Biflavonoid toxicarine, rotenoids and a flavanone from the roots of
Tephrosia toxicaria

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Abstract

For the first time the structural determination of the biflavonoid, 4-H-2,3-dihydro-1-benzopyran-4-one-6-[2-phenyl-8-(3-methyl-2-butenyl)-5, 7-dimethoxy-2-H-3,4-dihydro-1-benzopiran-4-yl]-5,7-dihydroxy-8-(3-methyl-2-butenyl)-2-phenyl, named toxicarine (1) is presented, joined the isolation of the flavanone 7-O-methylglabranine (2) and a mixture of the rotenoids tephrosin (3) and rotenolone (4) from the roots of Tephrosia toxicaria (Sw.) Pers. Their structures were established by spectroscopic methods, meanly spectral data obtained by analysis of 1H and 13C NMR (1D and 2D) spectra.

Keywords: Tephrosia toxicaria. Fabaceae. Flavonoids. Roots.

Biflavonóide toxicarina, rotenóides e uma flavanona das raízes de Tephrosia toxicaria

Resumo

O isolamento e a determinação estrutural do biflavonóide 4-H-2,3-diidro-1-benzopiran-4-on-6-[2-fenil-8-(3-metil-2-butenil)-5, 7-diidetoxi-2-H-3,4-diidro-1-benzopiran-4-il]-5,7-diidroxy-8-(3-metil-2-butenil)-2-fenil, denominada toxicarina (1) relatada pela primeira vez, a flavanona 7-O-metil glabranina (2) e a mistura de rotenóides tefrosina (3) e rotenolona (4) foram isolados e caracterizados, das raízes de Tephrosia toxicaria (Sw.) Pers. As estruturas foram determinadas por métodos espectroscópicos principalmente pelos espectros de 1H e 13C uni e bidimensionais.

Palavras-chave: Tephrosia toxicaria. Fabaceae. Flavonoides. Raízes.
Introduction

The genus *Tephrosia* of the family Fabaceae, is well-known to be a rich source of flavonoid compounds. This class of natural compounds presents a great variety of types and structures. Biological properties of flavonoids may be described by their capacity to act as antioxidants able to scavenge free radicals and reactive oxygen species (WILMSEN; DALLA SANTA; SALVADOR, 2005).

A review on the phytochemistry and pharmacology properties of this genus (TOUQUEER; AJAIB, 2013) show flavonoids and rotenoids as the main classes of compounds. 47 of *Tephrosia* species presented around 219 flavonoids and 65 rotenoids cited in this paper.

Biflavonoids are dimmers which present several biological and pharmacological activities, in relation to different unit linkage moieties. A study of 28 biflavonoids distributed in four types of linkage constituted a semi-empirical method to calculate inhibitory activity against *Mycobacterium tuberculosis*. The results suggested that it is possible to select the most promising molecules from a series of untested biflavonoid molecules (DIA; REBELO; ALVES, 2004).

Investigations were also conducted to develop more active anti-inflammatory biflavonoids having unique mechanisms of action. For this purpose, 5,7-dihydroxy[6,6”]biflavone was synthesized using C-C cross coupling reaction to link the different units (LIM et al., 2009).

Phytochemical study of *Rhus tripartitum* (Anacardiaceae) revealed the presence of myricetin and the new biflavonoid masazinoflavanone, 5, which was active in anti-inflammatory assays (MAHJOUB et al., 2010).

Tepicanol A, 6, was the first biflavonol with a 4,4”biflavanyl ether group to be isolated from *Tephrosia tepicana*, specially it presents the flavanol unit with the same pattern of the flavan part of toxicarine, described in this paper (GOMEZ-GARIBAY et al., 1997).

Previous phytochemical studies of *Tephrosia toxicaria* revealed the presence of flavanone, flavone, isoflavone, rotenoid and chalcone compounds. These compounds were isolated and tested for their potential chemopreventive properties (JANG et al., 2003), furthermore the structural and electronic characterization of the same isolated flavonoids showed that these effects are relevant to understand their biological activity (ARROIO et al., 2004).

Two other papers describe studies of the chemical composition of this plant. The first reports the essential oils composition of leaves, stems, and shoots using GC and GC/MS (RIBEIRO et al., 2006) and the second the chemical and biological study of the ethanol extracts from leaves, stems, pods and roots (VASCONCELOS et al., 2009).

*Tephrosia toxicaria* cited as *Tephrosia sinapou*, in a synonyms case (DOMINGUES et al., 2019) has been used in South American countries in traditional medicine to alleviate pain and inflammation. Regarding to this studies have shown the analgesic effect of extracts of this plant in mice models, also in this paper it was presented for the first time the isolation of the biflavonoid toxicarine (MARTINEZ et al., 2013; MARTINEZ et al., 2016).

Results and Discussion

A new biflavonoid, named as toxicarine (1), constituted by the units of the flavanone glabranine and flavan 5-o-methylisoderricin (respectively C-6:C-4a), the flavanone 7-o-methylglabranine (2) and a mixture of the rotenoids, tephrosin (3) and rotenolone (4), Figure 1, were isolated from the hexanic extract of the roots of *Tephrosia toxicaria*, through a combination of chromatographic techniques. The rotenoids were first identified with GC-MS analysis and and the structural confirmation was carried out by $^1$H and $^{13}$C NMR data and direct comparison with authentic samples isolated from *T. candida* (PEREIRA et al., 1998). The flavanone was identified by physical and spectral data comparison (mp, EIMS, IR, $^1$H and $^{13}$C NMR spectral values, (KISHORE et al., 2003). The position of the prenyl group was confirmed by HMBC correlations (Table 1), specially by heteronuclear interactions between C-5/H-5 (2JCH) and C-6/C-10/HO-5, (3JCH). This NMR experiment is very useful to assure flavonoid A rings substitution pattern and applied to this compound, it’s being reported for the first time. Compound 2 was first reported isolated from the roots of *Tephrosia* sp (CUCA-SUAREZ et al., 1980), and named as 5-hydroxysoderricin. Subsequently the name 7-O-methylglabranine was used, for the first time, when this compound was synthesized (DAYAL, 1980).

The biflavonoid toxicarine (1), was characterized by $^1$H, $^{13}$C ($^1$H- and DEPT), $^1$H-$^1$H-COSY, $^1$H-$^1$H-NOESY, $^1$H-$^{13}$C-COSY. $^n$JCH (n = 1, direct correlation via one bond, HMHC or HETCOR, n = 2 and n = 3, long range, HMBC or COLOC) NMR spectra.
Figure 1 – Flavonoids and rotenoids isolated from *Tephrosia toxicaria* (Sw.) Pers: 1-4 and the biflavonoids 5-6 with biological activities.

The flavanone unit was characterized by the chemical shifts of the carbonyl C-4 ($\delta_C 196.3$), CH-2 [$\delta_C 78.6$ and $\delta_H 5.42$, dd, $J = 13.2$ and $2.9$ Hz (H-2$_{ax}$)] and CH$_2$-3 [$\delta_C 43.7$ and $\delta_H 2.83$, dd, $J = 17.2$ and $2.9$ Hz, (H-3$_{eq}$) and $3.06$, dd, $J = 17.2$ and $13.2$ Hz, (H-3$_{ax}$)]; coherent with a ring C of a flavanone. COLOC spectra showed correlations among the carbons of the ring A at C-5 ($^2J$) and C-6 ($^2J$) with phenolic hydrogen HO-5 and C-7 ($^3J$) and C-8 ($^3J$) with HO-7, respectively. The correlations among C-7/C-9 ($^3J$) and C-8 ($^2J$) with H-1" indicated the position of the prenyl group at C-8. The B ring was characterized like as an unsubstituted ring. Table 1.

The flavan unit was characterized by the chemical shifts of the C ring. The saturated carbons CH-2a; CH$_2$-3a and CH-4a showed signals at $\delta_C 74.4$; 37.0 and 26.9, respectively, which revealed correlation in HMQC spectrum (Table 1) with the correspondents hydrogen signals.

Source: The authors.
Table 1 – NMR spectral data (\(^1\)H: 400 MHz; \(^{13}\)C: 100 MHz; in CDCl\(_3\)), including chemical shifts, \(^2\)J\(_{\text{CH}}\) and \(^3\)J\(_{\text{CH}}\) of toxicarine \(\Delta\), CDCl\(_3\) and TMS) and comparison with literature models. Chemical shifts in \(\delta\) (ppm) and coupling constants (\(J\) in parentheses) in Hz.

| C   | \(\delta\)\(_{\text{C}}\) (ppm) | \(\delta\)\(_{\text{H}}\) (ppm) | \(^2\)J\(_{\text{CH}}\) (Hz) | \(^3\)J\(_{\text{CH}}\) (Hz) | \(\delta\)\(_{\text{C}}\) (ppm) | \(\delta\)\(_{\text{H}}\) (ppm) |
|-----|---------------------------------|---------------------------------|-----------------------------|-----------------------------|---------------------------------|---------------------------------|
| 2   | 78.6                            | 5.42 (dd, 13.2, 2.9)            | H-3                         | H-2,5,6'                    | 79.0                            | 5.58 (dd, 13.3)                |
| 3   | 43.7                            | 2.83 (dd, 17.2, 2.9), 3.06 (dd, 17.2, 13.2) | 43.0                        | 2.80 (dd, 16.3), 3.13 (dd, 16.3, 13.9) | 4.06 (dd, 16.3, 13.9) |
| 4   | 196.3                            |                                 | H-3                         | 196.0                       |                                 |                                 |
| 5   | 159.3                            |                                 | HO-5                        | H-4a                        | 160.0                           |                                 |
| 6   | 109.8**                         | **\*                           | H-4a                        | 97.0                        | 6.04 (s)                        |                                 |
| 7   | 162.8                            |                                 | HO-7                        | H-1, H-4a                   | 162.0                           |                                 |
| 8   | 108.1                            |                                 | H-1"                        | HO-7                        | 106.0                           |                                 |
| 9   | 157.6                            |                                 | H-1"                        | 164.0                       |                                 |                                 |
| 10  | 102.8                            |                                 | HO-5, H-3                   | 103.0                       |                                 |                                 |
| 1'  | 139.1                            |                                 |                              | 135.0                       |                                 |                                 |
| 2'/6' | 125.9                          | 7.28-7.48 (m)                    | 126.0                       | 7.41-7.47 (m)               | 3.25 (d, 7.0)                   |                                 |
| 3'/5' | 128.3                          | 7.28-7.48 (m)                    | 126.0                       | 7.41-7.47 (m)               | 3.25 (d, 7.0)                   |                                 |
| 4'  | 21.8                            | 3.19 (t, 5.5)                   | 126.0                       | 7.41-7.47 (m)               | 3.25 (d, 7.0)                   |                                 |
| 5'  | 122.6                            | 5.15 (t, 6.8)                   | 3H-4"/3H-5"                 | 5.21 (like m)               |                                 |                                 |
| 6'  | 130.9                            |                                 | 3H-4"/3H-5"                 | 5.21 (like m)               |                                 |                                 |
| 7   | 17.8                            | 1.66 (s)                        | 1.61 (brs)                  | 1.61 (brs)                  |                                 |                                 |

*Substituted position. Number of hydrogens bound to carbon atoms deduced by comparative analysis of \(^1\)H and DEPT-\(^{13}\)C NMR spectra. Chemical shifts and coupling constants (\(J\) in parentheses) obtained from 1D-\(^1\)H NMR spectrum. 2D-\(^1\)H-\(^1\)H-COSY spectrum was also used in these assignments.

Source: The authors.
at $\delta_H$ 5.09 (brd, $J = 11.0$ Hz, H-2a$_A$), 2.35 (d, $J = 13.9$ Hz, H-3a$_B$), 2.22 (ddd, $J = 13.9$, 11.0, 4.5, 8.3a$_B$) and 4.69 (d, $J = 4.5$ Hz, H-4a$_B$). The ring A of this unit showed two methoxyl groups at C-5a and C-7a. This pattern of substitution was confirmed by the HMBC (Table 1) and NOESY spectra, the interaction of H-6a (6.17, s) with both MeO-5a ($\delta$ 3.77, s) and MeO-7a ($\delta$ 3.90, s) indicating spatial proximity. Carbon atoms of the ring A of this unit showed similar type correlations (heteronuclear long range coupling, $^2J_{CH}$ and $^3J_{CH}$) between the C-5a/C-7a ($^1J$) with methoxy hydrogens (MeO-5a and MeO-7a, respectively) and also C-7a/C-9a ($^3J$) and C-8a ($^2J$) with H-1$^+$ suggesting the of the prenyl group at C-8a. As the same of the flavanone moiety, the flavan presents also an unsubstituted ring B.

The linkage of the two units was established by long range correlations observed in the HMBC spectrum, that revealed coupling of the C-5, C-6 and C-7 ($\delta_C$ 159.3, 109.8 and 162.8, respectively) with H-4a ($\delta_H$ 4.66, d, $J = 4.5$ Hz), defining the linkage between C-6/C-4a.

The coupling constants of the signals of H-2 (dd, $J = 13.2$ and 2.9 Hz) and H-2a (brd, $J = 9.9$ Hz) were used to locate the phenyl groups at pseudo-equatorial position, since $J = 13.2$ and 9.88 Hz indicated trans interactions between H-2/2a (¢x) and H-3/3a (¢x).

The relative configuration of the CH-4a (flavon moiety) was postulated by constants coupling ($J = 4.0$ and 1.6 Hz) observed in the signal of the H-4a ($\delta_H$ 4.66, dd). These values of $J$ allow to eliminate the possibility of diaxial interaction involving this hydrogen H-4a. These arguments were used to locate the H-4a at pseudo-equatorial position and, consequently, the flavanon moiety in pseudoaxial position, Figure 2.

Finally, Table 1 shows the chemical shifts ($^1H$ and $^{13}C$), the correlations ($^2J_{CH}$ and $^3J_{CH}$) on COLOC spectra of toxicarine and comparison among chemical shifts ($^1H$ and $^{13}C$) with glabranin, (TANAKA et al., 1997; AGRAWAL; THAKUR; BANSAL, 1989), 5,7-dimethoxy-8-prenyllavand (GOMEZ et al., 1983) and 2H-furo[2,3-h]-1-benzopyran, 3,4-dihydro-5-methoxy-2,8-bis(4-methoxyphenyl) (BABA et al., 1986), that showed similar unit values in the comparison with the correspondent data of the flavonoid dimer 1.

Thus, structure of new flavonoid dimer was established and the NMR spectra (1D and 2D) were also used to attribute the complete $^1H$ and $^{13}C$ chemical shift assignments, summarized at Table 1.

The mass spectra (GC-MS/EI) showed a fragmentation pattern of a very stable molecule. Toxicarine (1) presented the peak correspondent to molecular ion at $m/z$ 660 (M$^+$, 100 %, base peak), and the other main fragments indicated loss of methoxy ($m/z$ 629), isobutenyl ($m/z$ 605) and isoprenyl ($m/z$ 592) radicals. The peak at $m/z$ 337 was attributed to the flavan moiety.

**Experimental**

**General experimental procedures**

$^1H$ (400 MHz), $^{13}C$ NMR (100 MHz) and HMBC spectra of 2 were recorded on a Bruker DRX-400 spectrometer (Chem. Dept. of University of Warwick, UK), $^1H$ (400 MHz), $^{13}C$ NMR (100 MHz), COSY, HMBC, and NOESY spectra of 1 were recorded on a JEOL Eclipse 400 spectrometer (LCQUI/ CCT-UENF) and $^1H$ (200 MHz), $^{13}C$ NMR (50 MHz), HETCOR and COLOC spectra of the mixture of 3 and 4 were recorded on a Bruker AC-200 spectrometer (Depto. de Química, ICE-UFRJ). All of the RMN spectra used CDCl$_3$ as solvent and TMS as internal standard. EIMS were obtained on a Shimadzu QP 5000 (GC/MS), using direct inlet with 70 eV ionization to obtain spectrum of compound 1 (CCT-LCQUI/UENF) and EIMS on a Shimadzu QP 5000 (GC/MS), using DB-1 column, 70 eV ionization toobtain spectra of compounds 2, 3 and 4 (LPMB/DQ-CCE/UEL); CC Silica gel (Merck 0.05-0.70 mm); TLC: silica gel GF/254 (Merck); spots were visualised by UV (254 nm), by exposure to iodine vapour and after spraying with vaniline/MeOH/H$_2$SO$_4$. Uncorrected melting points were obtained on a Kofer apparatus type.

**Plant material:** Roots of *Tephrosia toxicaria* (Sw.) Pers. were collected and identified in the Instituto Agronômico do Paraná, PR, Brazil, in January 1988. Voucher specimen are deposited at the herbarium of Instituto Agronômico, Londrina, PR, Brazil.

**Isolation and purification of constituents:** Exha-ustive extraction of the dry powdered roots (8.9 kg) with hexane at room temperature and the solvent removed under low pressure yielded 58.7 g of crude extract. 30g of hexane extract was applied to a silica gel (300 g) column, using hexane, hexane-CH$_2$Cl$_2$, CH$_2$Cl$_2$ and CH$_2$Cl$_2$-MeOH, as eluents, resulting in 20 groups, 8 oil and 12 solid, fractions. Column chromatography of the first solid fraction (1.86 g) using the same system of elution, re-
sulted in other 20 groups of refined fractions. Joined fractions 04-06 (1061.53 mg) with another CC, provided 825.3 mg of compound 2 and 35.4 mg of a mixture of 3 and 4. Similar chromatographic methodology of joined fractions 08 and 09 (75.9 mg), followed by TLC as a final purification step afforded 23.67 mg of compound 1.

**Isolated compounds**

*Toxicarine* (1): mp. 143 °C; $^1$H, $^{13}$C NMR and COLOC ($^2$J$_{CH}$ and $^3$J$_{CH}$) (Table 1); GC/EIMS m/z (rel. int. %): 660 (M$^+$, 100), 629 (5), 605 (5), 592 (10), 337 (48), 321 (32), 269 (30).

7-O-methylglabranine (2): mp. 123-125 °C; identified by $^1$H (400 MHz) and $^{13}$C (100 MHz) and HMBC NMR spectra and comparison with spectral data of literature (KISHORE et al., 2003).

*Tephrosin and rotenolone* (mixture of 3 and 4): identified by GC-MS analysis [tephrosin (3): 410 (M$^+$) 15 %; 392 (M$^+$-H$_2$O) 20 %; 377 (M$^+$-H$_2$O-Me) 13 %; 208 (RDA) 100 %] and [rotenolone (4): 410 (M$^+$) 42 %; 392 (M$^+$-H$_2$O) 37 %; 208 (RDA) 100 %] and direct comparison with authentic samples isolated from *Tephrosia candida*. Structures confirmed by $^1$H (200 MHz) and $^{13}$C (50 MHz) NMR spectra from the mixture.

**Acknowledgments**

This work was supported by CNPq research fellowships. The authors are grateful to Professor Dr. Oliver W. Howart, from the Chemistry Department of University of Warwick (UK), for the acquisition of NMR spectra ($^1$H, $^{13}$C and HMBC) of 7-O-methylglabranine, to Professor Dr. Jan Schipper, from LQUI/CCT, Universidade Estadual do Norte Fluminense for acquisition of GC and NMR ($^1$H, $^{13}$C, COSY, NOESY HMBC and HMQC) spectra of toxicarine (1), to Professor Dr. Mário Geraldo de Carvalho for acquisition of NMR spectra ($^1$H, $^{13}$C) of the mixture of tephrosin (3) and rotenolone (4) and to Dra. Sueli de Carvalho, from Instituto Agronômico do Paraná of Londrina, PR, Brazil, for collecting and identifying *Tephrosia toxicaria*.

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