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Identification and stability of a new bichalcone in *Achyrocline satureioides* spray dried powder

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A new chemical structure, the 4,2′,4″,2‴-tetrahydroxy-6′,6‴-dimethoxy-4′-*O*-4‴- bichalcone, named achyrobichalcone was isolated and identified from an *Achyrocline satureioides* spray-dried powder (SDP80). The thermal and photo stability of this new compound as well as that of the main polyphenols present in the spray dried powder, quercetin, luteolin, 3-*O*-metylquercetin and the corresponding kinetics of degradation are reported. In the long term testing ($30 \pm 2\,^{\circ}C/75 \pm 5\,\%$ RH, 12 months), the total polyphenols contained in SDP80 demonstrated to be stable, remaining higher than 90 % after a 12 month exposure. The photo stability testing revealed that all polyphenols were stable for 48 h when SDP80 was conditioned in amber or transparent flasks and exposed to UV-C radiation (light express LE UV, 254 nm, 30 W). In contrast, when unprotected, the polyphenols demonstrated to be sensitive to both, thermal stress testing ($80 \pm 2\,^{\circ}C$), for 14 days and to UV-C radiation. Luteolin showed to be the most stable against UVC light and 3-O-methylquercetin against temperature. The achyrobichalcone demonstrated to be the more unstable against both, temperature and light. The kinetics of polyphenol thermal degradation ($80 \pm 2\,^{\circ}C$, 49 days) and photodegradation (UV-C radiation, 96 h) followed, 2nd and 1st order reaction, respectively.

1. Introduction

Achyrocline satureioides (Lam.) DC., Asteraceae, a plant known as "marcela", is widely used in Brazilian folk medicine against inflammatory and gastrointestinal disorders. The flavonoids quercetin, luteolin and 3-*O*-methylquercetin have been reported as the main constituents of ethanol extracts from its inflorescences. Pharmacological activities like antioxidant, pro-oxidant, cytotoxic, anti-inflammatory, antispasmodic, analgesic, sedative, antiviral and immunomodulatory effects (Simões et al. 1988; Polydoro et al. 2004; Bettega et al. 2004; De Souza et al. 2007; Cosentino et al. 2008) have been reported for these compounds.

The interest in herbal spray-dried powders as an intermediary product for pharmaceutical dosage forms have been increasing in the field of phytopharmaceutical technology, mainly, due to its excellent stability, easy of handling and accurate dosage (Lemos-Senna et al. 1997). The relevant use of *Achyrocline satureioides* as a herbal medicine has motivated the study of spray-dried powders from this plant (Bassani et al. 2001) including the influence of various parameters as adjuvant, temperature and ethanol concentration of the extractive solution on the corresponding chemical composition on the biological activity (De Souza et al. 2007).

More recently, Holzschuh et al. (2007) reported the stability of a spray dried powder prepared from an ethanol 40 % (v/v) extractive solution (SDP40). The study revealed an anomalous behavior of quercetin, which improved its concentration after, 1 month at 50 °C or 2 days at 80 °C exposition, followed by its concentration decrease. The hypothesis that this phenomen is related to the simultaneous decreasing of a non-identified peak P3 present in the powder or to the hydrolysis of a non-identified precursor of quercetin like a quercetin heteroside was formulated. On the other hand, a spray dried powder prepared with ethanol 80 % (v/v) revealed a higher concentration of the main flavonoids than in ethanol 40 % (v/v) (De Souza et al. 2007) what motivated to prepare the corresponding spray dried powder (SDP80).

In this context, the present work was designed to investigate the thermal and photo stability of the polyphenols quercetin, luteolin, 3-*O*-methylquercetin and substance P8 present in the *A. satureioides* spray dried powder prepared from ethanol 80 % (v/v) extractive solution of the inflorescences (SDP80). Also, determination of the degradation kinetics of these constituents, represents, as far we know, the first report on the kinetics of degradation of chemical constituents present in a herbal spray-dried powder. Finally, the elucidation of the chemical structure of the substance P8 is reported analyzing its similarity to the substance P3 present in the SDP40.

2. Investigations, results and discussion

2.1. Thermal and photo stability tests

The long term thermal tests were performed according to the Brazilian official requirements for drugs (Brasil 2005), $30 \pm 2\,^{\circ}C$ temperature and $75 \pm 5\,\%$ relative humidity, during 12 months. Samples of 1 g of SDP80 were stored in transparent flasks and analyzed at 0, 3, 6, 9 and 12 months (3 samples each time).

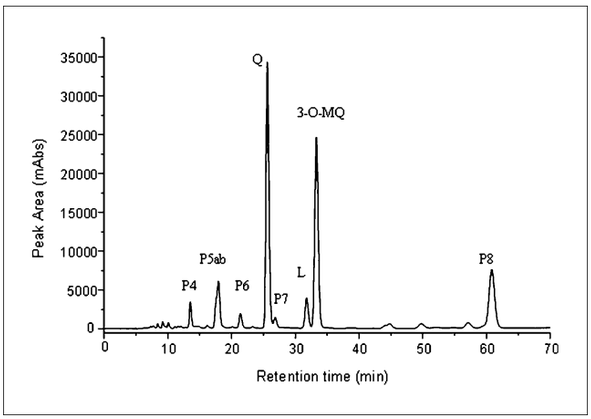

Fig. 1: Fingerprint LC chromatogram of the *Achyrocline satureioides* spray dried powder (SDP80) prepared from ethanol 80 % (v/v) extractive solution of the inflorescences. Q = quercetin, L = luteolin, 3-*O*-MQ = 3-*O*-methylquercetin, P8 = achyrobichalcone and not identified peaks P4, P5ab, P6, P7

In the final of the tests (12 months), the sensorial characteristics of SDP80 did not change its original properties, yellow fine powder and the peculiar smell. The loss on drying of the samples, measured before the polyphenol assay, was in the range of 2.12 % and 2.32 %, denoting that no significant influence was provoked by the presence of humidity.

Considering that polyphenols play an important role in the anti-inflammatory (Simões et al. 1988; De Souza et al. 2007; Cosentino et al. 2008) and antioxidant (Polydoro et al. 2004) activities of *Achyrocline satureioides*, these constituents were selected for evaluating the stability of SDP80.

The LC profile of SDP80 at time zero is presented in Fig. 1. The presence of the three major polyphenols quercetin, 18.46 μg/mg (Rt = 25.37), 3-*O*-methylquercetin, 42.52 μg/mg (Rt = 32.95) and substance P8, 8.38 μg/mg (Rt = 59.76) is observed. Luteolin, 2.80 μg/mg (Rt = 31.41) and the non-identified peaks P4 1.94 μg/mg (Rt = 13.42), P5ab 4.82 μg/mg (Rt = 17.80), P6 1.68 μg/mg (Rt = 21.22) and P7 1.45 μg/mg (Rt = 26.64) are also present, although in lower concentrations.

The long term thermal testing revealed that the total polyphenol concentration remained in an acceptable range until 12 months (Fig. 2) being within ± 10 % of the initial content (Hefendehl 1987; EMEA 2001; Lachman 2001). The stability of the main polyphenols, individually, corroborated this result. Quercetin, luteolin and 3-*O*-methylquercetin, presented good stability, remaining after 12 months at 94 %, 99 % and 97 % of their initial concentration when stored in transparent flasks. Substances P4, P6 and P7 showed slightly increased concentrations. After 12 months their concentrations were 111 %, 112 % and 108 % respectively. In contrast, substances P5ab and P8 were unstable, showing at the end of test concentrations of 78 % and 64 %, respectively (Fig. 2).

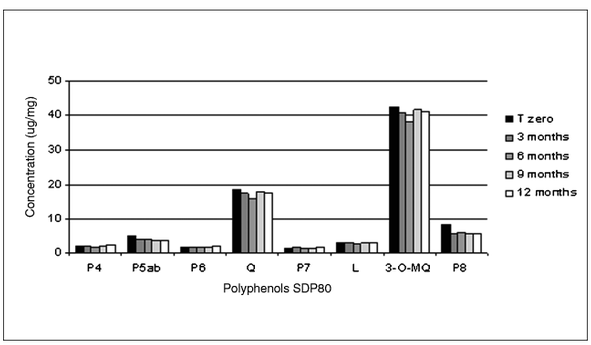

Fig. 2: Concentrations of the individual polyphenols present in SDP80 in long term testing (30 ± 2 °C/75 ± 5 % RH). Q = quercetin, L = luteolin, 3-O-MQ = 3-O-methylquercetin, P8 = achyrobichalcone and substances P4, P5ab, P6, P7

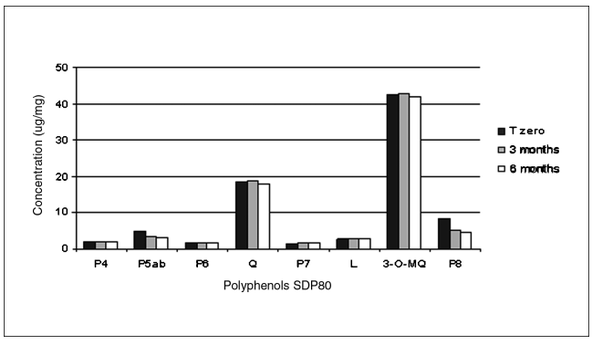

Fig. 3: Polyphenol concentration in SDP80 under accelerated conditions (40 ± 2 °C/75 ± 5 % RH). Q = quercetin, L = luteolin, 3-*O*-MQ = 3-*O*-methylquercetin, P8 = achyrobichalcone and substances P4, P5ab, P6, P7

The accelerated term testing was also carried out according to the Brazilian official requirements for drugs (Brasil 2005). Temperature of 40 ± 2 °C and 75 ± 5 % relative humidity were the conditions employed during the 6 months test. Samples of SDP80 (1 g) were stored in transparent flask containers and analyzed at 0, 3 and 6 month. No significant changes in the sensorial properties of SDP80 were observed during the test. The residual humidity was within the range of 2.12 % and 2.69 % for the samples maintained in transparent flask containers. The variation of the polyphenol concentration during the period of 6 months is shown in Fig. 3.

The accelerated term test demonstrated that the flavonoids quercetin, luteolin and 3-*O*-methylquercetin were stable under these conditions. Their concentrations at the end of the experiment were 99 %, 100 % and 99 %, from the initial value, respectively. Moreover, substances P4, P6 and P7 maintained their concentrations with 101 %, 105 % and 106 %, from initial value. However, the substances P8 and P5ab demonstrated instability under these conditions, decreasing, to 57 % and 62 %, from the initial value, respectively.

A temperature of 80 °C was chosen to evaluate the changes in the polyphenol constituents present in SDP80 under stress conditions. Samples of SDP80 (1 g) were stored in transparent tight flasks or in an unprotected open-dish. The residual humidity of SDP80 after 14 days of exposure was within the range of 2.12 % and 0.18 % for samples which were unprotected in open-dishes. When stored in transparent flasks, SDP80 did not change its sensorial characteristics of yellow fine powder and the peculiar smell of the inflorescences during 14 days. However, when stored in unprotected open-dishes, after 7 days, an intensified

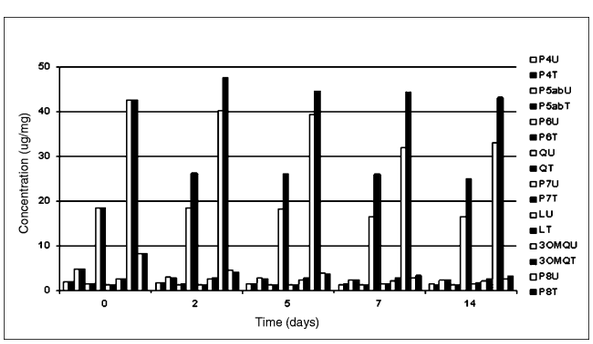

Fig. 4: Polyphenol concentration in SDP80 under stress conditions (80 ± 2 °C/14 days). Q = quercetin, L = luteolin, 3-*O*MQ = 3-*O*-methylquercetin, P8 = achyrobichalcone and substances P4, P5ab, P6, P7. U = unprotected and T = Transparent flask

smell was perceived suggesting chemical changes in the product. Polyphenol concentrations in the samples, after 14 days of exposure (80 °C) are shown in Fig. 4. The flavonoids luteolin and 3-*O*-methylquercetin demonstrated good stability, showing 100 % and 101 %, of the initial concentration after 14 days, when conditioned in transparent flasks. When kept in open-dishes, their concentrations decreased to 81 % and 77 %, respectively. While substances P4 and P6 showed an intermediary stability (76 %, and 84 %), P5ab and P8 revealed the poorest stability when conditioned in transparent flasks. At the end of the experiment their concentrations were 53 % and 39 % of the initial concentration, respectively.

Quercetin and substance P7 presented an anomalous behavior; their concentration increased to 134 % and 126 %, respectively, for samples conditioned in transparent flasks, after 14 days of experiment (80 °C). A similar behavior was first observed for an *Achyrocline satureioides* spray dried powder prepared from an extractive solution 40 % ethanol (SDP40) (Holzschuh et al. 2007) and the simultaneous decrease of the P3 substance (corresponding to P8 in SDP80) was thought as a possible explanation.

In the photo stability tests, the samples were submitted to UV-C radiation (254 nm, 30 W) in order to determine the stability of the polyphenol present in SDP80, they were also conditioned in amber flasks, transparent flasks and open-dishes. After a 48 h exposure, stored in amber or transparent flask, SDP80 maintained its sensorial characteristics of yellow fine powder and peculiar smell. When the samples were conditioned in unprotected open-dishes, after 48 h, the color was a very light yellow and the intensified smell suggested chemical degradation.

The loss on drying of the samples, measured at time zero of the stability test was 4.17 % (w/w). After 48 h exposure, the loss on drying, measured immediately before the polyphenol assay, was 4.41 % for the samples conditioned in amber flasks; 4.29 %, for the samples conditioned in transparent flasks and 6.49 % in open-dishes; denoting that no significant difference existed between amber and transparent flasks. In contrast, the samples conditioned in open-dishes showed a significant increase of the humidity content after 48 h exposure (4.17 % to 6.49 %).

The polyphenols in SDP80, when the samples were conditioned in amber or transparent flasks, were chemically stable during the 48 h of light exposure (Fig. 5). However, when SDP80 was stored in open-dishes the polyphenol concentration showed a significant decrease, especially of quercetin, 3-*O*-methylquercetin, P5ab and substance P8 (Fig. 5). Luteolin showed the highest stability against UV-C light radiation, as previously reported by Holzschuh et al. (2007).

Taken together, SDP80 demonstrated sensibility against UV-C light radiation when it was conditioned in open-dishes. Meanwhile, amber and transparent flasks demonstrated efficiency to protect SDP80 during 48 h of UV-C light exposure. This suggests that the presence of humidity and/or air is involved in the degradation.

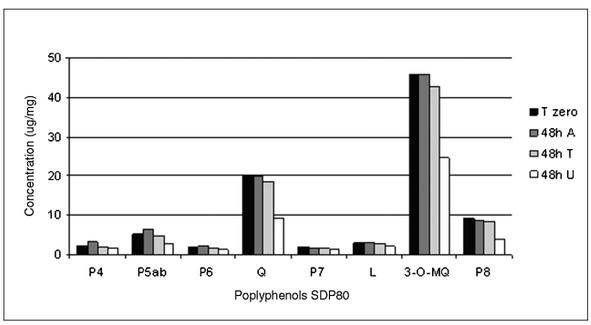

Fig. 5: Polyphenol concentration in SDP80 submitted to UV-C radiation (Light express LE UV, 254 nm, 30 W) initial value and after exposure in A = amber flask, T = Transparent flask and U = unprotected for 48 h. Q = quercetin, L = luteolin, 3-*O*-MQ = 3-*O*-methylquercetin, P8 = achyrobichalcone and substances P4, P5ab, P6, P7

Table 1: Parameters of kinetic degradation of quercetin, luteolin, 3-O-methylquercetin and P8 (achyrobichalcone) from SDP80, submitted to temperature of 80 °C, calculated as a second order reaction

	Parameters of kinetic of degradation		
Substances	k	$t_{0,5}$	$t_{10\,\%}$
Quercetin	$1{,}78 \times 10^{-3}$ d $^{-1}$	15,21 d	3,04 d
Luteolin	$4{,}42 \times 10^{-3}$ d $^{-1}$	40,34 d	8,06 d
3-*O*-Methylquercetin	$6{,}61 \times 10^{-4}$ d $^{-1}$	17,79 d	3,55 d
P8 (achyrobichalcone)	$9{,}81 \times 10^{-3}$ d $^{-1}$	6,07 d	1,21 d

* d = days

2.2. Degradation kinetics

In order to evaluate the kinetic of thermal and photo degradation of the main constituents of SDP80, quercetin, luteolin, 3-*O*-methylquercetin and substance P8, the samples were submitted to thermal stress (80 °C) for 49 days and to light stress conditions (UV-C 254 nm) for 96 h. The samples of SDP80 were analyzed by the LC method.

In the stability studies, the speed of reaction was determined by measuring the drug concentration (C) as function of time (t). Subsequently, the simple correlation (C x t) represents an order zero reaction. A logarithm correlation (Log x t) corresponds to a first order reaction. A second order reaction is represented by the correlation between the inverse of concentration in function of time (1/C x t). The reaction order is chosen by the most linear curve and the correlation index (Lachman 2001).

On the other hand, for pharmaceuticals, a degradation by 10 % of the initial concentration of the drug represents the accepted limit. Thus, the knowledge of the speed constant "k" allows estimating the amount of drug that degrades in a definitive period of time and the shelf-life of the preparation (Lachman 2001).

In the SDP80 thermal stress testing (80 °C, 49 days), the best correlation coefficients were obtained by ploting the inverse of polyphenol concentrations versus the time of exposure, (quercetin, $R^2 = 0.9866$, luteolin, $R^2 = 0.8259$, 3-*O*-methylquercetin, $R^2 = 0.9877$ and substance P8, $R^2 = 0.9547$), indicating a second order reaction profile. This profile indicates that the speed of reaction depends on the concentration of two reagents (A + B = C or $A^2 = C$) (Lachman 2001). Table 1 shows the main calculated parameters: degradation rate constant (k = 1/t (1/C-1/Co)), half-life ($t_{0,5}$ = Co/2k) and time of 10 % decomposition ($t_{10\,\%}$ = 0.9 Co/k) of the polyphenols.

In the SDP80 photo stability test (UV-C radiation for 96 h), the best correlation coefficients were obtained by ploting the logarithm of polyphenol concentrations *versus* the time of exposure (quercetin, $R^2 = 0.9640$, luteolin, $R^2 = 0.9672$, 3-*O*-methylquercetin, $R^2 = 0.9670$, and substance P8, $R^2 = 0.9163$) indicating a first order reaction profile, showing that the speed of reaction depends on the concentration of the reagent. In contrast to thermal degradation, in photo degradation the polyphenols should be decomposed in one or more products (A = products), the speed of the reaction being proportional to its concentration. Table 2 presents the following determined main parameters: degradation rate constant (k = 2.303/t (Log Co/C)), half-life time ($t_{0,5}$ = 0.693/k) and time of 10 % decomposition ($t_{10\,\%}$ = 0.152 $t_{0,5}$).

Table 2: Parameters of kinetic degradation of quercetin, luteolin, 3-O-methylquercetin and P8 (achyrobichalcone) from SDP80, submitted to UVC radiation, calculated as a first order reaction

	Parameters of kinetic of degradation		
Substances	k	$t_{0,5}$	$t_{10\,\%}$
Quercetin	$1{,}80 \times 10^{-2}\,h^{-1}$	38,36 h	5,75 h
Luteolin	$8{,}93 \times 10^{-3}\,h^{-1}$	77,59 h	11,64 h
3-*O*-Methylquercetin	$1{,}49 \times 10^{-2}\,h^{-1}$	46,35 h	6,95 h
P8 (achyrobichalcone)	$1{,}55 \times 10^{-2}\,h^{-1}$	44,54 h	6,68 h

* h = hours

The comparison between the results showed in table 1 and 2 reveals that the constant rates of degradation against UV-C radiation and temperature are different for the SDP80 polyphenols. Against photo exposure, the rate of reaction was $1.80 \times 10^{-2}\,h^{-1}$ for quercetin, $8.93 \times 10^{-3}\,h^{-1}$ for luteolin, $1.49 \times 10^{-2}\,h^{-1}$ for 3-O-methylquercetin, and, $1.55 \times 10^{-2}\,h^{-1}$ for substance P8. These results are higher than those observed after thermal exposure; quercetin: $1.78 \times 10^{-3}\,d^{-1}$, luteolin: $4.42 \times 10^{-3}\,d^{-1}$, 3-*O*-methylquercetin: $6.61 \times 10^{-4}\,d^{-1}$, and substance P8: $9.81 \times 10^{-3}\,d^{-1}$ corroborating the well known high photo sensibility of polyphenols. Among the four polyphenols, luteolin showed the highest stability against UV-C radiation and 3-*O*-methylquercetin was the most stable against heat. These results are in accordance with our previous report (Holzschuh et al. 2007).

2.3. Identification of substance P8

We have recently reported the thermal and photo stabilities of quercetin, luteolin and 3-*O*-methylquercetin present in a spray dried powder (SDP40) prepared from *Achyrocline satureioides* 40 % ethanol extract (Holzschuh et al. 2007). A non identified substance (P3) was detected in SDP40, its thermal degradation (80 °C) was coincident with the anomalous increase of quercetin concentration. In the present work, a similar chemical profile was found in SDP80, where substance P8 demonstrated a behavior similar than substance P3 in SDP40. The result of co-elution of SDP40 and SDP80 demonstrate that substance P3 present in SDP40 is identical to P8 present in SDP80 (Fig. 6). In order to enlighten if the hypothesis that substance P8 could be related to the increase of quercetin the identification of this substance was carried out.

The isolated and purified substance P8 was submitted to spectroscopic and spectrometric analysis. The UV spectrum displayed bands at 369.4 nm and 209.4 nm assignable to a chalcone system (Masesane et al. 2000; Mdee et al. 2003). The molecular formula $C_{32}H_{26}O_9$ was deduced from HRMS-ESI ($[M]^+$ 554.0947). ^{13}C NMR (3.9) showed 14 signals of carbon, whereas the mass analysis indicated the presence of 32 carbons. These data can suggest the existence of a biflavonoid, since the overlapping of signals is attributed to the symmetry of the molecule. The ^1H NMR data are shown in Table 3; it also presents the COSY ^1H-^1H and HMQC ^1H-^{13}C correlation. The singlet at δ 3.91 (6H) was attributed to the methoxyl groups at C6′ and C6″, singlet at δ 4.95 (4H) was attributed to the hydroxyl groups at C2′, C2″, C4 and C4″. Doublets at δ 5.96 (2H $J = 1.81$ Hz) and δ 6.04 (2H $J = 2.01$ Hz) were attributed to hydrogens at positions C3′, C3″, C5′ and C5″, respectively in A and A′ rings, whose coupling constants are characteristic of *meta* position. The doublet at δ 6.88 (4H $J = 8.45$ Hz) was attributed to the hydrogens at positions C3, C5, C3″ and C5″, respectively in B and B′ rings, whose coupling constants are in agreement to *ortho* position. The doublet at δ 7.46 (4H $J = 8.65$ Hz) was attributed to the hydrogens at positions C2, C6, C2″ and C6″, respectively in B and B′ rings. The doublet at δ 7.70 (2H $J = 15.7$ Hz) and the doublet at δ 7.77 (2H $J = 15.5$ Hz) were attributed to the hydrogens at positions C7′, C8′ and C7, C8, respectively. The coupling constant characterizes a *trans* geometry pattern of hydrogens of the double bonds, indicating the bichalcone nature of substance P8, similar to the bichalcone described by Masesane et al. (2000). The locations of the hydroxyl groups at C4 and C4″ were established from COSY ^1H-^1H data (Table 3).

Through structural elucidation, substance P8 was identified as 4,2′,4″,2‴-tetrahydroxy-6′,6‴-dimethoxy-4′-O-4‴-bichalcone (Fig. 7), named achyrobichalcone, and being reported for the first time herein.

In summary, the long term tests demonstrate that SDP80 presents good stability, remaining the total polyphenol concentration within the limits of 10 %, for a year, under storage conditions of 30 °C and 75 % of relative humidity. SDP80 was also stable for 48 h when stored in amber or transparent flasks, submitted to UV-C radiation. Similar results were previously reported for SDP40 (Holzschuh et al. 2007). The kinetic of degradation revealed that SDP80 polyphenols followed 2nd order reaction when submitted to thermal testing, and 1st order reaction in the photo degradation testing. For complex matrices as plant extracts, this is the first stability report which includes determination of the kinetics of degradation. All polyphenols showed sensibility against UV-C radiation and temperature. However, luteolin showed to be the most stable against light and 3-*O*-methylquercetin against temperature. Furthermore, P8 was identified as 4,2′,4″,2‴-tetrahydroxy-6′,6‴-dimethoxy-4′-O-4‴-bichalcone. We also demonstrated that substance P3 reported for SDP40 corresponds to Achyrobichalcone, therefore, P8 and P3 have the same chemical structure. Thus, the elucidation of the structure of P8 (and P3) ruled out the hypothesis that its degradation would explain the anomalous increase of

Table 3: Chemical shifts (ppm), multiplicities, coupling constant J (parentheses) and hydrogen attribution from 1H NMR and 13C NMR spectrum, 1H-1H COSY correlation of P8 (achyrobichalcone)

Chemical shifts (δ) ppm ^1H NMR	Multiplicities (*J*)	Number of protons	Attribution (Fig. 7)	^1H-^1H COSY	Chemical shifts (δ) ppm ^{13}C NMR
3.91	s	6	O-CH_3 e O-CH_3′		55.33
4.95	s	4	−OH (4)		−
5.96	d (1.81 Hz)	2	H3′ e H3‴		96.17
6.04	d (2.01 Hz)	2	H5′ e H5‴		91.10
6.88	d (8.45 Hz)	4	H3 e H3″	H2 e H2″	115.66
			H5 e H5″	H6 e H6″	
7.46	d (8.65 Hz)	4	H2 e H2″	H3 e H3″	129.75
			H6 e H6″	H5 e H5″	
7.70	d (15.7 Hz)	2	H7′ e H8′		123.77,142.17
7.77	d (15.5 Hz)	2	H7 e H8		123.77,142.17

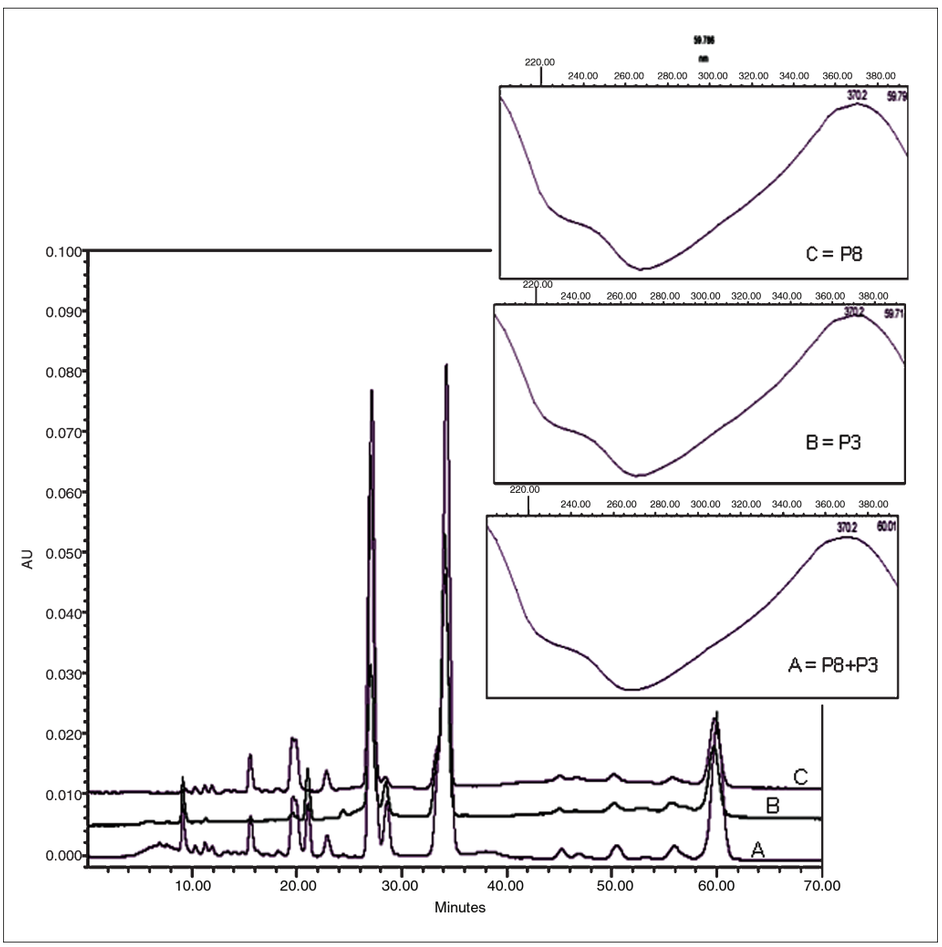

Fig. 6: LC chromatogram of the Achyrocline satureioides spray dried powder SDP40 prepared from ethanol 40 % (v/v) extractive solution plus SDP80 prepared from ethanol 80 % (v/v) extractive solution of the inflorescences (A), SDP40 (B) and SDP80 (C), with respective UV spectra of substances P3 + P8 (SDP40 + SDP80), P3 (SDP40) and P8 (SDP80)

quercetin by thermal degradation of SDP80 (or SDP40). Moreover, the new chemical structure opens new perspectives for *Achyrocline satureioides* investigation, since compounds with similar structures have been related to cytotoxic activity (Mdee et al. 2003).

3. Experimental

3.1. Chemicals

The following chemicals were used: methanol (LC grade, Merck, Darmstadt, Germany) and phosphoric acid (Merck, Darmstadt, Germany), water (Milli-Q system, Millipore, Bedford, MA, USA), polysorbate 80 (Delaware, Porto Alegre, Brazil), luteolin (Sigma, St. Louis, MO, USA), quercetin (Sigma, St. Louis, MO, USA), 3-*O*-methylquercetin obtained according to Schwingel et al. (2007), colloidal silicon dioxide (CSD) (Degussa, Düsseldorf, Germany), ethyl acetate, chloroform and methanol of analytical grade were obtained from Nuclear (Diadema, Brazil). Plant material *Achyrocline satureioides* Lam. (DC) was collected in Santo Antônio da Patrulha, RS, Brazil, voucher specimen ICN 12291.

3.2. Preparation of Achyrocline satureioides spray dried powder (SDP80)

The spray-dried powder was prepared following the procedure described in a Brazilian patent (Bassani et al. 2001). Briefly, inflorescences of *Achyrocline satureioides* Lam. (DC), Asteraceae, were extracted by maceration with

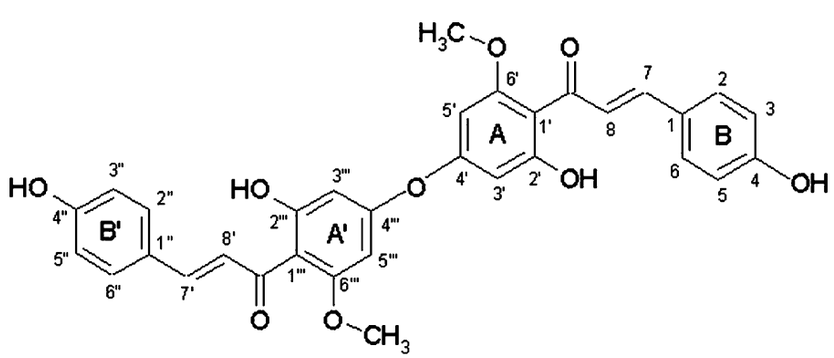

Fig. 7: Chemical structure of 4,2′,4″,2‴-tetrahydroxy-6′,6‴-dimethoxy-4′-*O*-4‴-bichalcone (achyrobichalcone)

ethanol 80 % (v/v) in order to obtain the extractive solution. The ethanol content was reduced by evaporation to 10 % (v/v). Colloidal silicon was added and the dispersion was dried using a Production Minor Spray Dryer plant NIRO A/S, under the following operating conditions: inlet/outlet temperature 180/110 °C; 10.400 rpm and feed rate 130 ml/min. The SDP80 contains 50 % of *A. satureioides* dry residue and 50 % of solid excipients.

3.3. SDP80 thermal stability

The thermal stability of SDP80 was carried out in triplicate at three different temperatures and relative humidity (RH) conditions:

- 30 ± 2 °C (75 ± 5 % RH), for 12 months (long term testing); this condition was obtained in a climatic chamber (Nova Ética mod. 420 CLD). The SDP80 LC analyses were carried out at 0, 3, 6, 9 and 12 months (ICH, 2003; Brasil, 2005).
- 40 ± 2 °C (75 ± 5 % RH), for 6 months (accelerated testing); this condition was obtained in a climatic chamber (Nova Ética mod. 420 CLD). The SDP80 LC analyses were carried out at 0, 3 and 6 months (ICH, 2003; Brasil, 2005).
- 80 ± 2 °C, for 14 days (stress testing); a conventional drying oven was used for obtaining this condition (Biomatic mod. 1305). The SDP80 LC analyses were carried out at 0, 2, 5, 7 and 14 days (Baertschi 2005).

Samples of SDP80 (1 g) were stored in transparent tight flask containers, for long term and accelerate testing, and also in unprotected open-dishes for stress testing.

3.4. SDP80 photo stability

The tests were performed under UV-C radiation (Light express LE UV, 254 nm, 30 W) in a light chamber, with internal mirror cover. Samples of SDP80 (1 g) were stored under three different conditions: transparent flasks, amber flasks and unprotected open-dishes. The polyphenol content was determined in all samples by LC method, taking into account the residual humidity after the stability tests in 0, 12, 24 and 48 h (Baertschi 2005).

3.5. SDP80 kinetic study

To evaluate the kinetics of SDP80 degradation, the samples were exposed to heat and UV light. Samples of SDP80 were stored in unprotected open-dishes.

- 80 ± 2 °C, for 49 days (thermal degradation); a conventional drying oven was used for obtaining this condition (Biomatic mod. 1305). The SDP80 LC analyses were carried out at 0, 5, 14, 21, 28, 35 and 49 days (Lachman 2001).
- UV-C radiation (Light express LE UV, 254 nm, 30 W) for 96 hours in light chamber, with internal mirror cover. The LC analyses were performed at 0, 12, 24, 48, 60, 72 and 96 hours (Lachman 2001).

3.6. Sensorial characteristics

The samples from thermal and photo stability tests were analyzed for their characteristics of smell, color and aspect in all time frames, before the LC analysis.

3.7. Loss on drying

The loss on drying determination was carried out using the gravimetric method, according to the Brazilian Pharmacopoeia, 1988.

3.8. LC analysis

3.8.1. Chromatographic conditions

To evaluate the constituents content in stability and kinetic studies the quantitative analysis was carried out using a HPLC Shimadzu equipped with a LC-10 AD pump, an auto sampler SIL-10 A and a UV-vis detector SPD-10 A controlled by a CLASS LC-10 software. Shim-pack CLC-ODS (M) RP-18, 5 μm, 250 × 4 mm i.d. column and a pre-column Waters (10 X 4 mm i.d.) packed with Bondapack C-18 10 μm (Waters, Milford, USA) were employed. The mobile phase consisted of methanol:phosphoric acid 0.16 M (53:47, v/v), filtered through a 0.45 (m membrane filter (Millipore-HVHP). The flow rate was 0.6 ml/min.; the injection volume was 20 μl and the peaks were detected at 362 nm. In order to check the peak purity of each compound a Waters Millenium DAD was used. The absorbance was measured from 200 to 800 nm every 1 s with 4.8 nm resolution (De Souza et al. 2002).

3.8.2. Standard curves

Quercetin, luteolin and 3-*O*-methylquercetin were used as external standards and dissolved in methanol:water (53:47,v/v) yielding concentrations of 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 μg/ml each. The linear equations were y = 126834x-50536 (R = 0.9993), y = 127422x-34478 (R = 0.9997), y = 45469x-9513.6 (R = 0.9997) for quercetin, luteolin and 3-*O*-methylquercetin, respectively. The content of each constituent was calculated by the corresponding linear equation. The concentrations of compounds P4, P5ab, P6, P7 and P8 were calculated by the linear equation of luteolin.

3.8.3. Sample preparation

SDP80 (0.5 g) was extracted with ethyl acetate for 2 h under magnetic stirring. The supernatant was filtered through a filter paper (grade 1:11 μm, Whatman, UK) and the volume was made up to 50 ml with the same solvent. An aliquot of 25.0 ml was evaporated; the residue was dissolved in methanol and transferred to a 25.0 ml volumetric flask. From this solution an aliquot of 1.0 ml was diluted to 20.0 ml with methanol 53 % (v/v). This solution was filtered through a 0.45 (m membrane filter (HVHP- Millipore) and analyzed by LC (De Souza et al. 2002). This procedure was repeated three times for each sample; moreover, each point was injected three times in the LC equipment.

3.9. Isolation and identification of bichalcone P8

SDP80 (1.0 g) was extracted with $CHCl_3$ for 2 h under magnetic stirring. The supernatant was filtered through a filter paper (grade 1:11 μm, Whatman, UK) and evaporated. The residue dissolved in 10 ml of $CHCl_3$ was applied in Si gel preparative TLC system using $CHCl_3$:MeOH (90:10) as mobile phase. After the elution, the yellow spot corresponding to P8 (Rf 0.6) was removed and exhaustingly extracted with $CHCl_3$:MeOH (90:10). The solution was concentrated to obtain purified substance P8 (55 mg). P8 ($CDCl_3$:CD_3OD) was submitted to NMR analysis in a Varian Inova and to a mass spectrometer analysis using Micromass Q-Tof Waters. See Table 3.
Substance P8: HR-EIMS 554.0947 ($C_{32}H_{26}O_9$), IR υ_{max}^{KBr} cm^{-1}: 3427, 2852, 1625, 1513, 1345, 1215. UV λ_{max}^{MeOH} nm: 209.4, 369.4. ^1H NMR (300 MHz, $CDCl_3$:CD_3OD) see Table 3. ^{13}C NMR (300 MHz, $CDCl_3$:CD_3OD) δ: 191.8 (2x C = O), 167.4 (C4′,C4‴), 164.4 (C6′, C6‴), 162.6 (C2′,C2‴), 159.3 (C4, C4″), 142.2 (C8, C8′), 129.7 (C2, C2″, C6, C6″), 126.4 (C1, C1″), 123.8 (C7, C7′), 115.6 (C3, C3″, C5, C5″), 105.0 (C1, C1‴), 96.2 (C3′, C3‴), 96.1 (C5′, C5‴), 55.3 (2x OMe).

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