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Antibacterial and Anti-Inflammatory activities of *Bunchosia armeniaca* (Cav.) DC. (Malpighiaceae)

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Abstract: *Bunchosia armeniaca* (*B. armeniaca*) is a native plant to America, and popularly called "cafezinho", "ciruela" or "falso-guaraná". In traditional medicine is used to treat different pathologies including infectious and inflammatory diseases. From ethanolic extract of *B. armeniaca* leaves (Malpighiaceae) afforded a flavonoid mixture consisting of rutin **1** (83.5%), isoquercitrin **2** (5.6%) and afzelin **5** (10.9%), which were identified and individually quantified as helpful for capillary electrophoresis and ^{1}H and ^{13}C NMR spectroscopy. The ethanolic extract showed an excellent antibacterial activity against *Staphylococcus aureus* (*S.aureus*) and moderate activity against *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*). The flavonoid mixture showed antibacterial activity, mainly against the gram negative bacteria. Moreover, this plant demonstrated significant anti-inflammatory action, inhibiting the leukocyte influx and exudate formation in pleural cavity caused by carrageenan. The inflammation mediators involved in this model study, myeloperoxidase, nitric oxide and tumor necrosis factor alpha were significantly inhibited by ethanolic extract and flavonoid mixture of *B. armeniaca*. The results show that *B. armeniaca* has a significant antibacterial and anti-inflammatory effects and that these effects is due, at least in part, to the presence of rutin, isoquercetrin and afzelin in large amounts. Hence, these compounds have potential as novel lead compounds for the future development of therapeutic interventions for the treatment of patients with infectious and inflammatory disorders.

Keywords: *B. armeniaca*; flavonoids; capillary electrophoresis; antibacterial; anti-inflammatory.

1. Introduction

The *Bunchosia* genus (*Malpighiaceae*) contains about 75 species, all of them native from America. In Brazil it is found predominately in Amazonia, Atlantic Forest and Pantanal [1]. *Bunchosia armeniaca* (*B. armeniaca*) is a native plant from the Andes, known as "cafezinho", "ciruela" or "falso-guaraná". In traditional medicine, this plant is used in endocrine, infectious, inflammatory, nutritional and metabolic disorder treatments and also some kind of cancer treatment [2].

Although the *Malpighiaceae* family has a large number of species, about 1,300 [3], only 2 % of these were studied under the chemical aspect. The most studied species of this genus are *Malpighiae marginata* and *Malpighia glabra* (acerola), which were identified as quercetin glycosides [4, 5], and

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some flavonoids which have been assigned various biological effects [6]. Other specie *Banisteropsis caapi*, known as "cipós da Amazonia" contains *β*-carboline alkaloids which exhibit hallucinogenic properties [7].

From *Byrsonima* species have been isolated some flavonoid compounds [8], mostly catechins and quercetin glycosides, although the triterpenes represent the most frequent natural substance classes [9], especially those with oleanane skeleton [10]. In *Camarea* genus has been reported free aglycone presences such as apigenin, crisoeriol, kaempferol and quercetin, as well as, 7-O-glycosides of apigenin and luteolin, 3-O-glycosides of quercetin and kaempferol [11]. However, until today it does not have scientific reports about the main constituents of *B. armeniaca*, moreover, also there is no scientific evidence about their biological proprieties, while there are reports of their use in Brazilian traditional medicine. Other species from *Malpighiaceae* family present several reports highlighting their antibacterial and anti-inflammatory effects [12, 13].

The increasing indiscriminate use of antibacterial drugs is reflected in the significant increasing of hospital bacterial resistance against traditional medicines. This fact has been led the researchers to search for new compounds or substances with antibacterial proprieties including the ones from natural products. Moreover, there is some inflammatory diseases that are still without effective treatment, such as glomerulopathies [14], vasculitis [15], rheumatoid arthritis [16], and psoriasis [17] and the searching for new medicines with the objective to increase the quality of life of affected patients remains in evidence.

Due to these facts, we decided to study the phytochemical profile of leaf extract and most of the compounds isolated from *B. armeniaca*, and also to evaluate its possible antibacterial and anti-inflammatory activities.

2. Material and Methods

2.1. Plant Material

B. armeniaca (Cav.) DC leaves were collected in February/2010 in Palhoça, Santa Catarina, Brazil (27.867°S, 48.604°W). A voucher specimen was identified by Prof. Dr. Daniel de Barcellos Falkenberg and deposited at Federal University of Santa Catarina Herbarium under the number of FLOR-41422.

2.2. General

Purchases happened as following: Muller Hinton broth and agar from Oxoid (Hampshire, UK); gentamicine from Laboratório Chile (Santiago, Chile); 2,3,5-triphenyltetrazolium chloride TTC fromVetec (São Paulo, SP, Brazil); organic solvents: acetone, chloroform, n-hexane, ethyl acetate, n-butanol, methanol, and ethanol (all analytical grade) from Synth (Diadema, SP, Brazil); sheep's blood (Newprov, Curitiba, PR, Brazil); Dimethylsulfoxide – DMSO, Carrageenan (degree IV), Evans blue dye, hidrogen peroxide, human neutrophil myeloperoxidase, vanadium (III) chloride, *o*-dianisidine •2HCl, phenol, sodium azide from Sigma–Aldrich (St. Louis, MI, USA), sodium nitroprussiate, naphthylethylendiamide dihydrochloride, sulphanilamide, phosphoric acid, dexamethasone (Ache pharmaceutical laboratories S.A., São Paulo, SP, Brazil), Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of mouse TNF-α (BD - Biosciences Pharmingen, San Diego, CA, USA; Cat. N. 559732) Other reagents used were of analytical grade and were obtained from various commercial sources.

Nuclear magnetic resonance spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a VARIAN NMR AS 400 spectrometer. Infrared (IR) spectra were recorded on ABB FTIR – FTLA2000 spectrometer. Thin layer chromatography (TLC) was performed on a pre-coated silica gel type-60 plate (Macherey-Nagel).

2.3. Vegetal material obtained

B. armeniaca (580.0 g) dried leaves were powdered and extracted with 96 % ethanol by maceration at room temperature for 7 days (three times). After filtration, the ethanol extract was further evaporated to dryness at 45 ºC under reduced pressure, yielding the crude extract, CE (14.11g / 100 g of leaves). This extract was dissolved in an ethanol solution of 20 % and after, the soluble part was partitioned by liquid-liquid extraction with solvents of increasing polarity to give hexane, ethyl acetate, buthanol, and water fractions. During the partitioning procedure with buthanol was observed a yellow precipitation formation as little grains (1.20 g).

2.4. Capillary electrophoresis analysis

The experiments were performed on an Agilent Technologies $HP^{3D}CE$ apparatus (Palo Alto, CA, USA), equipped with a diode array detector. The wavelength chosen for detection was 200 nm. Data acquisition and treatment were performed with HP Chemstation software.

Electrophoretic measurements were performed at 25 °C on an uncoated fused-silica capillary (L_{tot} 48.5 cm × L_{det} 40.0 cm × 75 μm I.D. × 375 μm O.D.) obtained from Polymicro Technologies (Phenix, USA). Prior to the first use, the capillary was conditioned with NaOH 1 mol/L and deionized water for 30 min, respectively. Daily, capillaries were conditioned with NaOH 1 mol/L, deionized water and BGE (background electrolyte, separation buffer) for 5 min, consecutively. The optimized BGE was composed of 20 mmol/L sodium tetraborate and 10% methanol, pH 9.3. Between the runs the capillary was flushed for 1.0 min with BGE. Standard solutions and samples were introduced from the inlet capillary extremity and injected hydro-dynamically at 50 mbar for 6 s (50 mbar = 4996.2 Pa). The applied separation voltage was 30 kV, positive polarity on the injection side.

The sample (12.5 mg) was prepared by dissolving 10 mL of methanol. Hydrolyzed and non-hydrolyzed samples were diluted two times with MeOH:H_2O (1:1, v/v) mixture before the injection in the capillary electrophoresis equipment.

In order to increase the analysis reliability it was performed standard additional tests on the samples. The proportion of each component in the sample was calculated from the respective peak areas shown in electropherograms, since this is proportional to the concentration. To minimize instrumental errors benzenesulfonic acid at concentration of 40 mg/L was used as internal standard.

2.5. Acid hydrolysis of the flavonoid mixtures

The yellow precipitation (FLV) was dissolved in 10 mL of HCl 2 mol/L in methanol and left under stirring and reflux for three hours. The reaction mixture was extracted with ethyl acetate and the solvent was evaporated, yielding a yellow solid.

For the capillary electrophoresis analysis, the acid hydrolysis was performed as following: 500 μL of the extract methanolic solution was added to 1.0 mL of H_2O and transferred to a test tube with cap. It was added to the sample 200 μL HCl 8 mol/L and the tube was placed in a stove at 100 °C for 1 h for hydrolysis. After cooling, it was added 2 mL H_2O and 2 mL of EtOAc for liquid-liquid extraction, twice. The organic phase was dried under a flow of N_2 and heated to 70 °C. The residue was dissolved in 200 μL of MeOH:H_2O (1:1, v/v) solution mixture for analysis.

2.6. Antibacterial assays

The microorganisms used were acquired from American Type Collection Culture (ATCC), *Staphylococcus aureus* ATCC 25923 (*S. aureus*), *Escherichia coli* ATCC 25922 (*E.coli*) and *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*). The strain identifications were confirmed by their biochemical profiles, according to the recommendation of the Clinical Microbiology Manual [18].

Antibacterial activity of the vegetal samples were evaluated by the minimum inhibitory concentration (MIC) determination [19, 20]. This test was performed in sterile 96-well micro-plates. The crude extract and flavonoid mixtures were dissolved in DMSO:H_2O (10 %, v/v) and transferred to

each micro-plate well in order to obtain a two-fold serial dilution on the original samples (crude extract 10000 μg/mL and flavonoid mixtures 1000 μg/mL). After that, each well was inoculated with 5 μL of suspension containing 10^8 CFU/mL of each tested bacteria. The antibiotics gentamicin and penicillin were included in the assays, as a positive control. The plates were incubated at 24 h at 37 ºC. Bacterial growth was performed by adding 10 μL of 2,3,5-triphenyltetrazolium chloride 5 mg/mL in sterile water, to each well. The plates were incubated again at 37 ºC for 1 h [20]. Bacterial growth in the wells was indicated by a red color, whereas clear wells indicated growth inhibition by the sample. MIC values were recorded as the lowest sample concentration showing clear wells. To crude extract, a MIC below 100 μg/mL was considered as an excellent effect, from 100 to 500 μg/mL as moderate, from 500 to 1000 μg/mL as weak, and over 1000 μg/mL as inactive. For isolated compounds a MIC below 10 μg/mL was excellent, 10 to 100 μg/mL was good, and over 100 μg/mL was inactive.

2.7. Anti-inflammatory assays

2.7.1. Animals

Swiss mice weighing 18 to 25 g were housed under standardized conditions (room maintained at 20 ± 2 °C, with alternating 12 h periods of light and dark) and were allowed free access to a standard mouse chow and water before use. The procedure was approved by the Committee for Ethics in Animal Research at University (protocol PP00757), and the experiments were performed according to Brazilian College norms of Animal Experimentation (COBEA).

2.7.2. Pleurisy Induction and analysis

The pleurisy was induced by a single intra-pleural (ipl.) injection of 0.1 mL of sterile saline (NaCl 0.95 %) plus carrageenan (Cg, 1%). Every inflammatory parameter was evaluated 4 h after pleurisy induction. After sacrificing the animals with a pentobarbital overdose (120 mg/kg, i.p.), the thorax was opened and the pleural cavity was washed with 1.0 mL of sterile phosphate buffered saline (PBS) (pH 7.6) containing NaCl (130 mmol/L), Na_2HPO_4 (5 mmol/L), and KH_2PO_4 (1 mmol/L) in distilled water containing heparin (20 IU/mL). Several pleural fluid samples were collected for further total and differential leukocytes determination, exudate concentrations, myeloperoxidase activity (MPO), nitric oxide products (NO_x), as well as tumor necrosis factor alpha levels (TNF-α).

The dose-response curves analyzed different animal groups and were treated with different crude extract (CE) (50, 100 and 200 mg/kg, i.p.) doses, and the flavonoid mixtures (0.1, 1.0 and 10 mg/kg, i.p.) 0.5 h before carrageenan administration. In these experiments, total leukocyte count and exudation levels in pleural fluid were analyzed. According to the results obtained in the experiments above, we chose CE lower doses and flavonoid mixtures that inhibited total leukocytes, as well as exudate concentrations. The CE (200 mg/kg) doses and flavonoid mixtures (1.0 mg/kg) were chosen to determine the other inflammatory parameters, such as MPO activity, NO_x and TNF-α levels. Further, the crude extract and flavonoid mixtures were only effective at inhibiting inflammatory parameters when it was administered 0.5 h before Cg injection (Data not shown). In parallel, different animal groups were treated with: 1) only with 0.1 mL of Cg (1 %, ipl.), considered the positive control group; 2) only with 0.1 mL of sterile saline or vehicle (NaCl 0.95%, ipl.), considered the negative control group; 3) treated dexamethasone (0.5 mg/kg, i.p., 0.5 h before) plus Cg (1%, ipl.), considered a reference anti-inflammatory treatment.

2.7.3. Quantification of leukocytes and exudate levels

Total and differential leukocyte counts were determined in a veterinarian automatic counter adjusted for mouse-specific parameters (MINDRAY, BC-2800 Vet, Nanshan, Shenzhen, China). All animals, except in the experiments that analyzed the MPO activity, NO_x and TNF-α levels, were previously challenged (0.5 h) with an Evans blue dye (25 mg/kg, i.v.) solution to evaluate the exudation extent into the pleural space. A fluid sample (500 μL) was collected from the pleural cavity, and the dye amount was estimated by colorimeter using an enzyme-linked immune-sorbent assay

(ELISA) plate reader (Organon Tecknica, Roseland, NJ, USA) at 620 nm by interpolation from Evans blue dye standard curve ranging from 0.01 to 50 μg/mL.

2.7.4. Quantification of myeloperoxidase activity (MPO)

In-house assays of MPO was used according to the methods developed by Rao et al. (1993) [21]. By using conventional reagents, the concentration of each enzyme was estimated in the fluid leakage of the pleural cavity by means of colorimetric measurements (absorbance of 450 nm) in an ELISA plate reader (Organon Tecknica, Roseland, NJ, USA). The results were expressed as mU/mL (MPO).

2.7.5. Quantification of nitric oxide products (NO_x)

Nitric oxide was measured as a breakdown of nitrite (NO_2^-) and nitrate (NO_3^-) products using Griess method [22]. The fluid leakage samples of the pleural cavity obtained from controls and treated animals were collected, separated, and stored at -70 ºC, and nitrate/nitrite levels were determined reducing as previously described [23]. The results were expressed in μM.

2.7.6. Quantification of tumor necrosis factor (TNF-α)

To analyze TNF-*α* levels, fluid leakage samples of pleural cavity were collected and immediately prepared for cytokine level analyses. This protocol used a commercially available kit with monoclonal specific antibodies against TNF-*α*. The cytokine levels were measured with an enzyme linked immune-sorbent assay Kit according to the manufacturer's instructions. The results were expressed in pg/mL.

2.7.7. Data analysis of anti-inflammatory tests

The data were reported as the mean ± SEM and the parameter comparisons between groups were performed by one-way variance analyses (ANOVA) followed by Dunnett's and/or Student's t-tests for post hoc analysis, as necessary. P values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Phytochemical study

The yellow precipitation (FLV) obtained during the liquid-liquid partitioning (14.7 mg/g CE), was recrystallized in methanol, showing m.p. 167.7–175.0 ºC. The Shinoda's test (HCl/Mg) showed the red color appearance, indicating flavonoid presences. The TLC analysis ($EtOAc/HCO_2/AcOH/H_2O$ 100:11:11:26) and revelation with $FeCl_3$ 5% (EtOH) showed three spots with R_f: 0.36, 0.43 and 0.64. The IR analysis showed absorption bands in 3428 cm^{-1} (O-H), 1655 cm^{-1} (C=O) 1602 cm^{-1} (C=C), 1297 cm^{-1} (C-O) and 2909 cm^{-1} (C-H).

The electropherogram of this precipitation shows three peaks and, by the standard UV-VIS comparison was possible to identify two glycoside flavonoids as rutin **1** (83.5%) and isoquercitrin **2** (5.6%), and a third flavonoid (10.95 %), that remained unknown (Figure 1a). The precipitation was submitted at acid hydrolysis and analyzed by capillary electrophoresis again in which only two peaks was obtained corresponding to quercetin **3** (87.0%) and kaempferol **4** (13.0%) (Figure 1b). From these analyses were possible to suggest that the flavonoids are of O-glycosides type [24], two of them composed by quercetin (rutin **1** and isoquercitrin **2**) and the other with kaempferol aglycone. The non-hydrolyzed sample was analyzed again adding standards to confirm the glycosyl flavonoids identity (Figure 2).

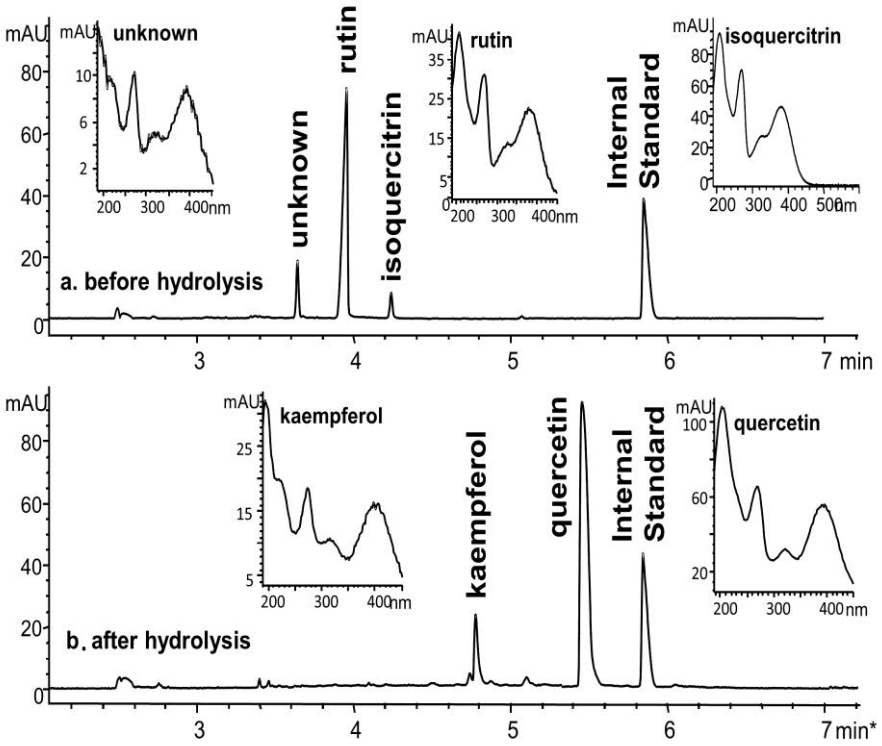

Figure 1. Flavonoid mixtures electropherograms before and after acid hydrolysis

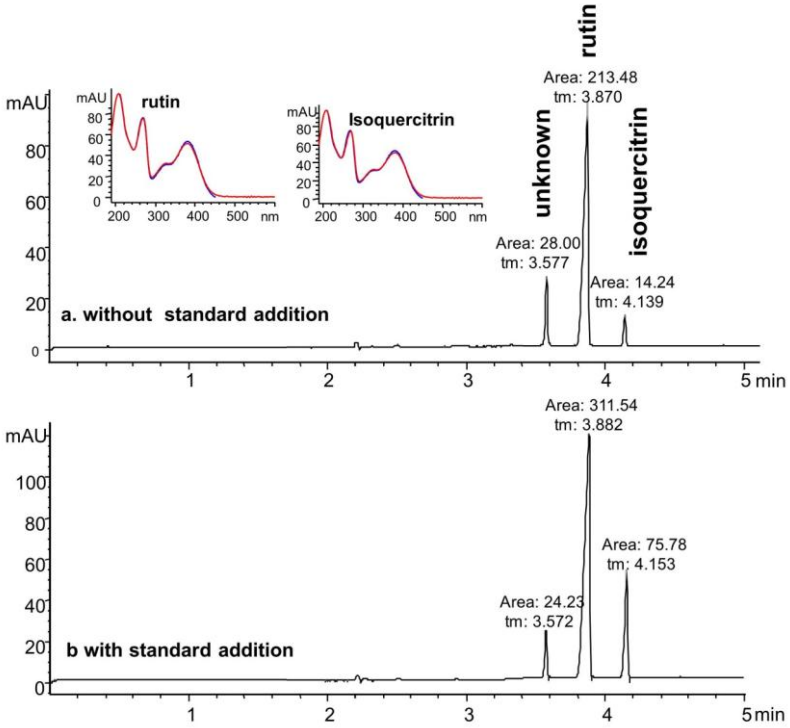

Figure 2. Flavonoid mixtures electropherograms with and without standard addition and UV-VIS spectra overlapping

The hydrolyzed precipitation was also analyzed by ^{1}H and ^{13}C NMR spectroscopy. The ^{1}H NMR spectrum showed two peak sets with different signal integrations (ratio about 1:7). The signals in the main ratio presented two doublets at δ_H 6.17 and 6.38 (J = 2.0 Hz, *meta* correlated) assigned to

H-6 and H-8, respectively, in the A ring of flavonoids. It was observed two doublets at δ_H 6.87 (J = 8.6 Hz, *ortho* correlated) and 7.73 (J = 2.1 Hz, *meta* correlated), and a doublet of doublets at δ_H 7.62 (J = 8.6 and 2.1 Hz), an ortho-meta coupling pattern corresponding to tri substituted aromatic ring. These peaks are assigned to H-5', H-2' and H-6' of B ring, respectively. These data are coherent with the flavonoid quercetin **3** [25, 26].

The minor integration signals showed two doublets at δ_H 6.12 and 6.32 (J = 1.8 Hz) assigned to H-6 and H-8, respectively, in the A ring of flavonoids and two doublets at δ_H 6.89 and 8.08 (J = 9.0 Hz), an *ortho* coupling pattern corresponding to *para* irreplaceable aromatic ring. These peaks are assigned both to H-3'/5' and H-2'/6' of B ring, respectively. These data correspond to kaempferol structure **4** [27, 28]. The ^{13}C confirmed 30 carbon presences for these two aglycones (Table 1).

After the capillary electrophoresis analysis was possible to confirm the corresponding peaks of quercetin3-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glycopyranoside or rutin **1** and quercetin 3-*O*-*β*-D-glycopyranoside or isoquercitrin **2** in the ^{1}H and ^{13}C NMR non-hydrolyzed sample spectra. Both compounds showed the same quercetin signal patterns as aglycone part. The ^{1}H spectrum of rutin **1**, the main compound, can be highlighted two anomeric hydrogen doublets at δ_H 5.10 (J = 7.6 Hz, *β*-anomer) and δ_H 4.51(J = 1.6 Hz, α-anomer). The anomeric carbons in ^{13}C NMR at δ_C 102.37 and 102.25 were assigned, respectively to glucose and rhamnose of rutinose. Additionally, can be cited the doublet at δ_H 1.11 (J = 6.2 Hz) and δ_C 17.87 assigned to rhamnose methy l group. The NMR isoquercitrin **2** spectra, the minor compound, can be cited the anomeric hydrogen doublet at δ_H 5.24 (J = 7.4 Hz, *β*-anomer), and at δ_C 102.28 assigned to glucose anomeric carbon (Table 1) [29].

Table 1. NMR spectral data of flavonoids from *Bunchosia armeniaca* before acid hydrolysis

	rutin **1**		isoquercitrin **2**		afzelin **5**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
C-2	158.39	-	*	-	159.3	-
C-3	135.59	-	*	-	135.54	-
C-4	179.34	-	*	-	179.31	-
C-5	162.87	-	*	-	*	-
C-6	99.89	6.20 (d, 2.1)	99.88	6.25 (d, 2.0)	99.95	6.15 (d, 2.0)
C-7	165.97	-	*	-	*	-
C-8	94.84	6.39 (d, 2.1)	94.79	6.44 (d, 2.0)	94.91	6.34 (d, 2.0)
C-9	158.43	-	*		158.39	-
C-10	104.64	-	104.63		105.57	-
C-1'	123.06	-	*		123.02	-
C-2'	115.99	7.66 (d, 2.1)	*	7.58 (d, 2.3)	132.36	8.05 (d, 9.0)
C-3'	145.78	-	*		116.11	6.88 (d, 9.0)
C-4'	149.76	-	149.71		161.42	-
C-5'	116.04	6.87 (d, 8.4)	*	6.82 (d, 8.6)	116.11	6.88 (d, 9.0)
C-6'	123.06	7.62 (dd, 8.4, 2.1)	*	7.57 (dd, 2.3, 8.6)	132.36	8.05 (d, 9.0)
		Glc		Glc		Rha
C-1"	102.37	5.10 (d, 7.6)	102.28	5.24 (d, 7.4)	102.32	4.46 (d, 1.6)
C-2"	75.68	*	75.63	*	71.39	*
C-3"	78.11	*	78.06	*	72.06	*
C-4"	71.34	*	69.65	*	73.86	*
C-5"	78.07	*	77.06	*	71.27	*
C-6"	68.5	3.80 (dd, 1.0, 10.7) 3.38 (dd, 6.1, 10.7)	*	*	17.83	1.06 (d, 6.2)
		Rha				
C-1'"	102.25	4.51 (d, 1.6)				
C-2'"	72.03	*				
C-3'"	72.19	*				
C-4'"	73.89	*				
C-5'"	69.68	*				
C-6'"	17.87	1.11 (d, 6.2)				

^{13}C NMR 100 MHz; ^{1}H NMR 400 MHz; methanol-D. Data: chemical shift / ppm (multiplicity – d=doublet, dd= doublet of doublets; coupling – *J* / Hz). * difficult to assign because of overlapping peaks. Glc = glucose, Rha = rhamnose.

Finally, it was possible to identify the third compound, by analyzing medium peak integrations as kaempferol 3-*O*-α-L-rhamnopyranoside or afzelin **5**. The ^{1}H NMR spectrum of this compound showed the same kaempferol signal patterns. In the sugar part can be highlighted the anomeric hydrogen at δ_H4.46 (*J* = 1.6 Hz) doublet, and the anomeric carbon at δ_C 102.32. Additionally, it can be cited the doublet at δ_H 1.06 (*J* = 6.2 Hz) and δ_C 17.83 assigned to rhamnose methyl group **(Table 1)** [30].

Therefore, from ethanolic leaf extract of *B. armeniaca* was obtained flavonoid mixtures formed to rutin **1** (83.5%), isoquercitrin **2** (5.6%) and afzelin **5** (10.9%), with a yielding of 1.47 % of leaf extract. This was the first report of afzelin presence in *Malpighiaceae* family species.

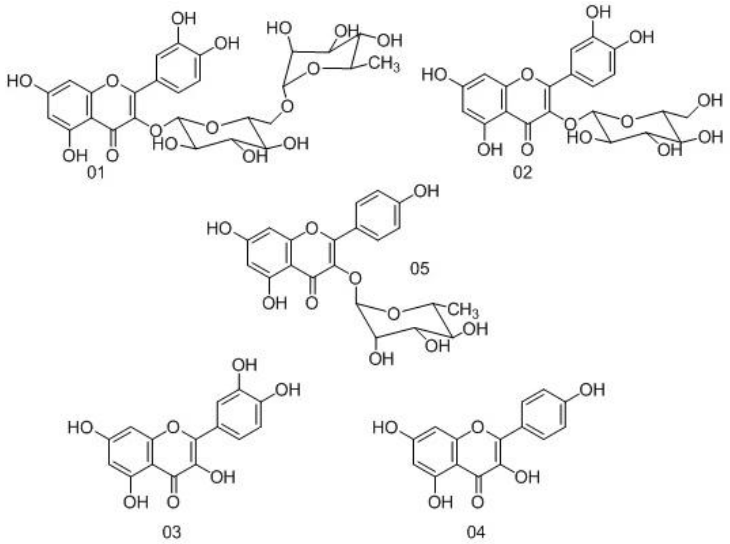

Figure 3. Flavonoids isolated from *Bunchosia armeniaca.* Compound 01: Rutin; compound 02: isoquercitrin; compound 03: quercetin; compound 04: kaempferol and compound 05: afzelin.

3.2. Antibacterial activity

Regarding the antibacterial activity, **Table 2** shows that *B. armeniaca* ethanolic extract has a moderate to excellent antibacterial activities against all microorganisms tested. The flavonoid mixtures showed an excellent effect against all tested bacteria. The ethanolic extract showed more active against *S. aureus* (MIC = 87.5 μg/mL), as expected, due to natural products to be more active against gram positive bacteria. This is based on different characteristics observed among gram positive and gram negative cell-wall, fact that limits the natural product effects against gram negative bacteria, that present outer-membrane permeability barrier which limits the compound accesses to their targets [31].

Table 2. Minimal inhibitory concentrations (MICs: μg/mL) of crude extract and flavonoid mixtures isolated from *B. armeniaca*

	S. aureus ATCC25923 (μg/mL)	*E. coli* ATCC 25922 (μg/mL)	*P. aeruginosa* ATCC 27853 (μg/mL)
Gentamicin	-	1.25	0.625
Penicillin	0.625	-	-
CE	87.5	175	350
FLV	3.0	1.5	1.5

CE = Ethanolic extract of leaves; FLV = Flavonoid mixtures. ATCC – *American type collection culture* (Data from three experiments).

Although, ethanolic extract results of *B. armeniaca* against gram negative bacteria cannot be neglected (MIC = 175 and 350 μg/mL), an unusual fact observed on natural products [32]. Some authors also reported significantly antibacterial activity of other *Malpighiaceae* family genus, such as *Mascagnia macroptera* hexane extract that had an activity against enterophatogenic bacteria [33], the methanolic and chloroformic extracts of *Byrsonima crassa* inhibit *H. pylori* growth *in vitro* [34], and ethyl acetate extract of *Byrsonima crassifolia roots* inhibit gram-positive (*S. aureus*) and gram-negative (*Salmonella typhi*) bacteria [35].

The flavonoid mixtures showed also a great antibacterial activity against all three bacteria studied, and these effects can be compared with the results obtained with the commercial antibiotics gentamicin and penicillin **(Table 2**). These results were surprising as it is not common the natural product to show excellent antibacterial activity against gram negative bacteria (*E. coli* and *P. aeruginosa*, MIC = 1.5 μg/mL). This fact, lead us to hypothesize that the effects observed in ethanolic extract studied is attributed, at least in part, to the flavonoids present in this extract. The flavonoids are natural products with recognized antibacterial activity [36], including all three identified in *B. armeniaca* mixture such as rutin [37], isoquercitrin [38] and afzelin [39].

The flavonoid antibacterial activities is mainly due to the DNA damage cased in the bacteria [40].These effects seem to be via different action mechanisms such as: *i*) complex with the bacterial cell wall and the decrease with microbial growth [41], *ii*) inhibiting DNA topoisomerase II (DNA gyrase) activity [42], and *iii*) inhibiting FtsZ protein, the tubulin bacterial analog, which mediates bacterial cell division [43].

3.3. Anti-inflammatory activity

3.3.1. Leukocyte influx and exudate

In our study *B. armeniaca* CE (200 mg/kg) significantly reduced the leukocytes influx to the pleural cavity when compared with the positive control group (Cg) (% of inhibition: 36.0±3.7) ($p<0.01$) **(Table 3)**. In another set of experiments flavonoid mixtures (FLV) also inhibited this same inflammatory parameter when administrated at 1.0 mg/kg (% of inhibition: 57.1±4.7) ($p<0.01$) dose **(Table 3).**

Table 3. Ethanolic Extract Effects and flavonoid mixtures of *B. armeniaca* upon cell migration, exudation and myeloperoxidase activity, in pleurisy mouse model induced by carrageenan.

Groups/doses (mg/kg)	Leukocytes (10^6)	Exudation (μg/mL)	Myeloperoxidase (μU/mL)
S	1.6 ± 0.3	1.7 ± 0.2	42.0±2.5
Cg	5.6 ±0.5	10.6 ± 0.6	461.0±50.2
Dex (0.5)	1.9 ±0.2**	6.7 ± 0.4	136.0±5.6**
CE (50)	5.7±1.1	9.9±0.8	425.0±10.5
CE (100)	4.8±1.5	8.9±1.2	400.0±55.6
CE (200)	3.6±0.5**	7.9±0.7**	230.0±30.5**
FLV (0.1)	5.8±1.3	10.0±0.8	440.5±86.0
FLV (1.0)	3.2±0.6**	8.4 ± 0.3*	320.0 ± 14.5**
FLV (10.0)	5.0±0.9	9.9±1.2	360.7±35.5*

S: animals treated with sterile saline (i.p.); Cg: animals treated with only carrageenan (1%, i.pl.); Dex: animals pre-treated with Dexamethasone (0.5 mg/kg, i.p.) before Cg administration; CE: animals pre-treated with *Bunchosia armeniaca* ethanolic extract (50-200 mg/kg, i.p.) before Cg administration; FLV: animals pre-treated with *B. armeniaca* flavonoid mixtures (0.1-10 mg/kg, i.p.) before Cg administration; $N = 5$ animals; * $P< 0.05$, ** $P< 0.01$.

Moreover, the crude extract also promoted a significant exudation reduction at the same dose (25.7±2.4%) ($p<0.01$) **(Table 3)**. At the same conditions the flavonoid mixtures (FLV) at 1.0 mg/kg dose administrated by intra-peritoneal route, also inhibited exudate concentrations by 21.3±3.1% ($p<0.05$) **(Table 3)**. These results showed that *B. armeniaca* have a potent anti-inflammatory action, by being able to reduce the two main inflammatory process signals (leukocyte influx and exudate increase), effects caused by carrageenan, a potent flogistic agent. The next step was to investigate, how *B. armeniaca* had these effects.

3.3.2. Myeloperoxidase activity

The inflammatory model used in our experiments (pleurisy induced by carrageenan) is characterized by a large leukocyte migration to pleural cavity, and these migration is almost all neutrophils [23].

The crude extract, CE, at the dose of 200 mg/kg reduced significantly the myeloperoxidase activity (25.7±2.4%) ($p<0.01$). At the same conditions, the flavonoid mixtures, at the dose of 1.0 mg/kg, also reduced significantly the myeloperoxidase activity in pleural cavity by 30.6±2.0% ($p<0.01$) **(Table 3)**. Therefore, the inhibitory effect upon leukocytes observed in previous experiments was associated decreasing myeloperoxidase activity. Abundantly expressed in primary neutrophils (PMNs) granules, the myeloperoxidase plays a pivotal role in inflammatory process and the increase is directly linked with PMNs activation [44]. With these results, we can concluded that the natural product studied not only reduced the leukocyte influx to pleural cavity due to inhibition of PMNs migration, as also inhibited their activation.

3.3.3. Nitrate/nitrite and TNF-α levels

The CE (200 mg/kg) and FLV (1.0 mg/kg) of *B. armeniaca* were able to decrease significantly the NO_x levels by 40.3±4.3% and 51.0±3.6% respectively ($p<0.01$) (Figure 4a). The NO is an important relaxant factor derivate from endothelium which maintains the vascular tonus and micro-vascular permeability. Moreover, it plays a pivotal function as inflammation mediator. The increase of NO levels mainly causes an increasing vascular permeability [45]. Thus, NO also plays an oxidative role in promoting edema formation mainly because of peroxynitrite formation [46].

This results suggest that the inhibition observed with animal treatments with *B. armeniaca* (CE and FLV) upon exudate could be attributed at least in part to its ability to decrease NO formation by inflammatory cells and endothelium [23].

In addition, the CE and FLV caused a significantly decrease in TNF-α levels. TNF-α inhibition in each treatment group was as following: CE (200 mg/kg) 43.5±13.0 and FLV (1.0 mg/kg) 63.5±13.5 ($p<0.05$) (Figure 4b). TNF-α is a pleiotropic cytokine and plays a key role in the innate and adaptive immune response, mainly released by resident immune cells. In the airway inflammatory diseases TNF-α plays an important role as chemo-attractant for neutrophils and increases the release of other cytokines and also, it is directly involved in T cell activations that are considered the inflammatory process manager [47].

This statement corroborates with the findings about the significant reduction observed in leukocyte migration to pleural cavity, fact that allows attributing the leukocyte influx reduction to the ability of *B. armeniaca* to inhibit TNF-α releasing by resident immune cells present in pleural fluid.

4. Conclusions

B. armeniaca ethanolic extract and flavonoid mixtures that are the main compounds present in this extract, displayed a significant antibacterial action against gram positive and negative bacteria. Moreover, this species also showed an important anti-inflammatory action by inhibiting the leukocyte influx and exudate increase and be able to reduce some inflammatory mediators, in the pleurisy murine model. These results confirm the traditional use of this plant and showed that the flavonoid mixture is the main responsible for antibacterial and anti-inflammatory effects attributed to *Bunchosia armeniaca*. However additional studies are needed, these compounds have potential as novel lead compounds for the future development of therapeutic interventions for the treatment of patients with infectious and inflammatory disorders.

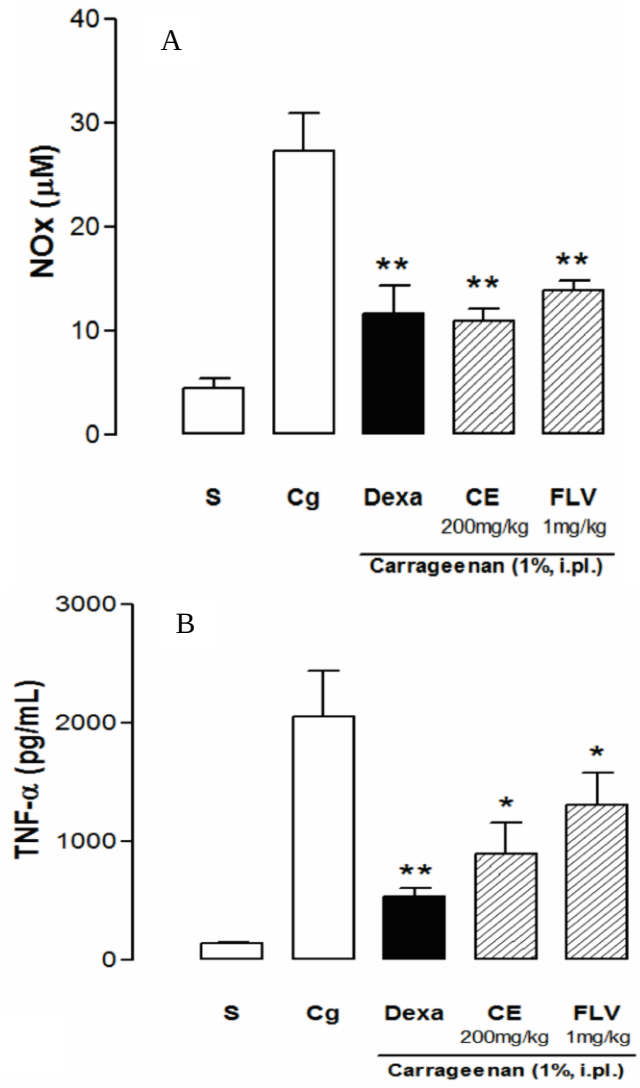

Figure 4. Ethanolic Extract Effects and flavonoid mixtures of *Bunchosia armeniaca* upon NO_x (a) and TNF-α (b) mouse pleurisy model levels induced by carrageenan.

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References

[1] A. Bonpland, A.V. Humboldt and K.S. Kunth (1821). Nova genera et species plantarum: quas in peregrinatione ad plagam æquinoctialem orbis novi collegerunt, descripserunt, partim adumbraverunt. Amat. Bonpland et Alex. de Humboldt ; ex schedis autographis Amati Bonplandi in ordinem digessit Carol. Sigismund. Kunth. v. 5., Lutetiae Parisiorum: sumtibus Librariae Graeco-Latino-Germanico.

[2] M. Giraldi and N. Hanazaki (2010). Uso e conhecimento tradicional de plantas medicinais no Sertão do Ribeirão, Florianópolis, SC, Brasil, *Acta. Bot. Bras.* **24**(2), 395-406.

[3] W.R. Anderson (2014). *Herbarium - University of Michigan - Malpighiaceae website*. [cited 2014 March, 07]; Available from: http://herbarium.lsa.umich.edu/herb/malpigh/.

[4] A. Bonpland, A.V. Humboldt and K.S. Kunth (2008). Survey of medicinal plants used in the region Northeast of Brazil, *Rev. Bras. Farmacogn.* **18**(3), 472-508.

[5] T. Hanamura, T. Hagiwara and H. Kawagishi (2005). Structural and functional characterization of polyphenols isolated from acerola (*Malpighia emarginata* DC.) fruit, *Biosci. Biotech. Bioch.* **69**(2), 280-286.

[6] M. Kawaguchi, H. Tanabe and K. Nagamine (2007). Isolation and characterization of a novel flavonoid possessing a 4,2 "-glycosidic linkage from green mature acerola (*Malpighia emarginata* DC.) fruit, *Biosci. Biotech. Bioch.* **71**(5), 1130-1135.

[7] M.C.M. Costa, M.C. Figueiredo and S.O.S. Cazenave (2005). Ayahuasca: uma abordagem toxicológica do uso ritualístico, *Rev. Psi. Clín.* **32**(6), 310-318.

[8] C. Dosseh, C, Moretti, A.M. Tessier and P. Delaveau (1980). Chemical study on the leaves of *Byrsonima verbascifolia* Rich. ex Juss, *Plan. Med. Phytother.* **14**(3), 136-142.

[9] O.R. Gottlieb, P. Henriques Mendes and M. Taveira Magalhaes (1975). Triterpenoids from Byrsonima verbascifolia, *Phytochem.* **14**(5-6), 1456-1456.

[10] F. Guilhon-Simplicio and M.D. Pereira (2011). Chemical and pharmacological aspects of *Byrsonima* (Malpighiaceae), *Quím. Nova.* **34**(6), 1032-1041.

[11] L.B. Motta, C. M. Furlan, A. Salatino and M.L.F. Salatino (2009). Flavonoids and the taxonomy of *Camarea* (Malpighiaceae), *Biochem. Syst. Ecol.* **37**(3), 201-205.

[12] F. Guilhon-Simplicio, C.C. de Souza Pinheiro, G.G. Conrado, G.S. Barbosa, P.A. dos Santos, M. M. Pereira and E.S.Lima (2012). Anti-inflammatory, anti-hyperalgesic, antiplatelet and antiulcer activities of *Byrsonima japurensis* A. Juss. (Malpighiaceae), *J. Ethnopharmacol.* **140**(2), 282-286.

[13] R.C. Santos, H. Kushima, C.M. Rodrigues, M. Sannomiyac, L.R.M. Rocha, T. M. Bauab, J. Tamashiroe, W. Vilegas and C.A. Hiruma-Lima (2012). *Byrsonima intermedia* A. Juss.: Gastric and duodenal anti-ulcer, antimicrobial and antidiarrheal effects in experimental rodent models, *J. Ethnopharmacol* **140**(2), 203-212.

[14] A.S. Bomback and G.B. Appel (2012). Pathogenesis of the C3 glomerulopathies and reclassification of MPGN, *Nat. Rev. Nephrol.* **8**(11), 634-642.

[15] M.S. Genta, R.M. Genta and C. Gabay (2006). Systemic rheumatoid vasculitis: A review, *Semin. Arthritis. Rheu.* **36**(2), 88-98.

[16] M. Meeus, S. Vervish, L.S. De Clerck, G. Moorkens, G. Hans and J. Nijs (2012). Central Sensitization in Patients with Rheumatoid Arthritis: A Systematic Literature Review, *Sem. Arthritis. Rheu.* **41**(4), 556-567.

[17] L. Puig, J.M. Carrascosa, E. Daudén, J. Sánchez-Carazo, C. Ferrándiz, M. Sánchez-regaña, M. García-Bustinduy, X. Bordas, J.C. Moreno, J.M. Hernanz, S. Laguarda and V. García-Patos (2009). Spanish Evidence-Based Guidelines on the Treatment of Moderate-to-Severe Psoriasis With Biologic Agents, *Actas. Dermosifiliogr.* **100**(5), 386-413.

[18] P.R. Murray, et al. (2003). Algorithms for identification of aerobic Gram-negative and Gram-positive bacteria, in Manual of clinical microbiology, E.J. Baron, Editor. ASM Press: Washington. p. 268 - 700 and 719 - 779.

[19] (CLSI), C.L.S.I., Performance standards for antimicrobial susceptibility testing : eighteenth informational supplement M100-S18. 2008, Wayne, PA.: Clinical and Laboratory Standards Institute.

[20] J.N. Eloff (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria, *Planta. Med.* **64**(8), 711-713.

[21] T.S. Rao, J.L. Currie, A.F. Shaffer and P.C. Isakson (1993). Comparative evaluation of arachidonic acid (AA)- and tetradecanoylphorbol acetate (TPA)-induced dermal inflammation, *Inflammation* **17**(6), 723-741.

[22] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum (1982). Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids, *Anal. Biochem.* **126**(1), 131-138.

[23] E.M. Dalmarco, G. Astolfi, C.M. de Córdova and T.S. Fröde (2012). Modulatory effect of mycophenolate mofetil on carrageenan-induced inflammation in the mouse air pouch model, *Int. Immunopharmacol* **13**(4), 476-482.

[24] K.R. Markham (1982). Techniques of flavonoid identification. *ed*: New York: Academic Press, London.

[25] M. Miyazawa and M. Hisama (2003). Antimutagenic activity of flavonoids from Chrysanthemum morifolium, *Biosci. Biotech. Biochem.* **67**(10), 2091-2099.

[26] P.K. Agrawal (1989). Carbon 13 NMR of flavonoids. Elsevier, Amsterdam

[27] T. Itoh, M. Ninomiya, M. Yasuda, K. Koshikawa, Y. Deyashiki, Y. Nozawa, Y. Akao and M. Koketsu (2009). Inhibitory effects of flavonoids isolated from *Fragaria ananassa* Duch on IgE-mediated degranulation in rat basophilic leukemia RBL-2H3, *Bioorg. Med. Chem.* **17**(15), 5374-5379.

[28] T. Okuyama, K. Hosoyama, Y. Hiraga, G. Kurono and T. Takemoto (1978). The Constituents of *Osmunda* spp. II. : A New Flavonol Glycoside of Osmunda asiatica, *Chem. Pharm. Bull.* **26**(10), 3071-3074.

[29] K. Kazuma, N. Noda and M. Suzuki (2003). Malonylated flavonol glycosides from the petals of *Clitoria ternatea*, *Phytochemistry* **62**(2), 229-237.
[30] T. Fossen, et al. (1999). Flavonoids from blue flowers of *Nymphaea caerulea*, *Phytochemistry* **51**(8), 1133-1137.
[31] J.B. Dalmarco, E.M. Dalmarco, J. Koelzer, M.G. Pizzolatti and T.S. Frode (2010). Isolation and identification of bioactive compounds responsible for the anti-bacterial efficacy of *Lotus corniculatus* var. São Gabriel, *Int. J. Green Pharm.* **4**(2), 108-114.
[32] H.A. Kirst (2013). Developing new antibacterials through natural product research, *Expert Opin. Drug. Dis.* **8**(5), 479-493.
[33] S. F. M. Salazar, A. E. Verdugo, C. C. López, E. B. Martínez, T. M. Candelas and R. E. Robles-Zepeda (2008). Activity of medicinal plants used by native populations from Sonora, Mexico, against enteropathogenic bacteria, *Pharm. Biol.* **46**(10-11), 732-737.
[34] C. Bonacorsi, M. S. G Raddi, I. Z. Carlos, M.Sannomiya and W. Vilegas (2009). Anti-Helicobacter pylori activity and immunostimulatory effect of extracts from *Byrsonima crassa* Nied. (Malpighiaceae), *BMC. Complem. Altern. M.* **9**, 1-7.
[35] M. Martinez-Vazquez, et al. (1999). Antimicrobial activity of *Byrsonima crassifolia* (L.) HBK, *J. Ethnopharmacol* **66,** 79-82.
[36] M. Daglia (2012). Polyphenols as antimicrobial agents, *Curr. Opin. Biotech.* **23**(2), 174-181.
[37] A.R. da Silva, S.M. de Morais, M.M. Marques, D.F. de Oliveira, C.C. Barros, R.R. de Almeida, I.G. Vieira and M.I. Guedes (2012). Chemical composition, antioxidant and antibacterial activities of two Spondias species from northeastern Brazil, *Pharm. Biol.* **50**(6), 740-746.
[38] J.R. Soberón, M.A. Sgariglia, D.A. Sampietro, E.N. Quiroga, M.G. Sierra and M.A. Vattuone (2010). Purification and identification of antibacterial phenolics from *Tripodanthus acutifolius* leaves, *J. Appl. Microbiol.* **108**(5), 1757-1768.
[39] L. Havyarimana, S.T. Ndendoung, J.D. Tamokou, A.T. Atchadé and J.M. Tany (2012). Chemical constituents of *Millettia barteri* and their antimicrobial and antioxidant activities, *Pharm. Biol.* **50**(2), 141-146.
[40] S. Kilani, M. B. Sghaier, I. Limem, I. Bouhlel, J. Boubaker, W. Bhouri, I. Skandrani, A. Neffatti, R. B. Ammar, M. G. Dijoux-Franca, K. Ghedira and L. Chekir-Ghedira (2008). *In vitro* evaluation of antibacterial, antioxidant, cytotoxic and apoptotic activities of the tubers infusion and extracts of *Cyperus rotundus*, *Bioresource. Technol.* **99,** 9004-9008.
[41] S. Urgaonkar , H. S. La Pierre , I. Meir, H. Lund , D. R. Chaudhuri and J. T. Shaw (2005). Synthesis of antimicrobial natural products targeting FtsZ: (+/-)-Dichamanetin and (+/-)-2 "'-hydroxy-5 "-benzylisouvarinol-B, *Org. Lett.* **7**(25), 5609-5612.
[42] T.P.T. Cushnie and A.J. Lamb (2005). Antimicrobial activity of flavonoids, *Int. J. Antimicrob. Ag.* **26**(5), 343-356.
[43] W. Vollmer (2006). The prokaryotic cytoskeleton: a putative target for inhibitors and antibiotics, *Appl. Microbiol. Biot.* **73**(1), 37-47.
[44] J. Arnhold and J. Flemmig (2010). Human myeloperoxidase in innate and acquired immunity, *Arch. Biochem. Biophys.* **500**(1), 92-106.
[45] A. Knethen, M. Soller and B. Brüne (2007). Peroxisome proliferator-activated receptor γ (PPARγ) and sepsis, *Arch. Immunol. Ther. Ex.* **55**(1), 19-25.
[46] H. Yoshida, H. Yanai, Y. Namiki, K. Fukatsu-Sasaki,N. Furutani and N. Tada (2006). Neuroprotective effects of edaravone: a novel free radical scavenger in cerebrovascular injury, *CNS. Drug. Rev.* **12**(1), 9-20.
[47] C. Brightling, M. Berry and Y. Amrani (2008). Targeting TNF-alpha: A novel therapeutic approach for asthma, *J. Allergy. Clin. Immun.* **121**(1), 5-10.