When animals are intra-peritoneally injected with acetic acid, a painful reaction and acute inflammation emerge in the peritoneal area. The stimulation of peritoneal nociceptors is indirect and occurs with the release of endogenous substances, which stimulate nervous endings (Berkenkopf and Weichman, 1988; Gyires and Torna, 1984). In this study, HEBMu inhibited significantly the number of writhing responses in mice intraperitoneally injected with acetic acid in dose 100 and 300 mg/kg, showing a significant anti-nociceptive activity. However, this writhing test alone does not substantiate the effect of HEBMu (Srinivasan et al., 2003). The peripheral analgesic activity displayed by both extracts is probably mediated via the inhibition of peripheral mediators which could be related with the antioxidant effects observed *in vitro* in the DPPH model.

The DPHH model demonstrated that HELMu and HEBMu exerted significant antioxidant activity with IC_{50} = 4.8 and 7.0 µg/ml, respectively. Similar results were found by Machado et al. (2014) for *M. urundeuva* with the same method, wherein found IC_{50} < 4.0 µg/ml for alcoholic extracts, emphasizing the strong antioxidant activity of this specie. Recent studies have shown that free radicals are responsible for mechanisms that cause inflammation and pain, and that high levels of reactive oxygen species (ROS) can induce pain. ROS are also involved in persistent pain, including neuropathic and inflammatory pain generated from many redox processes
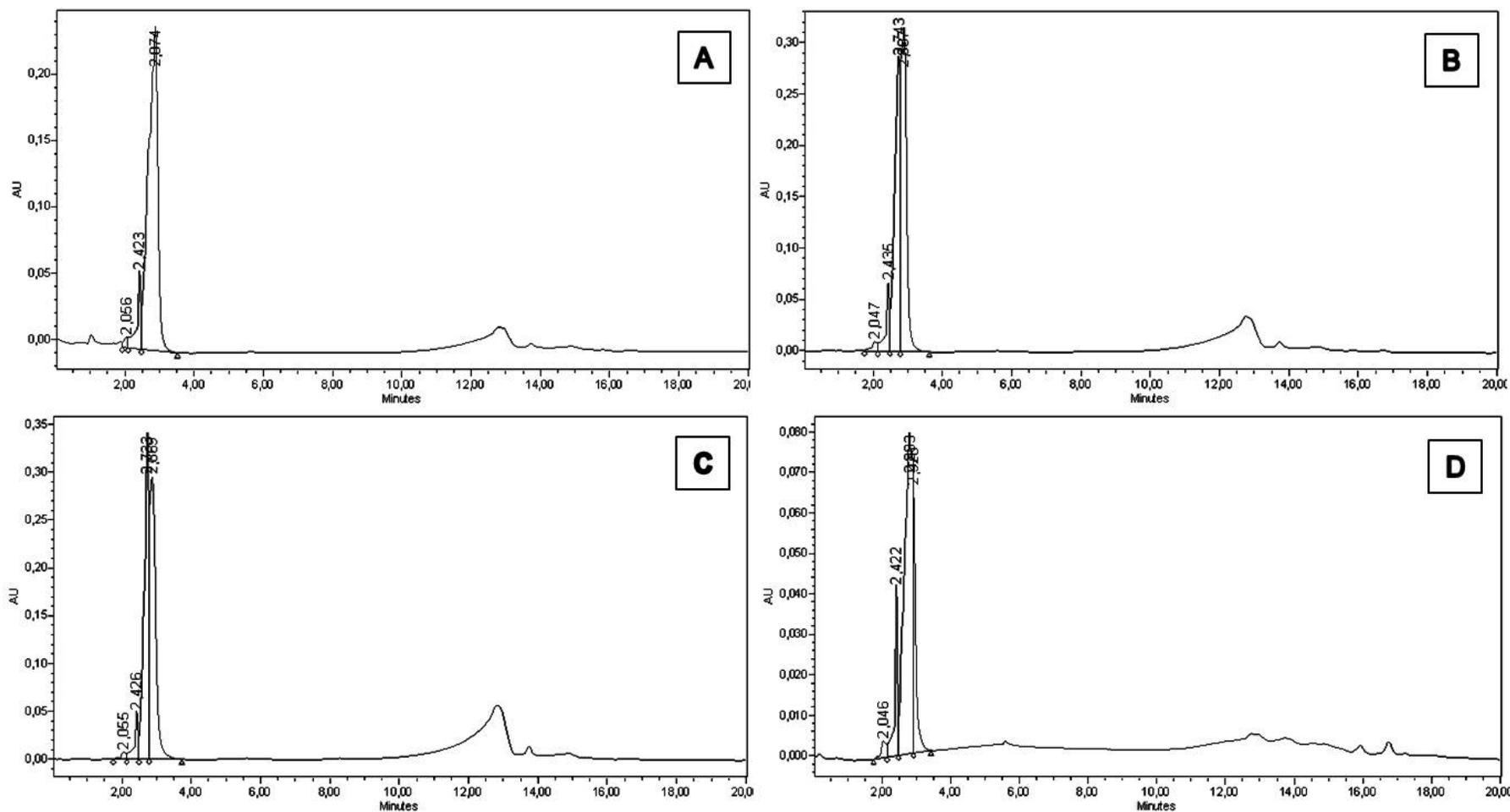
Figure 1. Chromatographic profile of the fractions of HELMu: hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) frations.

and are known to be major free radicals in the human body (Fidanboylu et al., 2011; Kumar, 2011). Antioxidants substances have the ability to scavenge these free radicals and thus prevent intracellular oxidative damage that results in pain (Valko et al., 2007). The activity antioxidant displayed by extracts can be related with presence of phenolic compounds that possess the capacity to neutralize free radicals (DPP▪) (Silva et al., 2007) and that differences in the structure or concentration of this compounds in the leaves and barks determine the difference obtained between the values of antioxidant activity of HELMu and HEBMu.

In the antimicrobial evaluation the HELMu proved be more efficient than HEBMu in disk diffusion and microdilution test. Only the HELMu inhibited growth of *S. aureus, E. faecalis, P. mirabilis*

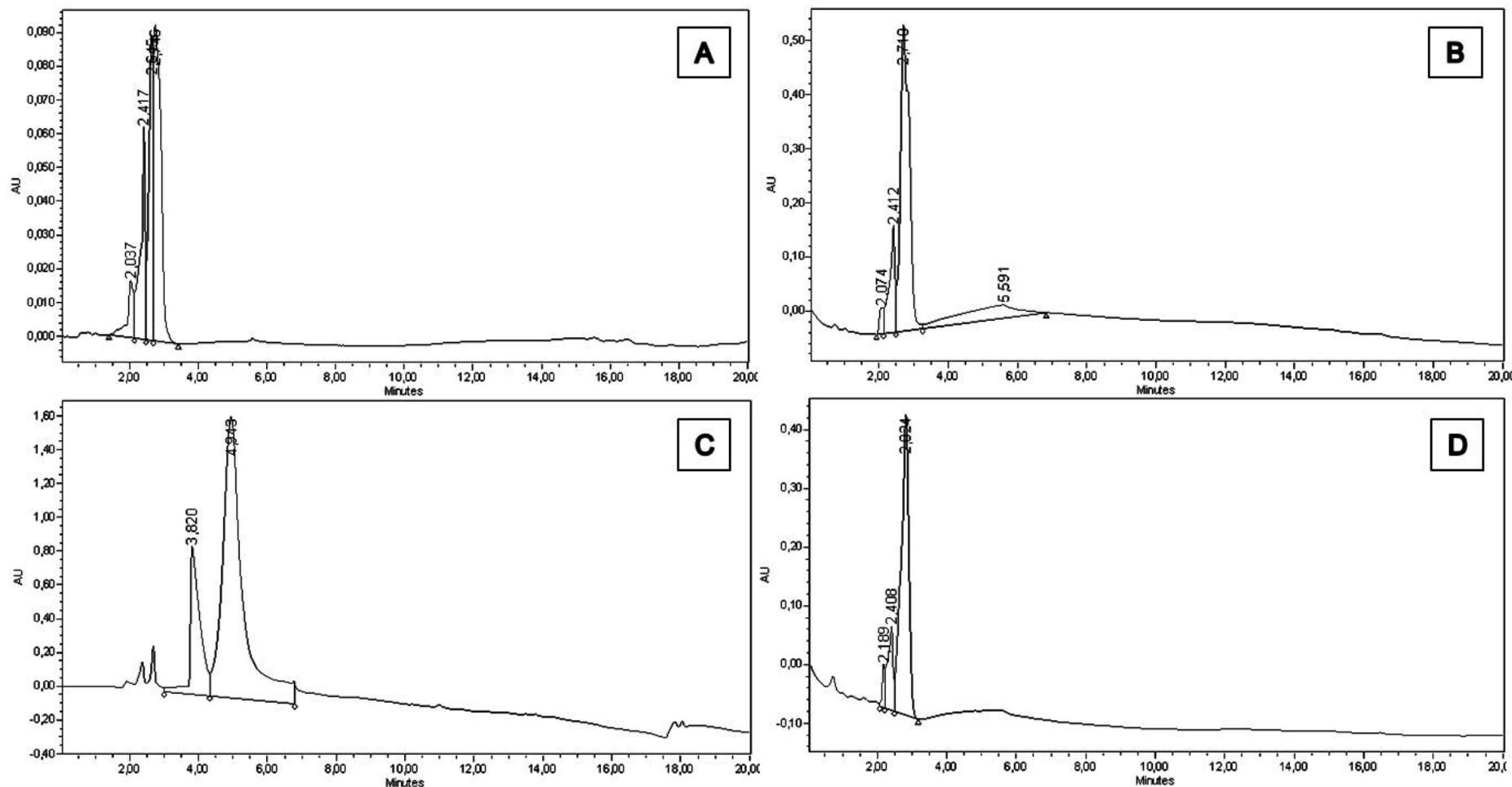**Figure 2.** Chromatographic profile of the fractions of HEBMu: hexane (A), dichlorometane (B), ethyl acetate (C) and isobutanol (D) fractions.

and *S. flexneri*. In relation to oral bacteria, the HELMu was more active with lower values of MIC and BMC (MIC ≤ 200 µg/ml and MBC ≤ 300 µg/ml against *S. oralis*) than the HEBMu that was not active against *S. salivarius* and *S. mutans*. The higher activity of HELMu can be associated with the presence of essential oils in the leaves in conjunction with saponnins and flavonoids (Petti and Scully, 2009; Orhana et al., 2010; Maatalah et al., 2012; Rapper et al., 2013

The toxicity of HELMu and HEBMu was evaluated using MTT assays on LLC-MK2 mammalian fibroblast cells. This test provides important information on the toxic effect of chemical compounds in direct contact with the cell culture. Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When
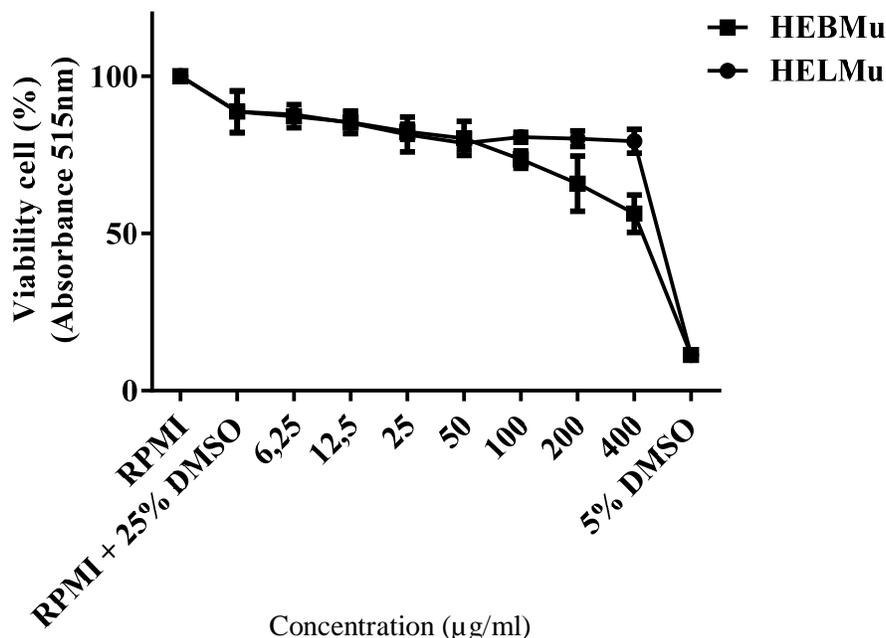
Figure 3. Effects of HELMu and HEBMu on the viability of LLC-MK2 mammalian fibroblast cells. The percentage cell viability was determined using MTT assay after 24 h treatment with the indicated concentrations.
Values are expressed by mean ± S.D. *p<0.05 vs. control indicates statistically significant differences (ANOVA followed by Tukey's Tests)
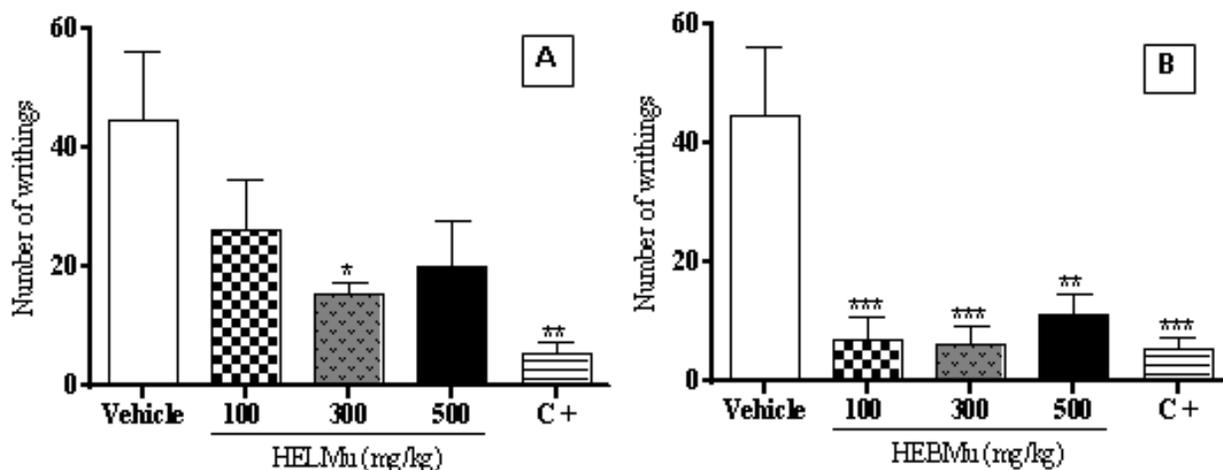

Figure 4. Effects of HELMu (A) and HEBMu (B) on the acetic acid-induced writhing assay. Vehicle (control), HELMu and HEBMu (100, 300 e 500 mg/kg), indomethacin (C+, 5mg/kg) were administered i.p. 0.5 h before acetic acid injection. Each column represents mean + SEM (n = 6). *p<0.01**p<0.001***p<0.0001 (ANOVA followed by Tukey's test).

When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall et al., 1995). It is one of the most commonly used tests for determining cytotoxicity of drugs (Riss et al., 2013). In this assay, both extracts no present toxicity at concentration evaluated with $IC_{50} > 400$ μg/ml, which is an estimated value, since the test was conducted up to 400 μg/ml showing that the actual value is greater.

In conclusion, HELMu exhibits strong antioxidant activity, moderated antimicrobial activity and moderated

nociceptive effect, and no toxicity, supporting the use of the leaves in traditional medicine instead of the bark.

Conflict of interest

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

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