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PHENOLIC COMPOUNDS FROM *BACCHARIS PAPILLOSA SUBSP. PAPILLOSA*

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ABSTRACT

Four phenolic compounds Ermanine **1**, Isokamferine **2**, Drupanine **3** and 5,7,5',4'- tetrahydroxy- 3-methoxyflavone **4** were isolated from the dry leaves of *Baccharis papillosa subsp. papillosa*. Their structures were determinate by spectroscopic methods and compared with bibliographic data. The plant material was collected in two different seasons; winter and summer, in both cases we determined the same compounds, but with different yields increasing the quantity of compounds **2**, **3** and **4** in summer. In addition, the pure compounds were submitted to an antioxidant evaluation using two methods: ABTS and FRAP determining a potent antioxidant activity for compound **4**, a significant antioxidant activity for compound **2** and a weak activity for the compounds **1** and **3**./ *Cuatro compuestos fenólicos fueron aislados Ermanine **1**, Isokamferine **2**, Drupanine **3** and 5,7,5',4'- tetrahidroxi- 3-metoxiflavona **4** a partir de las hojas secas de Baccharis papillosa subsp. papillosa. Sus estructuras fueron determinadas por métodos espectroscópicos y comparados con datos bibliográficos. El material vegetal fue colectado en dos estaciones; invierno y verano, en ambos casos fueron determinados los mismos compuestos pero con diferentes rendimientos, incrementandose la cantidad de los compuestos **2**, **3** y **4** en verano. Además, se evaluó la actividad antioxidante de cada compuesto puro mediante dos métodos: ABTS y FRAP, determinandose una potente actividad para el compuesto **4**, una significante actividad para el compuesto **2** y una débil actividad para los compuestos **1** y **3**.*

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INTRODUCTION

Baccharis (Astereae), one of the genera of the Compositae family comprises about 500 species restricted to the American continent, from United States until Argentina and Chile [1]. Among them, around 100 species have been described in Bolivia [2].

Figure 1. *Baccharis papillosa subsp. papillosa*

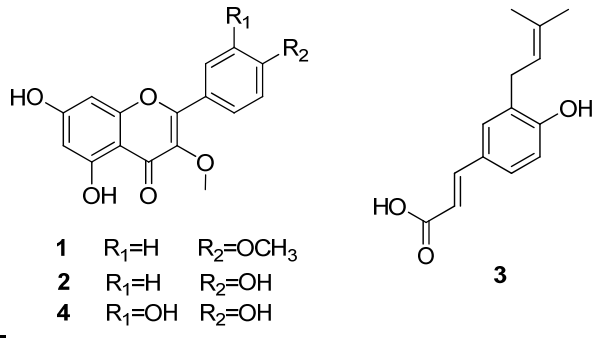

Figure 2. *Isolated compounds from B. papillosa subsp. papillosa*

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Baccharis papillosa subsp. Papillosa (Syn. *B. obtusifolia*), Fig. 1, is a shrub of 0.3 to 2 m. It is commonly named "Chua Chua", "Muyu thola", "Orko thola" and "Chilca redonda" [3]. These leaves are used popularly as tonic against stomach ailments because it shows a protective action to relieve stomach pains and also as healing, antiseptic and local anti-inflammatory [4]. The traditional use is in agreement with the anti-inflammatory *in vitro* study reported by Abad et al. [5] and with the anti-inflammatory *in vivo* studies developed by Gonzales et al. [6], who showed a significant activity for different extracts of *B. papillosa*. Chemically, several Baccharis species were studied showing mainly the presence of terpenoids and flavonoids [7][8]. In particular some compounds were isolated and elucidated from *B. papillosa*: drupanine **3,** Artepillin C, Rhamnocitrin and three ent-clerodanes [9].

A preliminary screening developed in our lab examined extracts of different polarity, determining that the ethanolic extract as the richest in phenolic compounds. In addition a preliminary antioxidant evaluation, using the ABTS [radical cation 2,2'-azino-bis(3-ethylbenzothiozoline-6-sulfonate)] and DPPH [1,1-diphenyl-2-dipicrylhydrazyl free radical] assays, suggest that the organic extract (CH_2Cl_2 extract 58.2 %I in DPPH and 98.0 in ABTS % at 2.7 mg/mL) contains scavengers of free radicals.In this paper, we report the isolation and structural elucidation of four compounds from *Baccharis papillosa* subsp. *papillosa*: Ermanine **1**, Isokamferine **2**, Drupanine **3** and 5,7,5',4'-tetrahydroxy- 3-methoxyflavone **4**. As well as the antioxidant evaluation as using the ABTS and FRAP assays for the pure compounds. In Figure 2 we see the structures of four isolated compounds.

RESULTS AND DISCUSSION

Baccharis papillosa subsp. Papillosa was collected in the surroundings of La Paz city (Bolivia) in two different seasons. The leaves were extracted with ethanol by maceration. The ethanolic extract was defatted with petroleum ether and then submitted to an acid-base process to obtain a CH_2Cl_2 extract rich in phenolic compounds. The extract rich in phenolic compounds was analyzed by diverse chromatographic techniques isolating four compounds: Ermanine **1**, Isokamferine **2**, Drupanine **3** and 5,7,5',4'- tetrahydroxy- 3-methoxyflavone **4.** The structures were determined mainly by NMR techniques, showing very similar signals for flavonoids **1**, **2**and **4** suggesting similar structures as we can see in Table 1. The ^{13}C NMR spectra for compounds **1**, **2** and **4** exhibed more than 15 signals that may suggesting a flavonoid structure with some methoxyl groups, the three spectra showed one carbonyl α, β insatured around 177 ppm which is caracteristic of flavons, by other hand 14 aromatic carbons between 98 and 164 ppm, 6 of these more deshielding belonging an oxygen; finally in the case of compound **2** and **4**, we have one methoxy group, and in the case of compound **1** we have two methoxy groups, they are in the aliphatic region with a shift between 55 and 60 ppm. Compound **1** was isolated as yellow crystals and the molecular formula $C_{17}H_{14}O_6$ was determined by MS (m/z 315.14 $[M^+H]^+$) that is in agreement with the 1D NMR data. The ^{13}C NMR spectrum (Table 1) exhibited seventeen carbons: one α,β-unsaturated carbonyl at δ 177.8, six sp^2 carbons joined to oxygen between 165 and 135 ppm, eight sp^2 carbons no substituted by oxygen or nitrogen between 130 and 93 ppm and finally two aliphatic carbons corresponding to two methoxy groups at δ 55.3 and 59.6. All the signals are in agreement with a tetra-substituted flavone. The ^1H NMR spectrum showed six aromatic protons coupling in two spin systems, the first one is an *ortho* system at δ 8.01 and 7.12 (J= 9.0 Hz) where each signal corresponds to two protons indicating a ring B *para* di-substituted. The second system showed two protons coupled in *meta* at δ 6.20 and 6.45 (J= 2.1 Hz) which indicate an A ring tetra-substituted. In addition, at δ 12.64 is present an OH proton located in C-5 because its chelated by the carbonyl group in C-4. Finally, we observed two singlets at δ 3.85 and 3.79 corresponding to the methoxy groups also determined in the ^{13}C NMR spectrum.

The methoxy group at δ 3.85 was located in C-4' due to the long range heteronuclear correlations between these protons and the carbon C-4', which also shows correlations with the protons H-2' and H-6'. The OMe group at δ 3.79 was located in C-3 because it shows a J-3 correlation with C-3 at δ137.8 which suffers a shielding effect for the carbonyl group at C-4. We can see these correlations more clear on HMBC spectrum, some of these are in Figure 3. The compounds **2** and **4** are very similar to **1**. Compound **2** has as unique difference the presence of one methoxy group instead of two, this group was located in C-3 for the same reasons discussed above for **1**. So, the difference is the presence of a hydroxyl group at C-4' which causes a shielding effect of protons H-2'/H-6' and H-3'/H-5' respect of **1**. The compound **4** showed a different spin system for the ring B. It is an *ortho*, *ortho-meta*, *meta* system at δ 7.44 *dd* (J = 8.5 and 2.3 Hz) H-2'; 6.90 *d* (J = 8.5 Hz) H-3' and 7.54 *d* (2.3 Hz) H-6', indicating a tri-substituted ring B. The 1D NMR spectra showed just one methoxy group located in C-3, for the previously discussed reasons.

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Table 1. *^{1}H and ^{13}C NMR data of compounds 1, 2 and 4*

No.	Compound 1		Compound2		Compound 4	
	^{1}H δ [ppm]	^{13}C δ [ppm]	^{1}H δ [ppm]	^{13}C δ [ppm]	^{1}H δ [ppm]	^{13}C δ [ppm]
2		155.1		155.5		155.5
3		137.8		137.5		137.5
4		177.8		177.8		177.8
5		156.3		156.3		156.2
6	6.20 *d* (2.1)	98.5	6.19 *d* (2.1)	98.5	6.19 *d* (2.1)	98.4
7		164.1		164		164
8	6.45 *d* (2.1)	93.7	6.43 *d* (2.1)	93.6	6.40 *d* (2.1)	93.5
9		161		161.1		161.1
10		104.2		104.1		104.4
1’		122.1		120.5		120.4
2’	8.01 *dd* (9.0)	129.9	7.93 *d* (8.9)	129.9	7.44 *dd* (8.5, 2.2)	120.7
3’	7.12 *dd* (9.0)	114.1	6.94 *d* (8.9)	115.5	6.90 *d* (8.5)	115.6
4’		161.2		160		148.6
5’	7.12 *dd*(9.0)	114.1	6.94 *d* (8.9)	115.5		145.1
6’	8.01 *dd*(9.0)	129.9	7.93 *d* (8.9)	129.9	7.54 *d* (2.21)	115.3
3-OMe	3.79 *s*	59.6	3.78 *s*	59.6	3.78 *s*	59.5
4'-OMe	3.85 *s*	55.3				

Solvent: DMSO-D_6 Equipment: NMR Bruker 500 MHz.

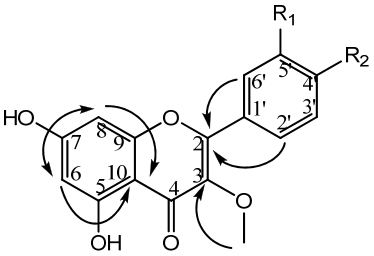

Figure 3. *Some HMBC correlations for **1**, **2** and **4**.*

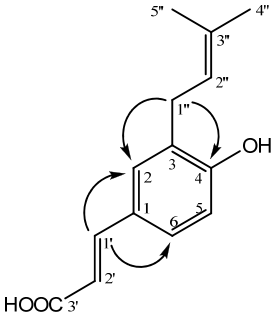

Figure 4. *Some HMBC correlations for **3**.*

Then, two hydroxyl groups were located at C-4’ and C-5’ in agreement with the C^{13} data. Finally all the 1D NMR data were compared with bibliographic data [10][11][12] confirming the proposed structures. The skeleton of compound **3** is completely different as shows Table 2, it was resolved with the ^{13}C NMR, which shows 14 signals, 5 of these belonging to quaternary carbons, 6 methynic carbons, 1 methylenic carbon and finally 2 methylic carbons. The signal most deshielding to 167.88 ppm, is an acid group and the most shielded belonging to two methyl groups between 17 y 26 ppm. In the spectrum ^{1}H NMR we identified three spin-spin systems, one of these corresponding a prenyl group, another to an olefinic group α – carbonyl and the last was an aromatic system. The total structure was building according to the HMBC spectrum which showed principally the correlations that we can see in the Figure 4. The plant was collected in two seasons, the first collection was done in July (dry season) and the second in January of the next year (wet season). The compounds **1**, **2**, **3**, and **4** were present in both seasons; however the yields were very different, as is shown in Figure 4. The difference among yield results may be due to the difference in solar radiation received in La Paz principally in January, in which the major compound **3** may be acting as protector against the high UV solar radiation, which the plant is exposed, because it has a high absorbance in the UVB (290-320) region (λ_{max} 312.0 nm), Figure 5. The 5.7-dihydroxy 3,4 '-dimethoxyflavone **1** called "Ermanin" [13] was isolated in 1976, it is a derivative of kaempferol. This compound according to inflammatory studies shows a stronger activity than indomethacin [14] it was tested on mice [15] and over some inflammatory processes showing interesting results [16].

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Table 2. *^{1}H and ^{13}C NMR data of compound 3*

No.	^{1}H δ [ppm]	^{13}C δ [ppm]
1		125.2
2	7.34 *d* (2.2)	129.6
3		128.1
4		157.3
5	6.80 *d* (7.9)	115
6	7.32 *dd*(7.9 ,2.2)	127.5
1'	7.45 *d* (15.8)	144.4
2'	6.23 *d* (15.9)	115.2
3'		167.9
1''	3.21 *d* (7.3)	27.9
2''	5.29 *dddd*(7.3,7.3,1.2,1.2)	122.4
3''		131.5
4''	1.68 *s*	25.5
5''	1.68 s	17.6

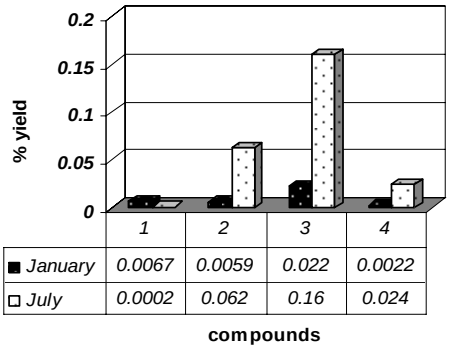

Figure 4. *Yield of each pure isolated compound*

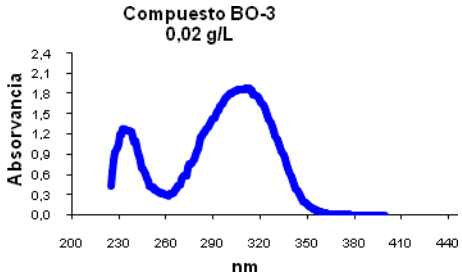

Figure 5. *UV spectrum of compound 3*

In the National Cancer Institute in America found also antineoplastic activity in various tumor systems, like: adenocarcinoma 75, sarcoma180, leukemia L-1210 [17] and leukemia HL-60 [18]. Other essays showed strong antitumor inhibition against Epstein-Barr virus and cytomegalovirus or CMV [19]. Were also performed cytotoxicity studies in human cells HT-1080 fibrosarcoma and murine colon 26-L5 carcinoma cells [20]. By DPPH method was determined this low antioxidant activity [21], while other studies showed strong hepatoprotective activity [22]. The Isokaempferide (4',5,7-trihydroxy-3-methoxyflavone) **2** is a derivative of kaempferol, popularly used in Brazil as an bronchodilator [23]. This compound inhibits tumor cell growth more potent than kaempferol [24]. Recently was determined as a anti-inflammatory [25]. Moreover, **2** shows lethal action against epimastigotes of Trypanosomacruzi, which is an agent of Chagas disease [26]. It showed a significant hepatoprotective effect [27]. The 5, 7, 5', 4' tetrahydroxyde 3-methoxyflavone **4** is a derivative of quercetin, it shows mainly an anti-inflammatory activity as indometacin [28]. It acts inhibiting the overproduction of NO; peroxynitrite trapping [29][30] and tumor necrosis factor-α (TNF-α) [31], now a day it is applied in Alzheimer's disease because shows an antiamnesic activity [32]. It has a lower antioxidant activity of quercetin, but higher than Luteolin, this is due to the presence of OH and an H in the position of the C-3 [33][34]. Several biological activities were reported for 3-prenyl-4-hydroxycinnamic acid **3**, (also known as drupanine) [35]. Drupanine inhibited cell growth in human tumor cells, according to studies conducted in Japan by Yukihiro Akao, presents an inhibitory effect of 50% in 72 hours against colon cancer, stomach cancer and cancer of the blood [36], also this compound shows an inhibitory effect on the growth of prostate cancer tumors [37][38]. On the other hand **3** showed antifungal activity against *Microsporum canis, Epidermophyton floccosum, Trichophyton rubrum and Trichophyton mentaraphytes* [39], which are part of a group of fungi called

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dermatophytes responsible for dermatophytosis. In addition it resulted active against *Candida albicans* fungus that causes vaginal and intestinal disorders [40]. In 2007 was determined a weak activity against *Aspergillus niger* which in high concentrations can cause pulmonary abnormalities [41]. This molecule is also reported as a good antioxidant [42][43], based on tests with DPPH and ABTS [44][45][46] made in Brazil. It presents some studies of structure/antioxidant activity, where it is compared with its derivatives come to see the importance of replacing a hydroxyl group at C-4, because when this replaced by a H or Me, the activity decreases notoriously [47]. In addition to the reported data, our research group have done the antioxidant evaluation of the pure compound using two common methods FRAP and ABTS, the results are shown in the follow tables and are in agreement with the previously reported data.

Tabla 4. *Total Antioxidant Capacity TAC, measured by the methods FRAP and ABTS.*

Compound	TAC by FRAP µmol/l	TAC by ABTS µmol/l
1	0.23 +/- 0.002	0.12 +/- 0.001
2	0.08 +/- 0.005	0.08 +/- 0.002
3	0.70 +/- 0.001	0.39 +/- 0.002
4	1.71 +/- 0.003	1.05 +/- 0.003

Control compound: Trolox TAC µmol/l =1

EXPERIMENTAL SECTION

All organic solvents were of distilled-inglass grade (Laboratorio de Bioorganica-IIQ-UMSA – La Paz – Bolivia). UV spectra were obtained in MeOH on a HELIUS ALFA 254 – 365 nm instrument. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance 500 at 500 and 120 MHz, respectively, and chemical shifts are shown in δ(ppm) with TMS as an internal reference. EIMS and were recorded on a MICROMASS Q-TOF MICRO spectrometer. The melting points were recorded on a Digital FISATOM 430 D. Silica gel 60 (15-40 μm) for column chromatography, were purchased from Macherey-Nagel, and Sephadex LH-20 was obtained from Pharmacia Biotech.

Plant material

The leaves of *Baccharis papillosa subsp. Papillosa* were colected twice. The first time was in July 2006 and the second in January 2007; in both cases was colected at Cota Cota (3800 m.s.n.m.) wich is an area belonging to La Paz, Bolivia; it was identify by Lic. Esther Valenzuela, a botanist of the Bolivian National Herbarium where we can find a model of the specimen.

Extraction and isolation

The dry leaves were first extracted with ethanol 96 % for 15 min, these features were previusly as the optimum for our objetives in the Laboratory of Natural Products from the UMSA. Then these dry ethanolic extract was extracted with methanol and petroleum ether getting two phases; we chose the methanolic phase and over this we made another more selective extraction with a mixture (CH_2Cl_2:MeOH 80:20), finally we got the medium polarity extract. According our objetives we made an acid bases extraction, firstly with KOH on the medium polarity extract, getting two phases; on the acuos phase we added HCl getting a precipited, that we called as Phenolic of Medium Polarity Extract (PMPE). After these extraction we made the fraccionamient inicialy in a VLC column, and finally to get the puris compounds we have used sephadex.

Measurement of TAC

Total antioxidant capacity (TAC) was measured by ABTS and FRAP methods by use of spectrophotometry performed using an Ultrospec 3000 (Pharmacia Biotech, Lund, Sweden) at 25°C. As a standard Trolox was used, a water-soluble analogue of alpha-tocopherol.

Method ABTS

To oxidize the colorless ABTS to the blue-green $ABTS^{\cdot+}$by the addition of potassium persulphate (2.42 mmol/l) and kept for 12-16 hours at room temperature in the dark. This reagent was stable for 2-3 days stored in the dark. On the day of analysis the $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. After the addition of 1 ml of $ABTS^{\cdot+}$ solution to 100 µl of sample the mixture was stirred for 30 seconds and the absorbance

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reading was started after another 30 seconds and finished after six minutes. The readings were performed at 734 nm and 25°C. The percent inhibition ofthe sample was then compared with a standard curve made from the corresponding reading of Trolox (20-200 µmol/l). The data were expressed as µmol Trolox per litre of sample.

Method FRAP

The yellow Fe^{+3}-TPTZ complex is reduced to the blue Fe^{+2}-TPTZ complex by electron donating substances under acidic conditions. Any electron donating substance with a half reaction of lower redox potential than Fe^{+3}/Fe^{+2}TPTZ will lead the formation of the blue complex. The FRAP reagent was a mixture of 0.1 mol/L sodium acetate buffer (pH 3.6), 10 mmol/L TPTZ and 20 mmol/L ferric chloride (10:1:1 v/v/v). To 900 µl of reagent 90 µl of water and 30 µl of sample were added. The absorbance reading were performed at 593 nm for 10 min. The blank consisted of 120 µL of water and 900 µl of reagent. The final absorbance of each sample was compared with the standard curve made using Trolox (100-1000 µmol/L). The data were expressed as µmol Trolox per litre of sample.

Compound 1 (5,7-dihydroxy 3,4'-dimethoxyflavone)

Yellow cristals of mp. 218-221 ºC; $C_{17}H_{14}O_6$ (MW 314.29 g/mol); UV (c. 0.02 g/L. in MeOH); λ_{max} 219.0 (absorbance 1.85); λ_{max} 267.0 (absorbance 1.75); λ_{max} 344.0 (absorbance 1.49), ^{1}H NMR (500 MHz, in DMSO-D_6), Table 1; ^{13}C NMR (500 MHz in DMSO-D_6), Table 1; ESI-HMRS m/z 315,0800. $[M+H]^+$ (calculated for $C_{17}H_{15}O_6$, 315.2800).

Compound 2 (4',5,7-trihydroxy-3-methoxyflavone)

Yellow cristals, mp. 293 – 294 ºC; $C_{16}H_{12}O_6$ (MW 300.3 g/mol); UV (c. 0.02 g/L. in MeOH); λ_{max} 219.0 (absorbance 1.42); λ_{max} 267.0 (absorbance 1.25); λ_{max} 347.0 (absorbance 1.08), ^{1}H NMR (500 MHz, in DMSO-D_6), Table 1; ^{13}C NMR (500 MHz in DMSO-D_6), Table 1; ESI-HMRS m/z 301.0634. $[M+H]^+$ (calculated for $C_{16}H_{13}O_6$, 301.2629).

Compound 3 (4-hydroxy, 3-prenylcinámic acid)

White crystals, mp.149 – 151 ºC; $C_{14}H_{16}O_3$ (MW 232.3 g/mol); UV (c. 0.02 g/L. en MeOH); λ_{max} 236.0 (absorbance 1.26); λ_{max} 312.0 (absorbance 1.88); ^{1}H NMR (500 MHz, in DMSO-D_6), Table 2; ^{13}C NMR (500 MHz in DMSO-D_6), Table 2; ESI-HMRS m/z 233.1099 $[M+H]^+$ (calculated for $C_{14}H_{17}O_3$, 233.2750).

Compound 4 (5,7,3',4'- tetrahydroxy-3- methoxyflavone)

yellow crystals, mp. 263 – 267 ºC; $C_{16}H_{12}O_7$ (MW 316.2g/mol); UV (c. 0.02 g/L. en MeOH); λ_{max} 228.0 (absorbance 2.15); λ_{max} 258.0 (absorbance 2.32); λ_{max} 356.0 (absorbance 2.12), ^{1}H NMR (500 MHz, in DMSO-D_6), Table 1; ^{13}C NMR (500 MHz in DMSO-D_6), Table 1; ESI-HMRS m/z 317.0583 $[M+H]^+$ (calculated for $C_{16}H_{13}O_7$, 317.2623)

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