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Article

PAMPA Permeability, Acetylcholinesterase Inhibition and Antioxidant Activity of Pyranoisoflavones from *Polygala molluginifolia* (Polygalaceae)

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Uma investigação fitoquímica de *Polygala molluginifolia* (raiz, caule, folhas e flores) resultou no isolamento de quatro piranoisoflavonas das quais a isoflavona **1** é desconhecida na literatura. As isoflavonas **2**-**4**, embora sejam compostos sintéticos conhecidos são descritos neste trabalho como novos produtos naturais. Os compostos isolados foram avaliados em termos do seu efeito antioxidante e o potencial para inibir a enzima acetilcolinesterase. As isoflavonas **1** e **4** inibiram a acetilcolinesterase, exibindo valores de IC_{50} de 68 μmol L^{-1} e 84 μmol L^{-1}, respectivamente. No ensaio de DPPH, os mesmos compostos e a rutina **5** apresentaram valores de EC_{50} de 61 μmol L^{-1}, 55 μmol L^{-1} e 16 μmol L^{-1}, respectivamente. Além disso, a permeabilidade das compostos **1**-**4** foi testada através do ensaio de permeabilidade em membrana artificial paralela (PAMPA) com as isoflavona **3** (9.25×10^{-6} cm s^{-1}) e **4** (3.48×10^{-6} cm s^{-1}) exibindo as maiores permeabilidades.

A phytochemical investigation of *Polygala molluginifolia* (root, stem, leaves and flowers) resulted in the isolation of four pyranoisoflavones, of which isoflavone **1** was previously unknown in the literature. Isoflavones **2**-**4**, although they are known synthetic compounds, are described in this work as new natural products. The isolated compounds were evaluated for their antioxidant effects and potential for inhibiting the acetylcholinesterase enzyme. Isoflavones **1** and **4** inhibited acetylcholinesterase, displaying IC_{50} values of 68 μmol L^{-1} and 84 μmol L^{-1}, respectively. In the DPPH assay, the same compounds and rutin **5** exhibited EC_{50} values of 61 μmol L^{-1}, 55 μmol L^{-1} and 16 μmol L^{-1}, respectively. Moreover, the permeability of compounds **1**-**4** was evaluated using parallel artificial membrane permeability assay (PAMPA) with isoflavones **3** (9.25×10^{-6} cm s^{-1}) and **4** (3.48×10^{-6} cm s^{-1}) exhibiting the highest permeabilities.

Keywords: Polygalaceae*, Polygala molluginifolia*, isoflavones, *in vitro* assays

Introduction

In the course of our research on the chemotaxonomy and biological activity of the genus *Polygala*, we investigated *P. molluginifolia* (A. St.-Hil.), a small herb found in grassland formations along the Atlantic Forest of southern Brazil.[1] No previous investigations have been reported regarding this species. The genus *Polygala* has been chemotaxonomically characterized by an accumulation of xanthones,[2,3] coumarins,[4] saponins,[5,6] flavonoids,[7,8] lignans,[9] and more rarely, isoflavones.[10] In our previous studies, we detected an unusual occurrence of styrylpyrones in *P. sabulosa*.[11]

The *Polygala* species have significant ethnopharmacological value. For example, *P. cyparissias* is used as a local anesthetic due to the presence of methyl salicylate in its roots,[12,13] and *P. tenuifolia* is used in traditional Chinese medicine to improve memory and intelligence.[14] Biological studies have reported that *P. tenuifolia* is utilized in the treatment of Alzheimer's disease,[15] and also applied as an expectorant and sedative.[16] *P. caudate* is widely employed for treating cough and hepatitis and is used as a sedative.[17] Moreover, *P. radix* exhibits neuroprotective effects in Parkinson's patients,[18] and *P. japonica* demonstrates anti-inflammatory activity.[19] Our pharmacological studies indicated that *P. paniculata* possess neuroprotective effects,[20] whereas *P. sabulosa*

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exhibits anti-depressant, anxiolytic and anti-convulsant activity.[21] We now report the isolation and structural elucidation of four isoflavones from *P. molluginifolia*, their antioxidant activity, acetylcholinesterase inhibition and PAMPA (parallel artificial membrane permeability assay) permeability.

Experimental

General experimental procedures

Melting points were determined on a Microquímica APF-302 apparatus and are uncorrected. The infrared (IR) spectral data were acquired using Perkin-Elmer FT 16PC and Varian 3100 FT-IR instruments with KBr disks. UV spectra was measured with a Hewlett Packard 8452A instrument. The 1D and 2D nuclear magnetic resonance (NMR) data were acquired on a Varian Unity plus NMR spectrometer operating at 9.4 Tesla, observing ^{1}H and ^{13}C at 400 and 100 MHz, respectively. Thin-layer chromatography was performed on a pre-coated silica gel type-60 plate (Merck), and the spots were located by spraying with sulfuric-anisaldehyde followed by heating. For the standard chromatographic fractionation, 70-230-mesh silica gel was used, and for flash chromatographic fractionation, 230-400-mesh silica gel was employed. The electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOFMS) measurements were performed with a micrOTOF Q-II (Bruker Daltonics) mass spectrometer equipped with a KD Scientific automatic syringe pump for sample injection. The ESI-QTOF mass spectrometer was run at 4.5 kV and a desolvation temperature of 180 °C. The mass spectrometer was operated in the positive ion mode using a standard electrospray ion (ESI) source to generate the ions. The samples were injected using a constant flow rate (3 μL min^{-1}), and the solvent was an acetonitrile/methanol mixture. The ESI-Q-TOF MS instrument was calibrated in the *m/z* 50-3000 range using an internal calibration standard (low concentration tuning mix solution) supplied by Agilent Technologies. The data were processed using Bruker data analysis software version 4.0. The acetylcholinesterase was measured using acetylthiocholine iodide (ATCI), 5,5′-dithiobis-[2-nitrobenzoic acid] (DTNB), acetylcholinesterase (AChE) type VI-S from an electric eel, [tris(hydroxymethyl)aminomethane] Tris-HCl buffer, bovine serum albumin (BSA) and magnesium chloride hexahydrate supplied by Sigma Aldrich (USA). For the 2,2-diphenyl-1-picrylhydrazyl (DPPH) analysis, DPPH (Sigma Aldrich), methanol (Vetec) and ethanol (Synth) were used. The bioassays were performed on a Perkin Elmer Lambda S spectrophotometer. High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from J. T. Baker, and Milli-Q pure water was obtained from a Milli-Q Plus ultra-pure water purification system purchased from Millipore. The PAMPA experiments were performed using phosphatidylcholine, dimethyl sulfoxide and dodecane purchased from Sigma Aldrich and multiscreen filter plates from Millipore. The donors, acceptors and equilibrium solutions were analyzed using a Shimadzu HPLC system equipped with an LC-10AT pump and a UV detector. The analysis was performed on a Phenomenex C_{18} column (150 × 4.6 mm; 5 μm), and elutions were obtained in isocratic mode [25% H_2O (v/v) and 75% acetonitrile (v/v)] at a flow rate of 1 mL min^{-1}, with detection at 280 nm.

Plant material

Polygala molluginifolia was collected in December, 2010, near the Pitangui River, at coordinates UTM 593095 and 7231847, in the region of Campos Gerais, Ponta Grossa, Paraná State, Brazil. The species was identified by Raquel Lüdtke (UFPel), and a sample specimen was deposited at the FLOR Herbarium (UFSC) registered under number 39551 by Rafael Trevisan.

Extraction and isolation

The dried whole-plant material (37.0 g) was extracted (three times for seven days each) with EtOH at room temperature. The resulting extracts were combined, filtered and concentrated under reduced pressure at 50 °C to yield the crude alcoholic extract (10.45 g), which was then solid-liquid partitioned with hexane and EtOAc, and the solvents removed under reduced pressure to yield the respective extracts. The residue from the extraction was denominated water-soluble fraction. The EtOAc-soluble fraction (3.66 g) underwent chromatographic fractionation on a silica gel column and eluted with a gradient of EtOAc in hexane (1:9, 2:8, 3:7, 4:6, 5:5, 7:3), and then a gradient of EtOH in EtOAc (2:8, 4:6), to yield 41 fractions. Fraction 6 (1:9 EtOAc-hexane) underwent a successive chromatographic fractionation on a flash silica gel column and eluted with a hexane:acetone 8:2 gradient to yield isoflavones **2** (7.2 mg) and **3** (11.7 mg). Fractions 19 (3:7 EtOAc-hexane) and 31 (EtOAc) were recrystallized with MeOH to yield compounds **4** (16.4 mg) and **1** (38.7 mg).

The water-soluble fraction (3.96 g) underwent chromatographic fractionation on a silica gel column and was eluted with a scaled gradient of 7:3 hexane:EtOAc; 5:14:1 hexane:EtOAc:MeOH; 1:19:13 hexane:EtOAc:EtOH and finally MeOH to yield 30 fractions. Fractions 15 and 19

(1:19:13, hexane:EtOAc:EtOH) were combined to generate compound **5**. Fraction 28 (methanol) was recrystallized with MeOH to yield compound **6**.

3′,4′-dihydroxy-6″,6″,6‴,6‴-tetramethylbis(pyrano[2″,3″:5,6::2‴,3‴:7,8]isoflavone (**1**)

Yellow solid; mp 205–207 °C; IR (KBr) υ_{max} / cm^{-1} 3416, 1654, 1595, 1507, 1452; UV (MeOH) λ_{max} / nm (ε) 340 (2265), 302 (25604), 292 (25335), 258 (24798); for NMR data, see Table 1; HRMS (ESI+) *m/z* calculated for $C_{25}H_{22}O_6$ $[M+H]^+$: 419.1495; found, 419.1495.

3′,4′-dimethoxy-5-hydroxy-6″,6″-dimethylpyrano[2″,3″:7,6]isoflavone (**2**)

Yellow solid; mp 136–138 °C; IR (KBr) υ_{max} / cm^{-1} 3434, 1665, 1622, 1583, 1509; for NMR data, see Table 2; HRMS (ESI+) *m/z* calculated for $C_{22}H_{20}O_6$ $[M+H]^+$: 381.1338; found, 381.1328.

3′-methoxy-5,4′-dihydroxy-6″,6″-dimethylpyrano[2″,3″:7,6]isoflavone (**3**)

Yellow solid; mp 155-158 °C; IR (KBr) υ_{max} / cm^{-1} 3434, 1662, 1624, 1581, 1516; for NMR data, see Table 2; HRMS (ESI+) *m/z* calculated for $C_{21}H_{18}O_6$ $[M+H]^+$: 367.1182; found, 367.1179.

5,3′,4′-trihydroxy-6″,6″-dimethylpyrano [2″,3″:7,6]isoflavone (**4**)

Yellow solid; mp 165–167 °C; IR (KBr) υ_{max} / cm^{-1} 3386, 1656, 1621, 1573, 1519; for NMR data, see Table 2; HRMS (ESI+) *m/z* calculated for $C_{20}H_{16}O_6$ $[M+H]^+$: 353.1025; found, 353.1022.

Acetylcholinesterase activity

The enzymatic activity was measured using an adaptation of the method described by Mata *et al.*[22] Briefly, 325 µL of 50 mmol L^{-1} Tris-HCl buffer, pH 8.0, 100 µL of a buffer solution containing the sample at five different concentrations dissolved in EtOH and 25 µL of an AChE solution containing 0.28 U mL^{-1} (50 mmol L^{-1} Tris-HCl, pH 8.0 buffer, 0.1% BSA) were incubated for 15 min. Then, 75 µL of an acetylthiocholine iodide solution (0.023 mg mL^{-1} in water) and 475 µL DTNB (3 mmol L^{-1} in Tris-HCl, pH 8.0 buffer, 0.1 mol L^{-1} NaCl, 0.02 mol L^{-1} $MgCl_2$) were added, and the final mixture was incubated for another 30 min at room temperature. The absorbance of the mixture was measured at 405 nm. A control mixture containing ethanol instead of the sample was considered to have 100% AChE activity. The inhibition (%) was calculated as follows: $I\ (\%) = 100 - (A_{sample}/A_{control}) \times 100$ in which A_{sample} is the absorbance of the vegetal sample and $A_{control}$ is the absorbance without sample. The tests were performed in triplicate, and a blank containing Tris-HCl buffer was used instead of the enzyme solution. The sample concentration with 50% inhibition (IC_{50}) was determined by plotting the inhibition against the sample solution concentrations. Reminyl-containing galantamine was used as the positive control.

Antioxidant activity

The antioxidant activity was measured using the DPPH free-radical scavenging methodology described in the literature with adaptations.[23] A 0.004% DPPH solution in methanol was added to the test solutions in various concentrations, and the absorbances were determined using a UV-VIS (517 nm) spectrophotometer after 30 min. The absorbance at the initial time (A_0) was obtained by measuring the absorbance of a DPPH solution. The analysis was performed in triplicate. The EC_{50} values of the samples were obtained by plotting the decreasing DPPH absorbance against the sample solution concentrations.

PAMPA permeability assay

PAMPA experiments for compounds **1**-**4** were performed according to Wohnsland and Faller with modifications using a 96-well filter plate.[24] The filters were coated with 10 µL of a 1% (m/v) dodecane solution of phosphatidylcholine. The donor solutions of compounds **1**-**4** were prepared by diluting the DMSO stock solutions (1000 ppm) in 50% (v/v) phosphate buffered saline and stirring overnight. Then, 150 µL of the donor solutions were added to the filter plate wells, and 300 µL of the acceptor solutions (50% DMSO in phosphate buffer) were added to the receiver plate wells. The filter plate was coupled to the receiver plate and incubated in the dark for 5 h with agitation and then 20 min at room temperature. Next, 150 µL of each donor solution was added to 300 µL of the acceptor solution to yield the respective equilibrium solutions. The experiments were performed in quadruplicate. After incubation, the filter plate and the receiver plate were separated, and the donor, acceptor and equilibrium solutions were analyzed via HPLC.

The PAMPA permeability (*Papp*) was calculated using the following equation obtained from Wohnsland and Faller:[24]

$$Papp = \frac{V_D V_A}{(V_D + V_A)At}(-\ln(1 - r))$$

in which V_D is the volume in the donor well (cm^3), V_A is the volume in the acceptor well, A is the area of the membrane

(cm^2), *t* is the incubation time (s) and *r* is the ratio between the compound concentrations in the acceptor well and equilibrium solution. The compound concentrations were obtained by integrating the HPLC peaks.

Results and Discussion

In our investigation of the ethyl acetate fraction from *Polygala molluginifolia*, four isoflavones (**1**-**4**) were isolated and their structures elucidated. In addition, rutin (**5**) and sucrose (**6**) were isolated from the water-soluble fraction (Figure 1).

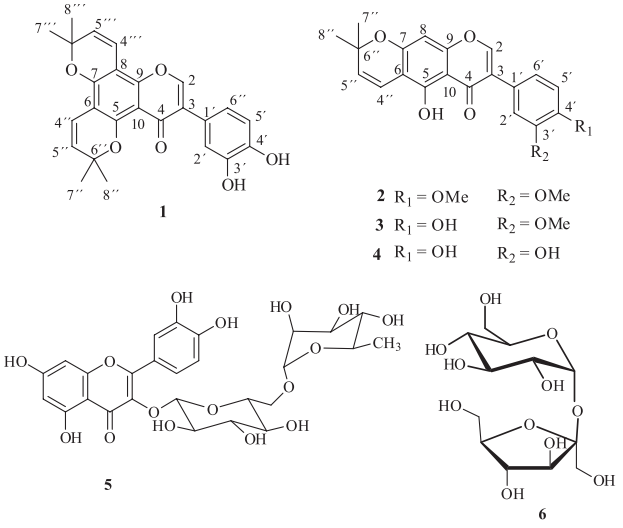

Figure 1. Structures of isolated compounds **1**-**6**.

The infrared spectra of compounds **1**-**4** demonstrated characteristic absorptions for the hydroxyl group, conjugated carbonyl, conjugated double bonds and aromatic rings. Compound **1**, a yellow solid, had the molecular formula $C_{25}H_{22}O_6$, as determined by HRMS (ESI+) analysis and NMR spectroscopy. The UV spectrum showed absorption maxima at 258, 292, 302 and 340 nm. The ^{1}H NMR spectrum of compound **1** (Table 1) indicated the presence of an ortho-meta system at δ_H 6.79 (d, 1H, *J* 8.0 Hz, H-5′), 6.77 (dd, 1H, *J* 2.0, 8.0 Hz, H-6′) and 6.95 (d, 1H, *J* 2.0 Hz, H-2′), four methyl groups at δ_H 1.47 (s, 12H, H-7″, H-8″, H-7‴, H-8‴), four *cis*-coupled olefinic hydrogens at δ_H 6.60 (d, 1H, *J* 10.0 Hz, H-4″), 5.66 (d, 1H, *J* 10.0 Hz, H-5″), 6.73 (d, 1H, *J* 10.0 Hz, H-4‴) and δ_H 5.69 (d, 1H, *J* 10.0 Hz, H-5‴), and a singlet at δ_H 7.95 (s, 1H, H-2) corresponding to a proton attached to a sp^2 carbon. The ^{13}C NMR and DEPT spectroscopic data (Table 1) revealed twenty-three carbons: two quaternary sp^3 carbons, two signals related to methyl groups, eight methine carbons, and eleven sp^2 quaternary carbons, five of which are oxygenated and one is a carbonyl. These data, in addition to HRMS, indicated a pentacyclic scaffold. Directly connected hydrogens and carbons were assigned via HMQC analysis. The long-range ^{1}H-^{13}C correlation map obtained from the HMBC NMR experiment (Table 1) exhibited a $^2J_{CH}$ correlation H-2 (δ_H 7.95)/ C-3 (δ_C 124.7), and $^3J_{CH}$ correlations H-2/C-1′ (δ_C 126.9) and H-2/C-4 (δ_C 177.4). The olefinic hydrogens H-4″ (δ_H 6.60) and H-5″ (δ_H 5.66) exhibited $^3J_{CH}$ correlations with C-5 (δ_C 155.5) and C-6 (δ_C 108.5), respectively, indicating that one dimethylpyran ring was fused to the A ring at C-5 and C-6. The remaining dimethylpyran ring was fused to the A ring at carbons C-7 (δ_C 153.8) and C-8 (δ_C 103.3), as verified by the $^3J_{CH}$ long-range correlations of these carbons with hydrogens H-4‴ (δ_H 6.73) and H-5‴ (δ_H 5.69), respectively. The B ring is disubstituted with two hydroxyl groups located at C-3′ and C-4′, as demonstrated by HMBC correlations $^3J_{CH}$ C-3′ (δ_C 146.0)/H-5′ (δ_H 6.79), $^2J_{CH}$ C-3′/H-2′ (δ_H 6.95), and $^3J_{CH}$ C-4′ (δ_C 146.5)/H-2′. The overall analysis of HMQC and HMBC experiments, and MS data indicated that compound **1** refers to 3′,4′-dihydroxy-6″,6‴,6‴,6‴-tetramethylbis (pyrano[2″,3″:5,6::2‴,3‴:7,8]isoflavone (Figure 1), which is reported herein for the first time.

Table 1. NMR data (400 MHz, CD_3OD) for compound **1**

Position	**1**			
	δ_H (mult., *J* / Hz)	δ_C / ppm	HMBC	
			$^2J_{CH}$ / Hz	$^3J_{CH}$ / Hz
2	7.95 (s)	152.3	-	-
3	-	124.7	H-2	H-2′
4	-	177.4	-	H-2
5	-	155.5	-	H-4″
6	-	108.5	-	H-5″
7	-	153.8	-	H-4‴
8	-	103.3	-	H-5‴
9	-	154.2	-	H-2
10	-	109.9	-	H-8
1′	-	126.9	-	H-5′, H-2
2′	6.95 (d, *J* 2.0)	117.6	-	H-6′
3′	-	146.0	H-2′	H-5′
4′	-	146.5	-	H-2′
5′	6.79 (d, *J* 8.0)	116.1	-	-
6′	6.77 (dd, *J* 2.0, 8.0)	121.9	-	H-2′
4″	6.60 (d, *J* 10.0)	116.4	-	-
5″	5.66 (d, *J* 10.0)	129.8	-	H-7″, H-8″
6″	-	79.5	H-5″, H-7″, H-8″	H-4″
7″, 8″	1.47 (s)	28.3	-	-
4‴	6.73 (d, *J* 10.0)	115.7	-	-
5‴	5.69 (d, *J* 10.0)	128.9	-	H7‴, H8‴
6‴	-	79.2	H-5‴, H-7‴, H-8‴	H4‴
7‴, 8‴	1.47 (s)	27.9	-	-

The ^{1}H NMR data of compounds **2**-**4** agree with those published for synthetic isoflavones 3′,4′-dimethoxy-5-hydroxy-6″,6″-dimethylpyrano[2″,3″:7,6] isoflavone (**2**),[25] 3′-methoxy-5,4′-dihydroxy-6″,6″-dimethylpyrano[2″,3″:7,6]isoflavone (**3**),[26] and 5,3′,4′-trihydroxy-6″,6″-dimethylpyrano[2″,3″:7,6] isoflavone (**4**).[25] These structures were here confirmed by ^{13}C NMR, DEPT, HMQC, HMBC, NOESY, and HRMS spectra (Table 2 and experimental).

Compounds (**5**) and (**6**) were identified by comparison of their NMR data with those described in the literature.[27,28]

The isolation of these isoflavones has considerable chemotaxonomic importance because few studies have examined isoflavones in the Polygalaceae family. This family is closely related to the Leguminosae, in whose subfamily, Papilionoideae, isoflavones are common.

Moreover, reports suggest that isoflavones have multiple protective functions, such anti-anxiety properties,[29] and neuroprotective effects associated with antioxidative and hippocampal β-secretase activities that decrease the formation and deposition of insoluble β-amyloid debris.[29-36] Isoflavones can also strengthening the antioxidation ability of the body, reducing neural damage from free radicals, enhance the activity of the antioxidant enzymes superoxide dismutase and glutathione peroxidase effectively postponing the degeneration and apoptosis of neurons.[30,37] Isoflavones can be used to treat Alzheimer's disease by their ability to decrease acetylcholine hydrolysis, thereby aiding the maintenance of learning and memory functions.[30,37] These findings have led to the assertion that isoflavones represent a potential new therapeutic strategy for Alzheimer's disease.

Thus, we investigated isoflavones **1**-**4** for their ability to inhibit the acetylcholinesterase enzyme (AChE), and antioxidant effects. Rutin (**5**) was also evaluated for its antioxidant activity.

As shown in Table 3, compounds **1**-**4** demonstrated a inhibition effect towards of the acetylcholinesterase enzyme. Among these, isoflavones **1** and **4** showed the best results with IC_{50} values of 68 and 84 μmol L^{-1}, respectively. These compounds exhibit hydroxil groups in positions 3′ and 4′ in the B ring of the isoflavone scaffold, which suggests that these groups may enhance the acetylcholinesterase inhibition.

The addition of a second dimethylpyran ring fused to the A ring also increased the inhibition, as observed for compound **1** when compared to **4**. These findings suggest that both the hydrophobicity and the presence of a catechol group play important roles in the isoflavones ability to inhibit acetylcholinesterase. These results followed the same trend as the antioxidant activity by scavenging DPPH. Compounds with a catechol group in the B ring, such as

Table 2. NMR data for compounds **2** (400 MHz, $CDCl_3$), **3** (400 MHz, $CDCl_3$) and **4** (400 MHz, C_3D_6O)

Position	**2**				**3**				**4**			
	δ_H (mult., *J* / Hz)	δ_C / ppm	HMBC		δ_H (mult., *J* / Hz)	δ_C / ppm	HMBC		δ_H (mult., *J* / Hz)	δ_C / ppm	HMBC	
			$^2J_{CH}$ / Hz	$^3J_{CH}$ / Hz			$^2J_{CH}$ / Hz	$^3J_{CH}$ / Hz			$^2J_{CH}$ / Hz	$^3J_{CH}$ / Hz
2	7.86 (s)	152.6	-	-	7.81 (s)	154.6	-	-	8.13 (s)	154.7	-	-
3	-	123.6	H-2	H-2′	-	124.3	H-2	H-2′	-	123.5	-	H-6′
4	-	180.8	-	H-2	-	181.9	-	-	-	182.0	-	H-2
5	-	156.9	-	-	-	157.8	-	H-4″	-	157.9	-	H-4″
6	-	106.1	-	-	-	106.1	H-4″	H-8	-	106.2	H-4″	H-8, H-5″
7	-	159.7	H-8	H-4″	-	160.3	H-8	H-4″	-	160	H-8	H-4″
8	6.35 (s)	94.8	-	-	6.31 (s)	95.5	-	-	6.33 (s)	95.6	-	-
9	-	157.2	H-2	H-8	-	158.9	H-8	H-2	-	158.3	H-8	H-2
10	-	106.08	-	H-8	-	106.8	-	H-8	-	106.9		H-8
1′	-	123.3	-	H-5″, H-2	-	123.4	-	H-5′, H-2	-	124.3	H-2′	H-2
2′	7.10 (d, *J* 2.0)	112.2	-	H-6′	7.10 (d, 2.0)	115.8	-	H-6	7.13 (d, *J* 2.0)	117.4	-	-
3′	-	148.8	MeO-3′, H-2′	H-5′	-	145.8	MeO-3′, H-2	H-5′	-	145.8	H-2′	H-5′
4′	-	149.2	MeO-4′, H-5′	H-2′	-	146.5	-	H-2′, H-6′	-	146.5	H-5′	H-2′, H-6′
5′	6.95 (d, *J* 8.0)	111.2	-	-	6.95 (d, *J* 8.0)	116.0	-	-	6.87 (d, *J* 8.0)	116.1	-	-
6′	7.04 (dd, *J* 2.0, 8.0)	121.2	-	H-2′	6.93(dd, *J* 2.0, 8.0)	121.5	-	H-2′	6.93 (dd, *J* 2.0, 8.0)	121.7	-	H-2′
4″	6.75 (d, *J* 10.0)	115.4	-	-	6.70 (d, *J* 10.0)	117.2	-	-	6.65 (d, *J* 10.0)	116.0	-	-
5″	5.65 (d, *J* 10.0)	128.1	-	H-7″	5.60 (d, *J* 10.0)	129.6	-	H-7″, H-8″	5.74 (d, *J* 10.0)	129.6	-	H-7″, H-8″
6″	-	78.0	H-5″, H-7″, H-8″	H-4″	-	78.09	H-7″, H-8″, H-5″	H-4″	-	79.0	H-5″	H-4″
7″, 8″	1.48 (s)	28.3	-	H-5″	1.45 (s)	28.5	-	H-5″	1.45 (s)	28.6	-	H-5″
HO-5	13.17 (s)	-	-	-	13.14 (s)	-	-	-	13.44 (s)	-	-	-
MeO-3′	3.94 (s)	55.97	-	-	3.92 (s)	56.0	-	-	-	-	-	-
MeO-4′	3.92 (s)	55.99	-	-	-	-	-	-	-	-	-	-

Table 3. Inhibitory activity of **1-4** toward acetylcholinesterase, and antioxidant effects of **1-5** in DPPH free-radical scavenging

Compounds	Acetylcholinesterase inhibition	DPPH
	IC_{50} / (μmol L^{-1})	EC_{50} / (μmol L^{-1})
1	68	61
2	132	838
3	126	208
4	84	55
5	-	16
Reminyl®	32	-
Ascorbic acid	-	52
Quercetin	-	21

rutin (**5**) and isoflavones **1** and **4**, exhibited the best results, with EC_{50} values of 16, 61 and 55 μmol L^{-1}, respectively, compared with ascorbic acid (52 μmol L^{-1}) and quercetin (21 μmol L^{-1}).

The strong antioxidant activity for rutin is in accordance with that reported in the literature,[38] indicating that rutin is a multifunctional agent capable of inhibiting Aβ aggregation and cytotoxicity, which can prevent mitochondrial damage, reduce free-radical and proinflammatory cytokine production and increase the levels of antioxidant enzymes.[38]

Given the significant acetylcholinesterase inhibition and DPPH activity of isoflavones **1**-**4**, the permeability of these compounds was also evaluated using the PAMPA assay. A high-throughput screening technique, PAMPA, was used to assess the passive permeability characteristics of molecules with potential pharmacological activity to predict passive transcellular molecule absorption.

In most cases, according to this assay, the hydrophobic isoflavones exhibited lower permeability than the polar isoflavones, a result consistent with that of Dreassi *et al.*[39] Of the compounds analyzed, isoflavone **1** exhibited the lowest permeability (1.50×10^{-6} cm s^{-1}), possibly due to the presence of two dimethylpyran rings, which increased the hydrophobicity of the molecule.

Regarding isoflavones **2** and **4**, which differ only in their B ring substitution, isoflavone **2** had the lowest permeability (2.01×10^{-6} cm s^{-1}), probably due to the presence of two methoxyl groups. Isoflavone **3** demonstrated the best permeability (9.25×10^{-6} cm s^{-1}), although it is more hydrophobic than compound **4** (3.48×10^{-6} cm s^{-1}). According to Siegel, increasing the number of hydroxyl groups can decrease the compound permeability.[40]

Conclusions

This study reports the acetylcholinesterase inhibition, antioxidant activity and permeability of four isoflavones (**1**-**4**) isolated and identified from *Polygala molluginifolia*. Of the compounds analyzed, isoflavones **1** and **4**, exhibited the best antioxidant activity and acetylcholinesterase inhibition, while PAMPA experiments indicated that the isoflavones **3** and **4** demonstrated the highest permeability. Results suggest that the isoflavones isolated from *P. molluginifolia* could be of interest for the treatment of neurodegenerative diseases.

Supplementary Information

Supplementary data, including IR, MS and 1D/2D NMR, are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgements

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Supplementary Information

PAMPA Permeability, Acetylcholinesterase Inhibition and Antioxidant Activity of Pyranoisoflavones from *Polygala molluginifolia* (Polygalaceae)

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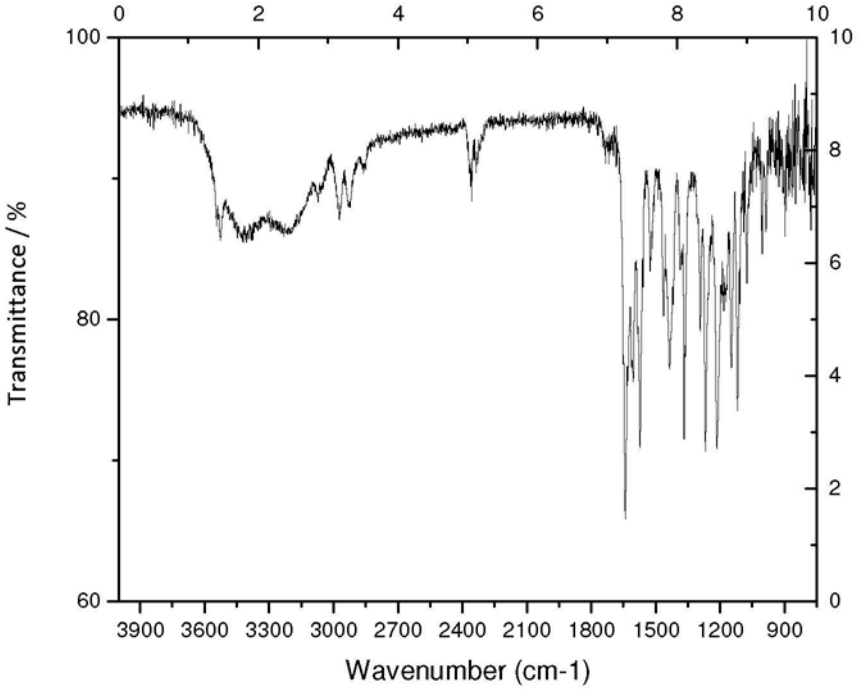

Figure S1. IR spectrum (KBr disks) of isoflavone **1**.

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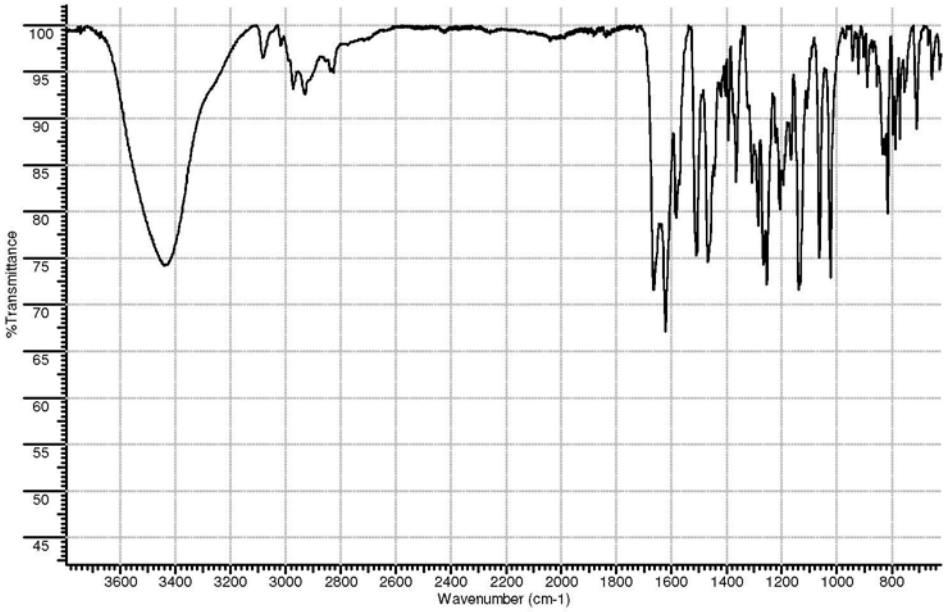


Figure S2. IR spectrum (KBr disks) of isoflavone **2**.

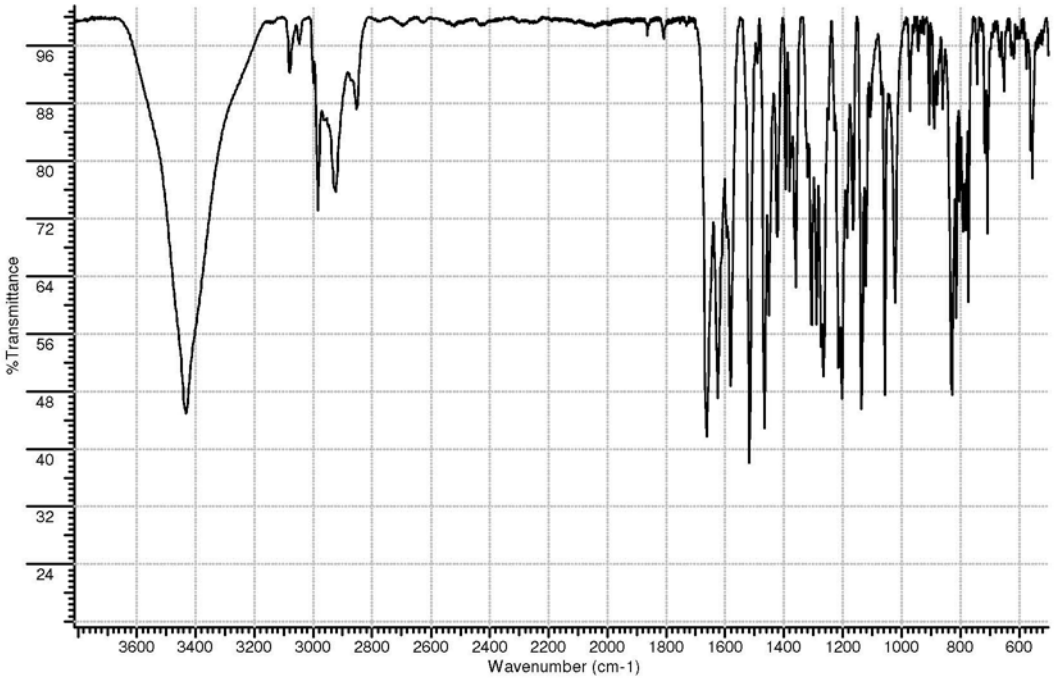


Figure S3. IR spectrum (KBr disks) of isoflavone **3**.

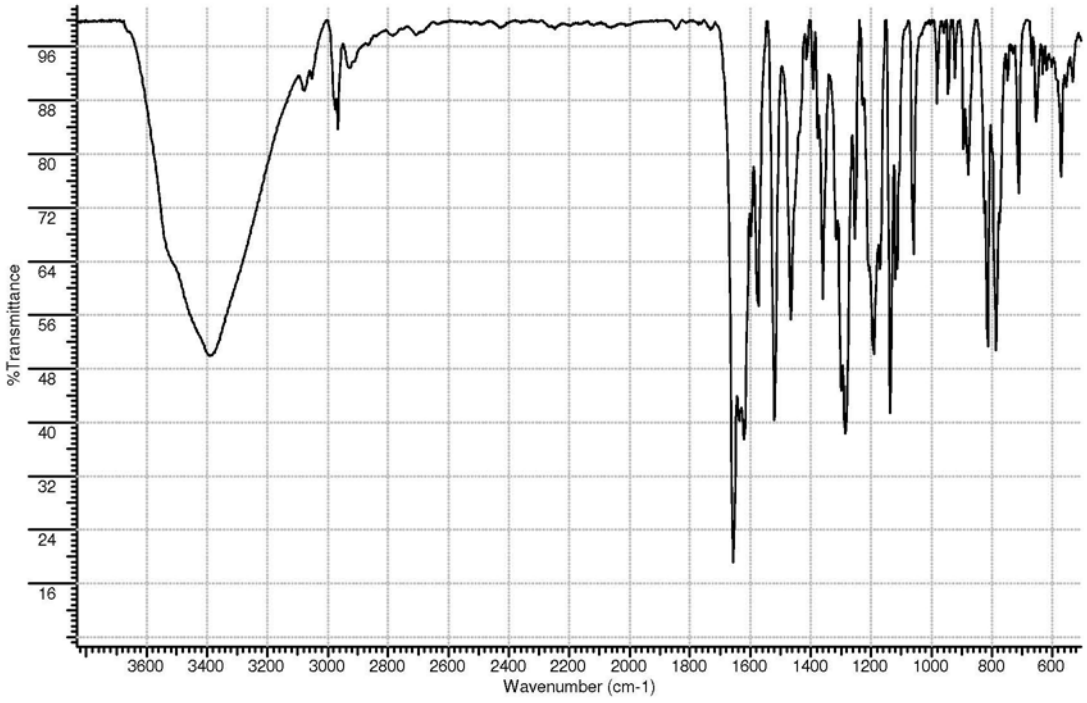


Figure S4. IR spectrum (KBr disks) of isoflavone **4**.

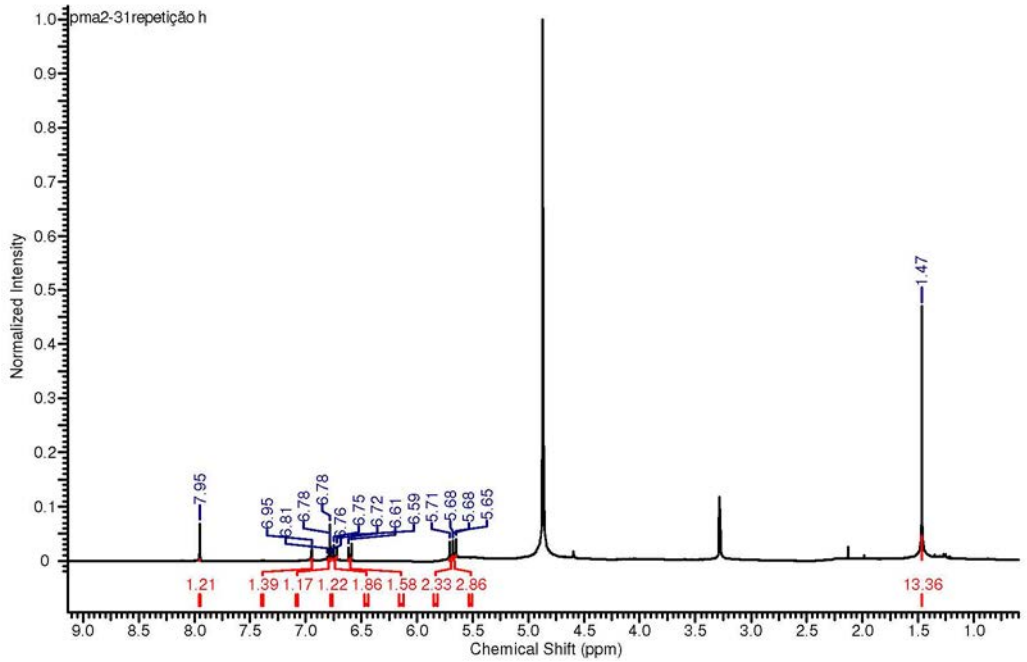


Figure S5. ^{1}H NMR spectrum (400 MHz, CD_3OD) of isoflavone **1**.

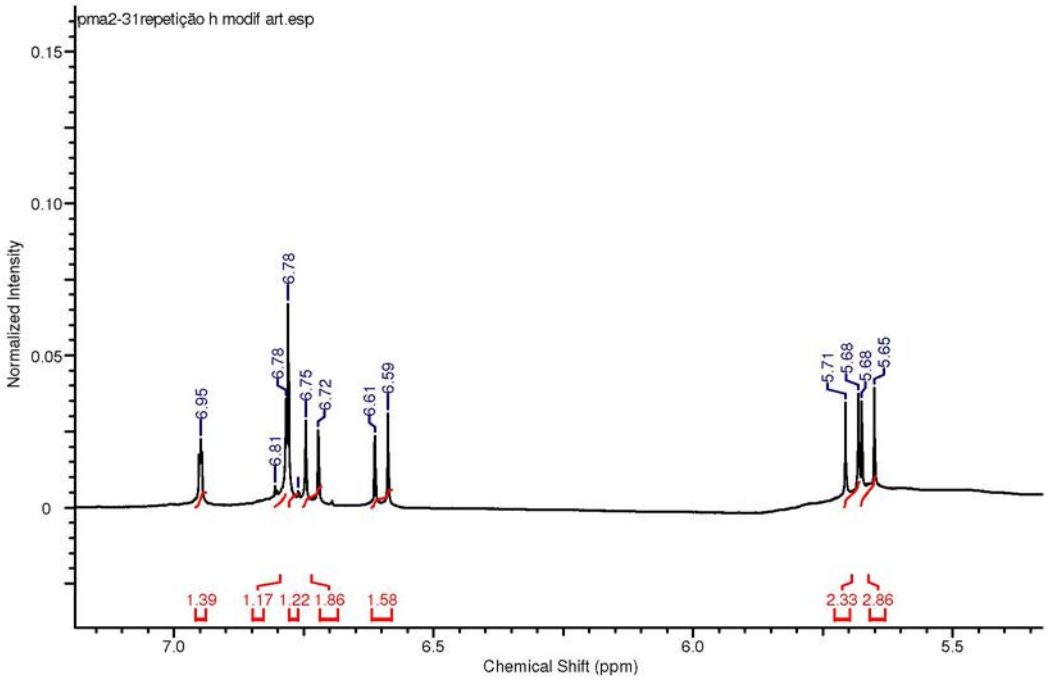


Figure S6. Expanded ^{1}H NMR spectrum (400 MHz, CD_3OD) of isoflavone **1**.

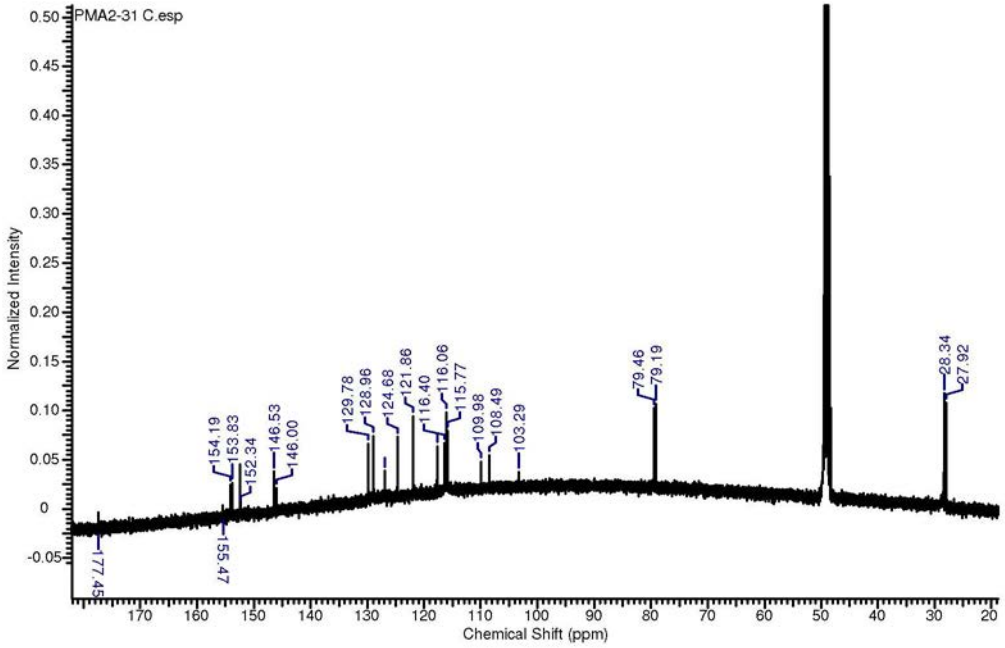


Figure S7. ^{13}C NMR spectrum (100 MHz, CD_3OD) of isoflavone **1**.

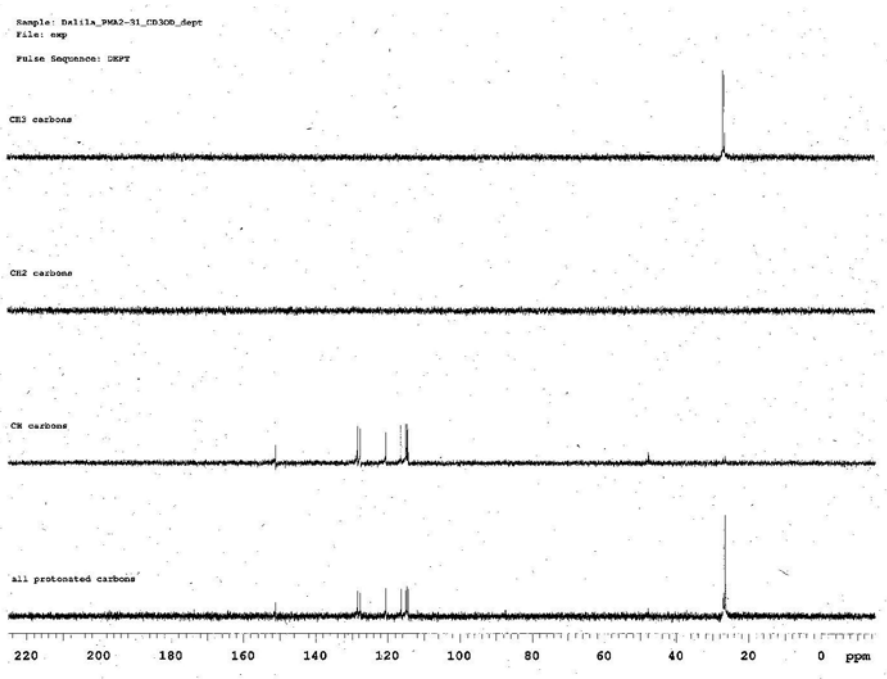


Figure S8. DEPT spectrum (100 MHz, CD_3OD) of isoflavone **1**.

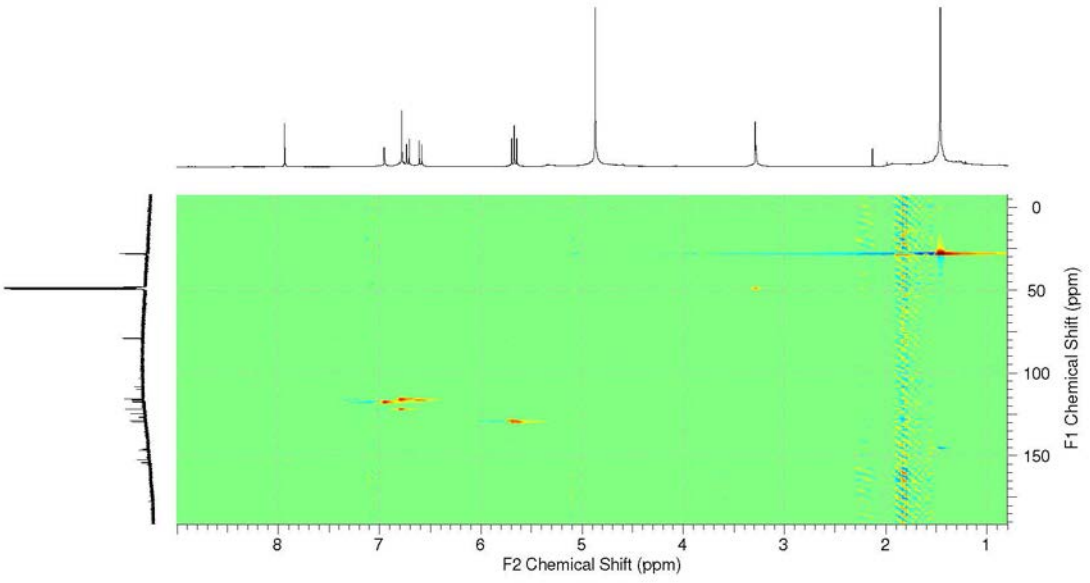


Figure S9. HMQC spectrum (400 MHz, CD_3OD) of isoflavone **1**.

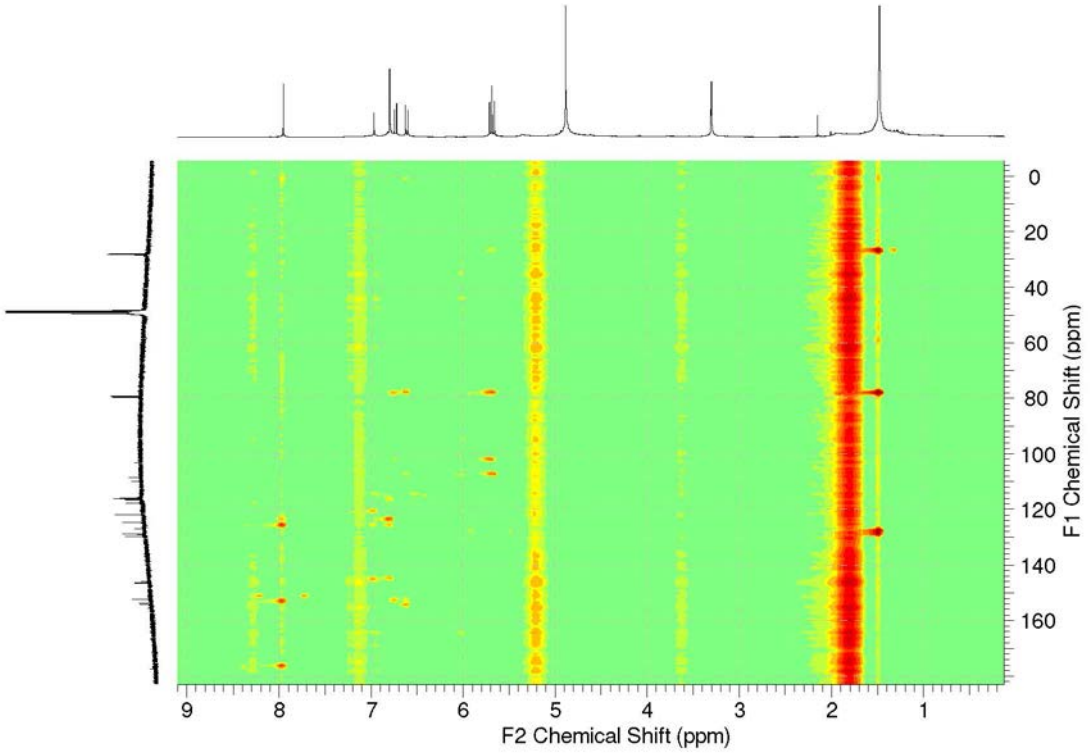


Figure S10. HMBC spectrum (400 MHz, CD_3OD) of isoflavone **1**.

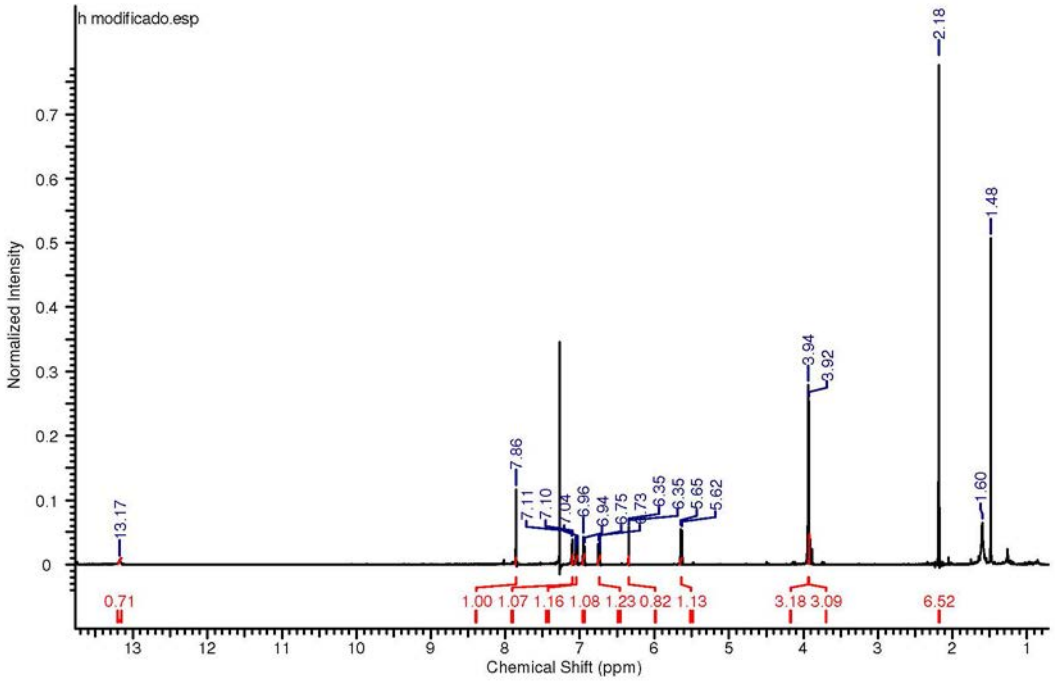


Figure S11. ^1H NMR spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.

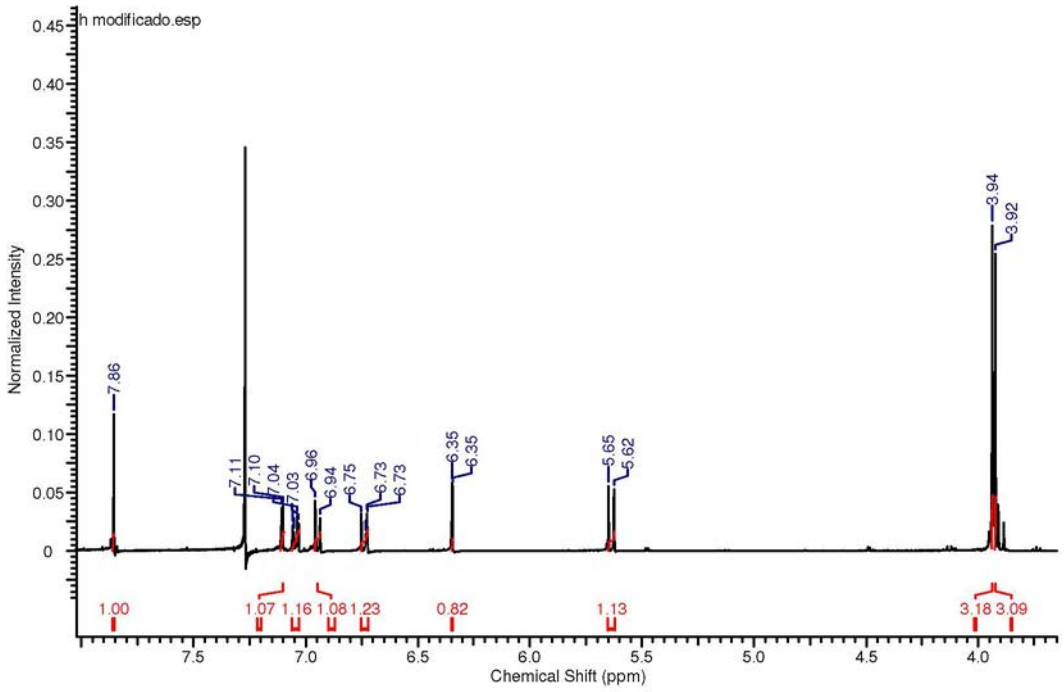


Figure S12. Expanded ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.

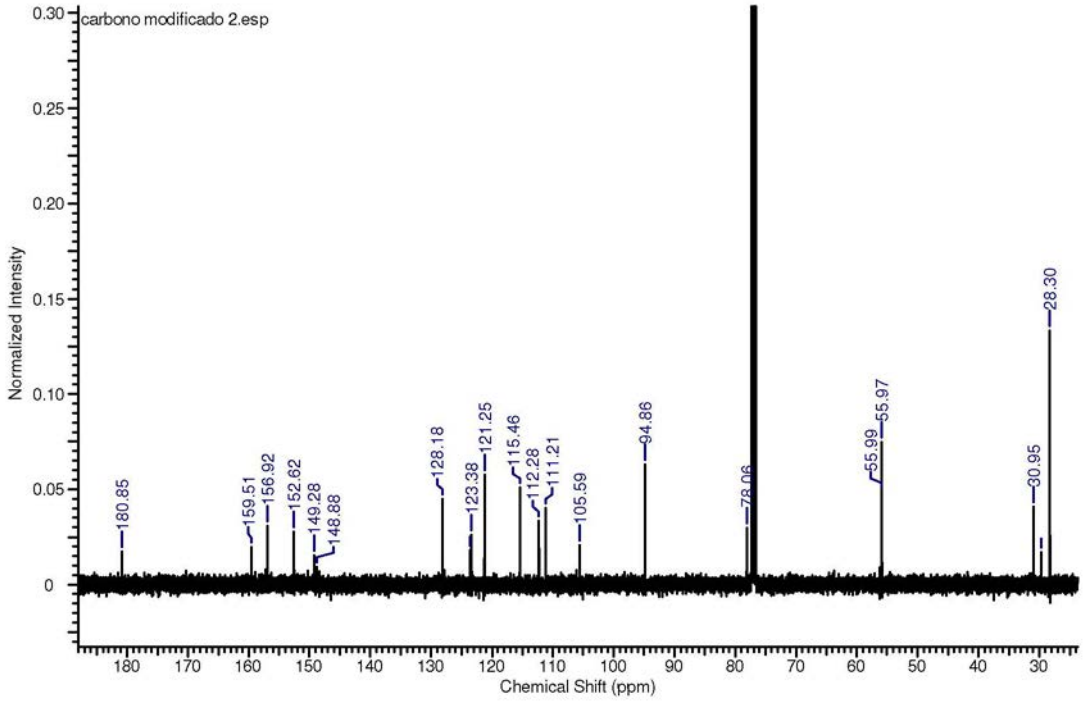


Figure S13. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of isoflavone **2**.

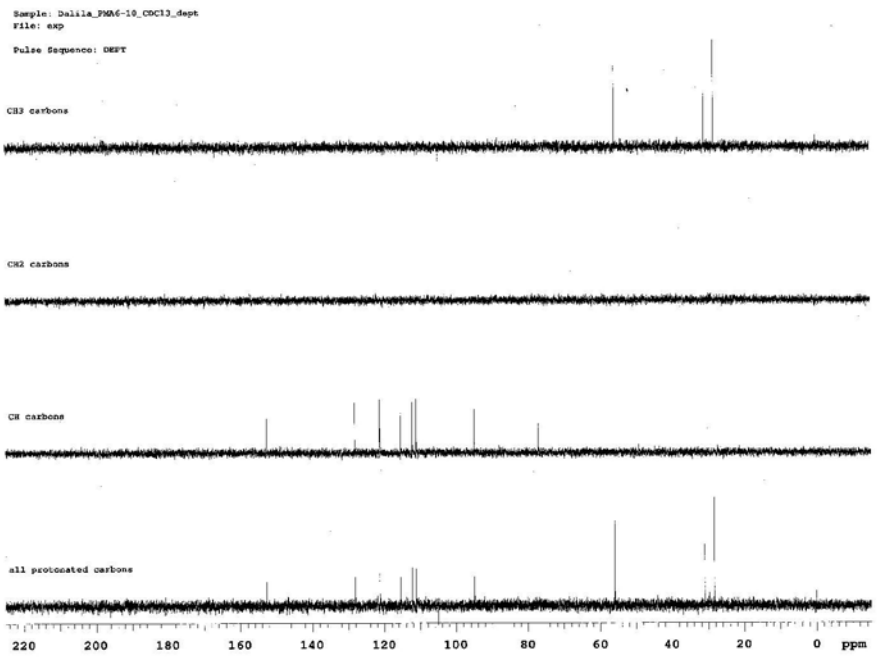


Figure S14. DEPT spectrum (100 MHz, $CDCl_3$) of isoflavone **2**.

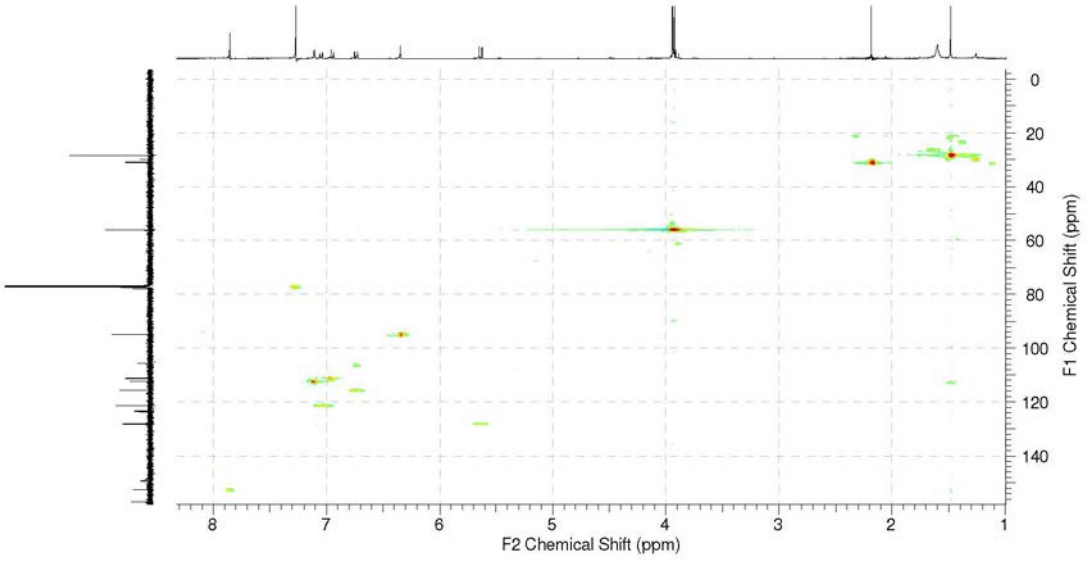


Figure S15. HMQC spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.

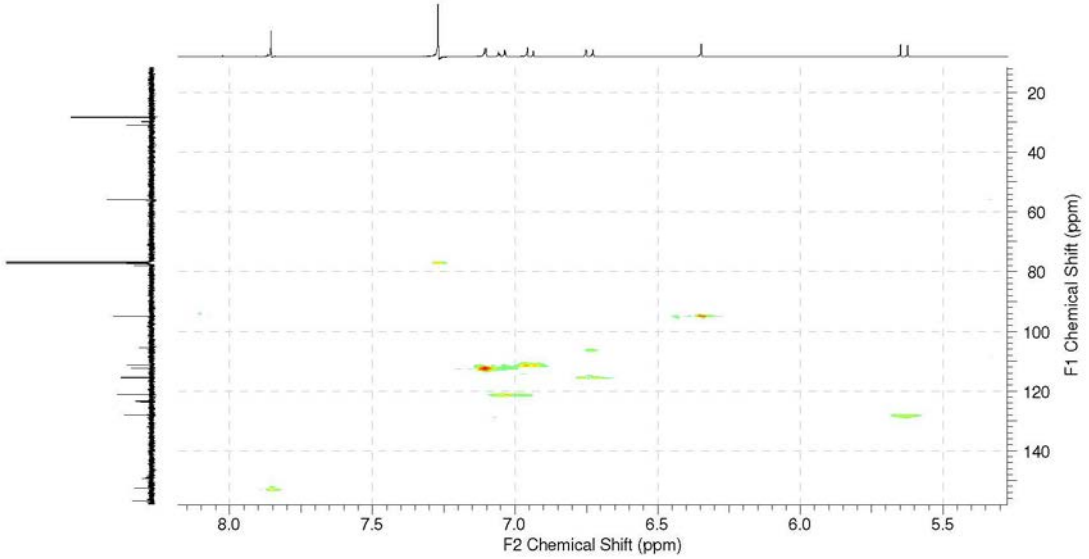


Figure S16. Expanded HMQC spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.

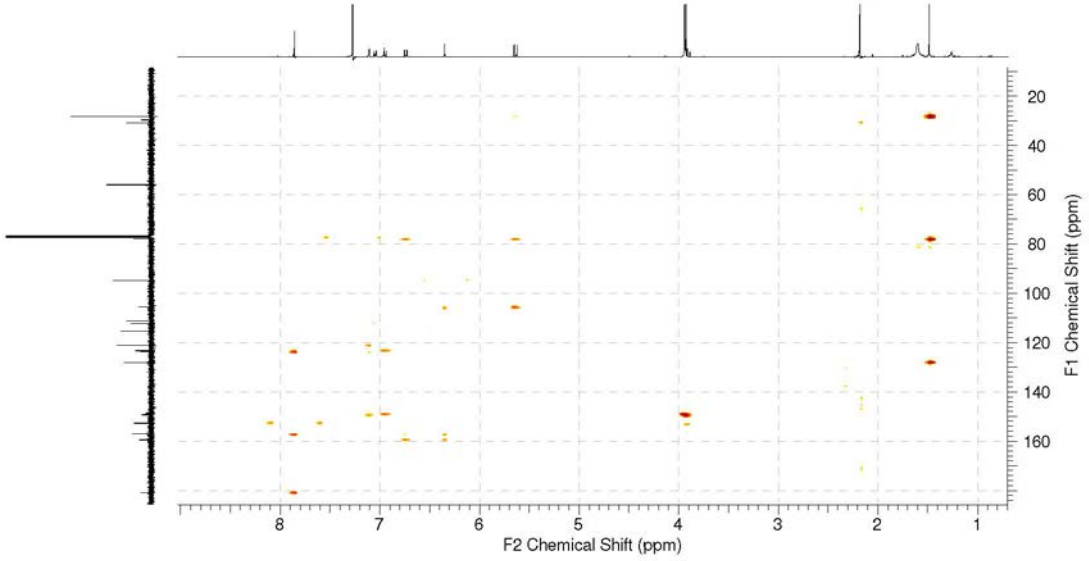


Figure S17. HMBC spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.

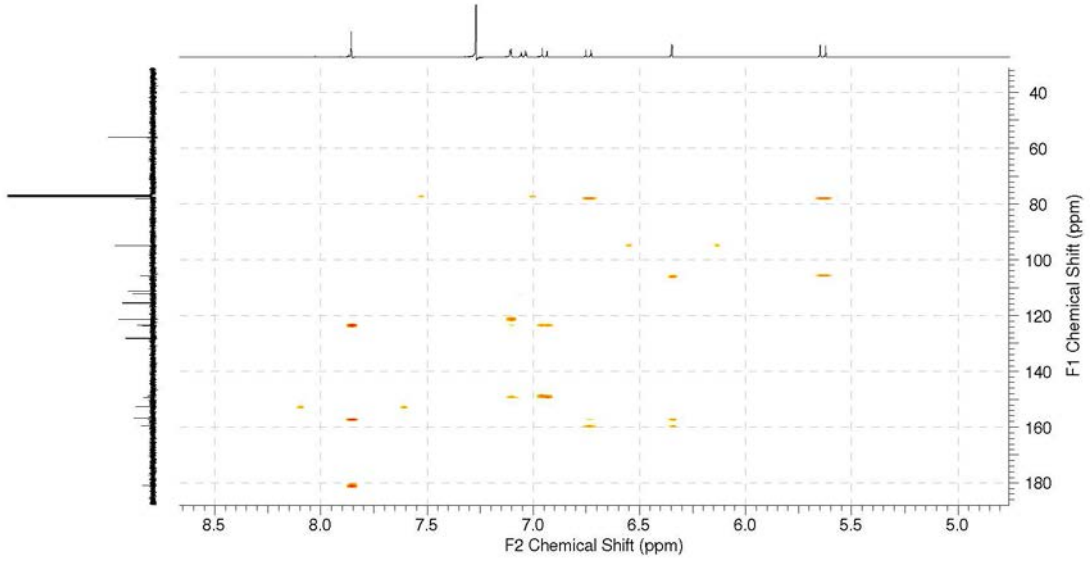
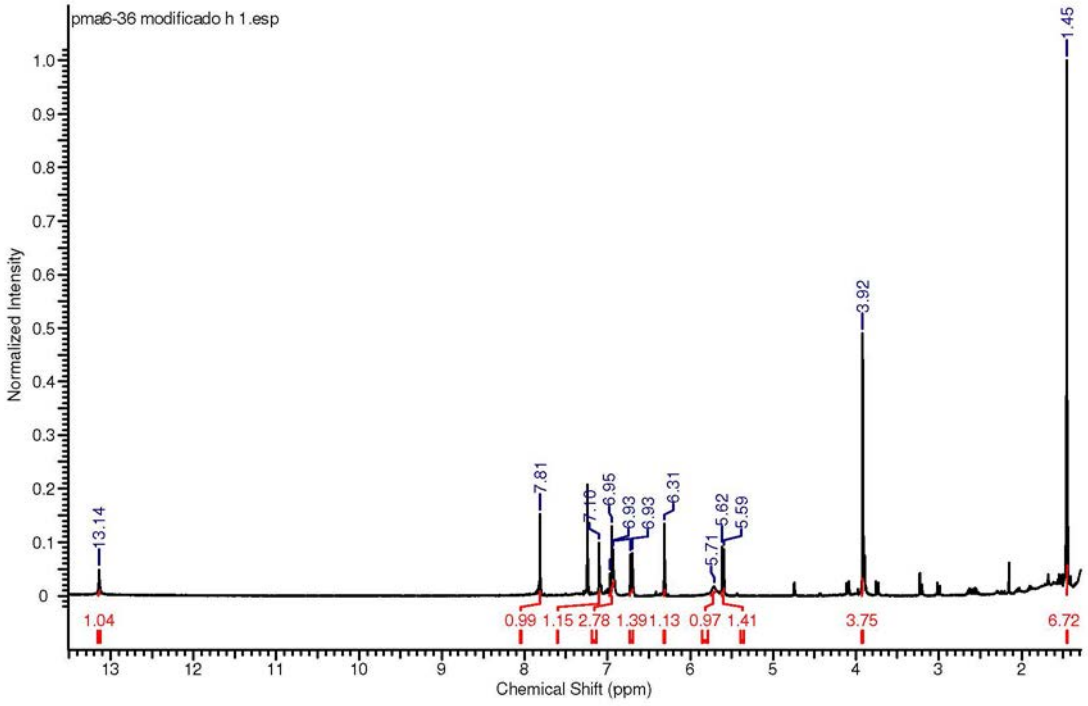


Figure S18. Expanded HMBC spectrum (400 MHz, $CDCl_3$) of isoflavone **2**.



FigureS 19. ^1H NMR spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

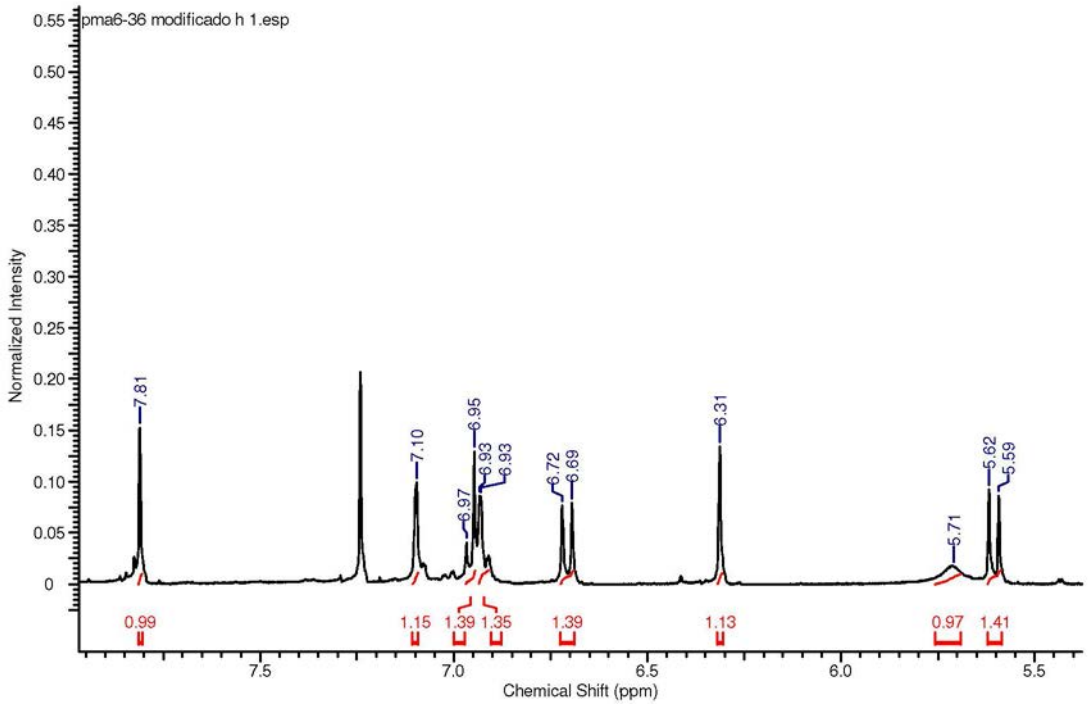


Figure S20. Expanded ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

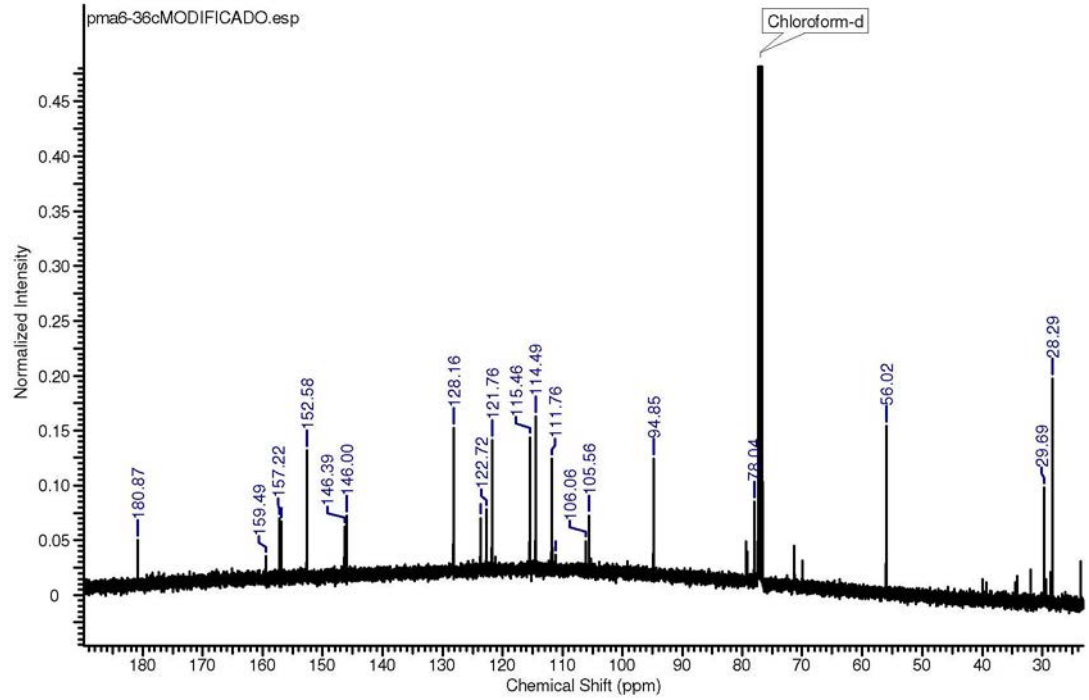


Figure S21. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of isoflavone **3**.

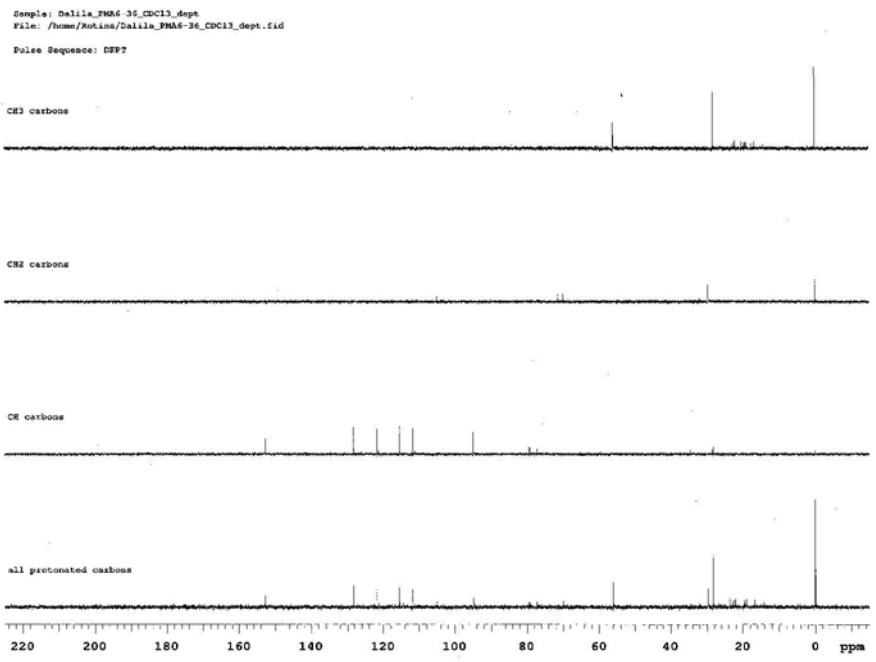


Figure S22. DEPT spectrum (100 MHz, $CDCl_3$) of isoflavone **3**.

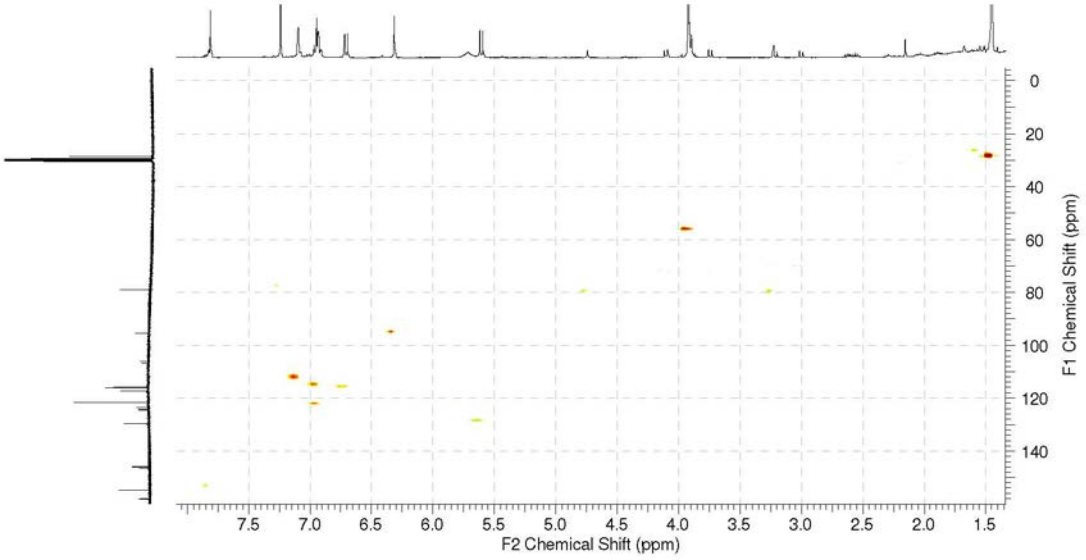


Figure S23. HMQC spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

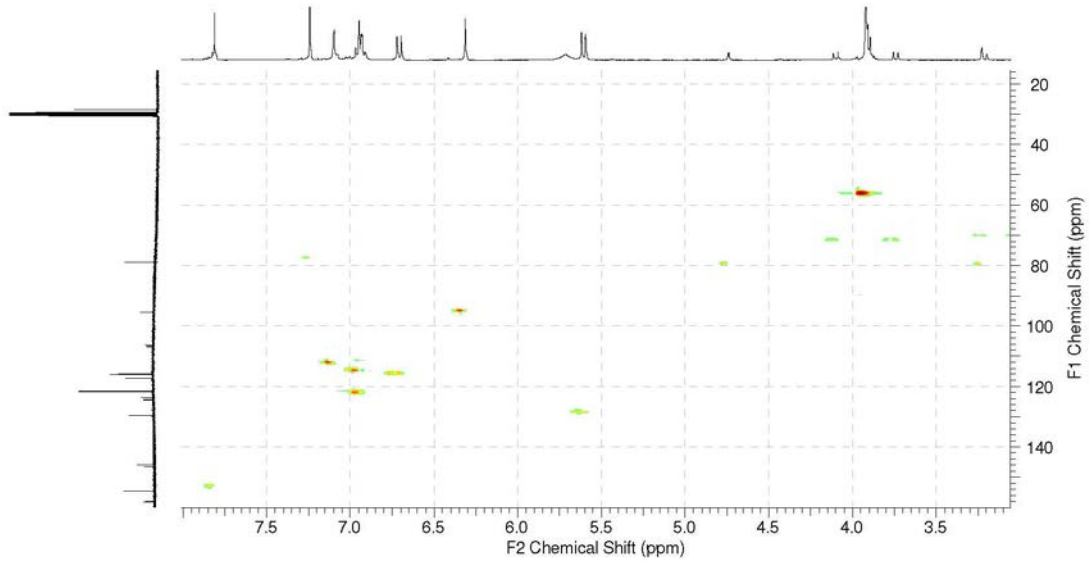


Figure S24. Expanded HMQC spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

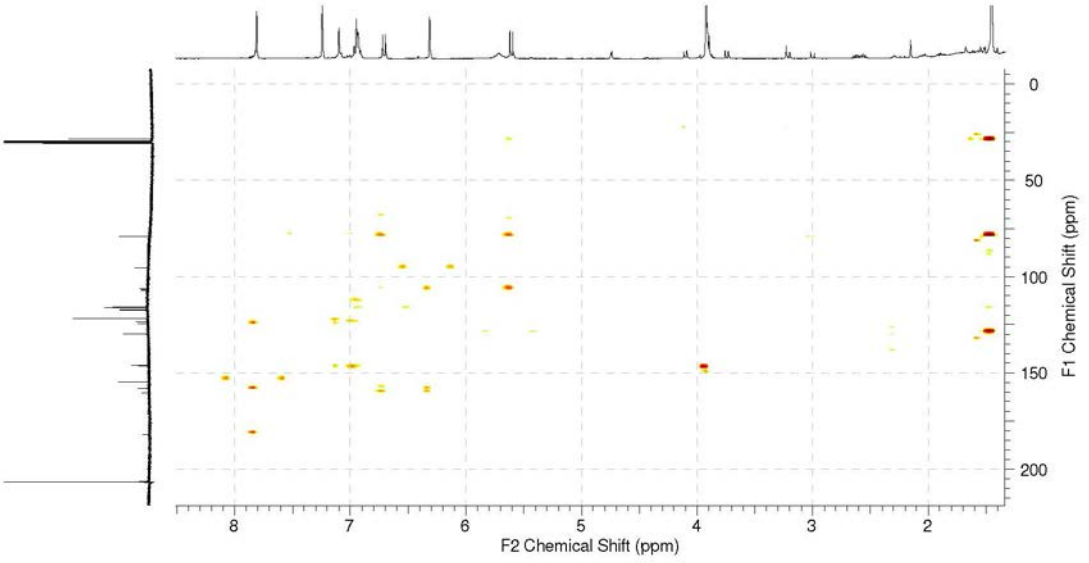


Figure S25. HMBC spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

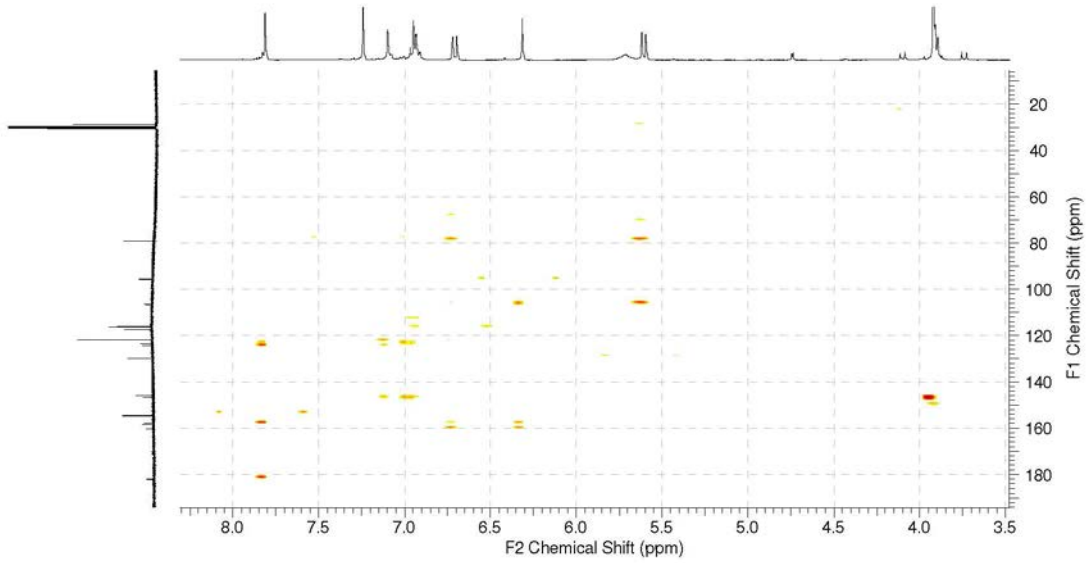


Figure S26. Expanded HMBC spectrum (400 MHz, $CDCl_3$) of isoflavone **3**.

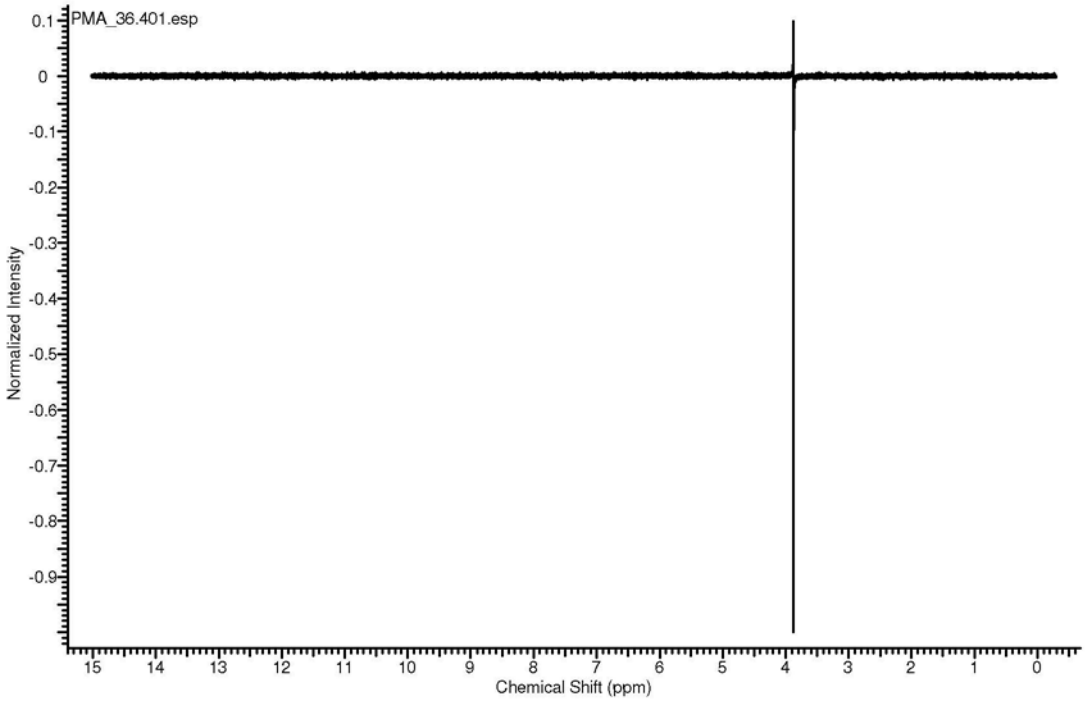


Figure S27. NOE spectrum (400 MHz, $CDCl_3$) of isoflavone **3** (H-2´ irradiated).

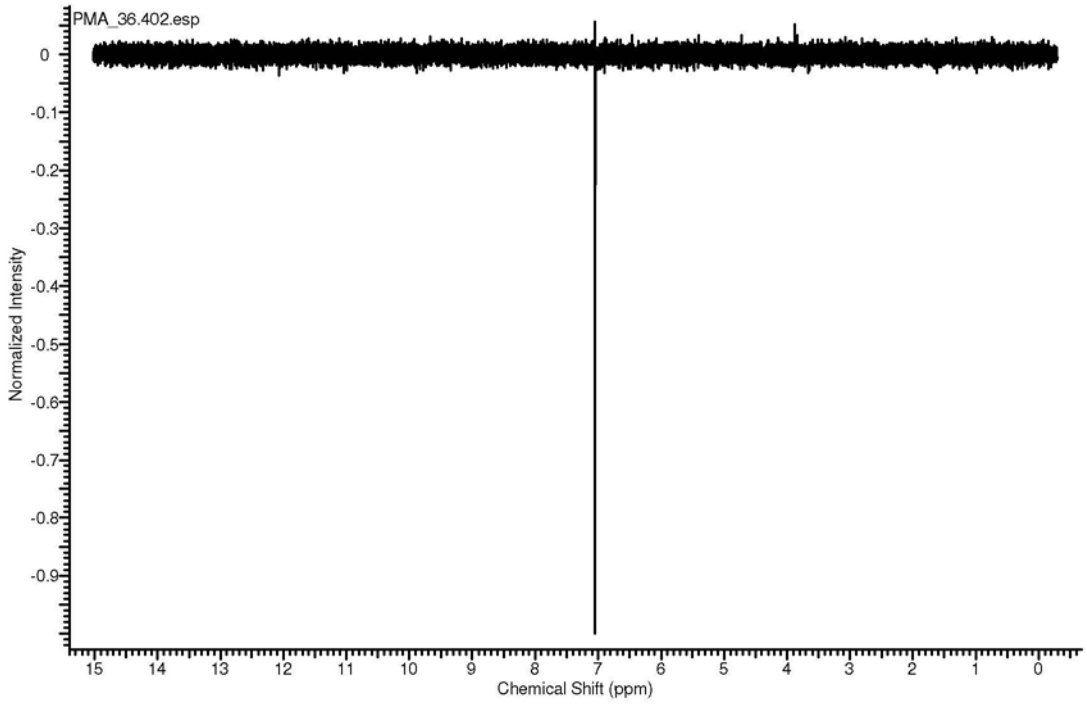


Figure S28. NOE spectrum (400 MHz, $CDCl_3$) of isoflavone **3** (OME-3´ irradiated).

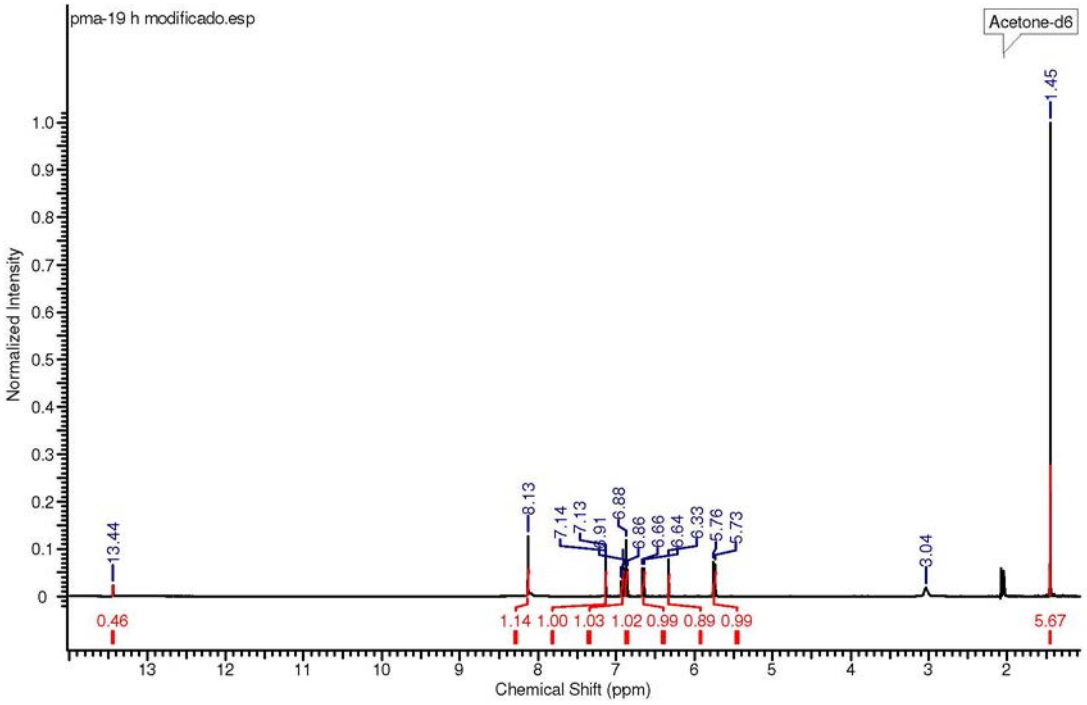


Figure S29. ^{1}H NMR spectrum (400MHz, C_3D_6O) of isoflavone **4**.

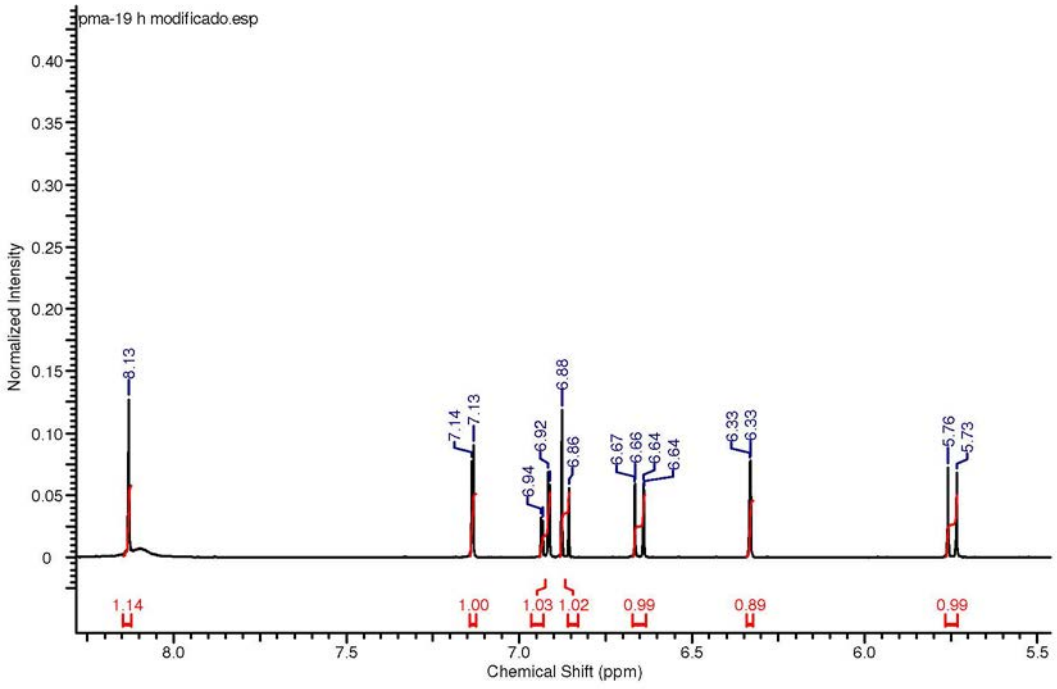


Figure S30. Expanded ^{1}H NMR spectrum (400MHz, C_3D_6O) of isoflavone **4**.

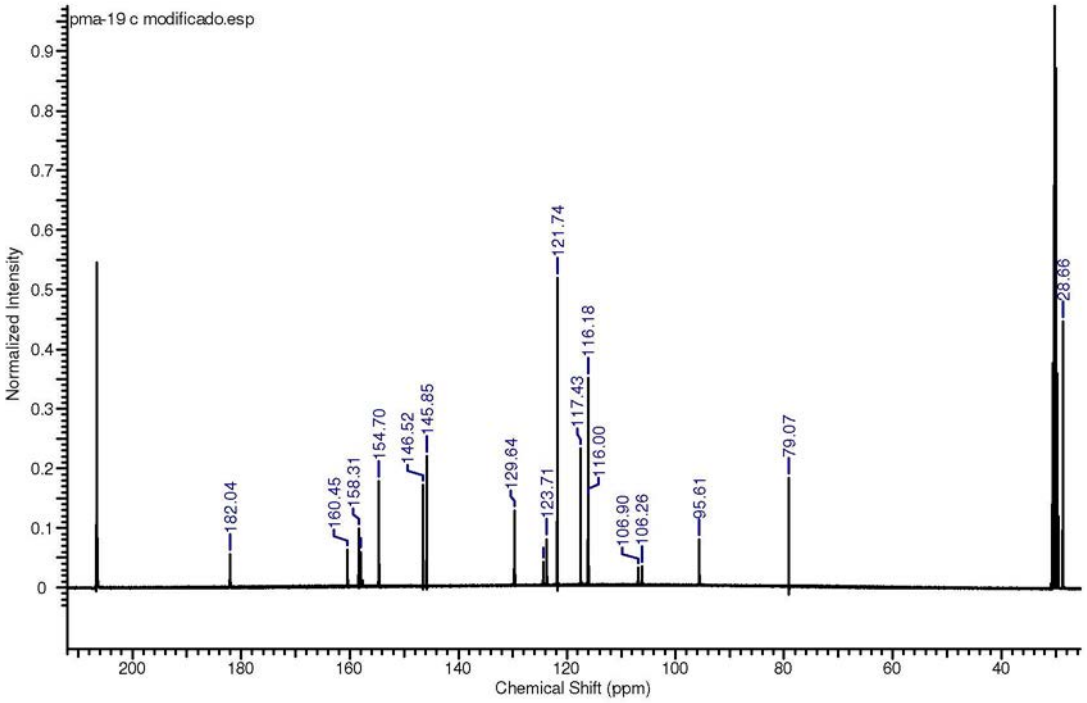


Figure S31. ^{13}C NMR spectrum (100MHz, C_3D_6O) of isoflavone **4**.

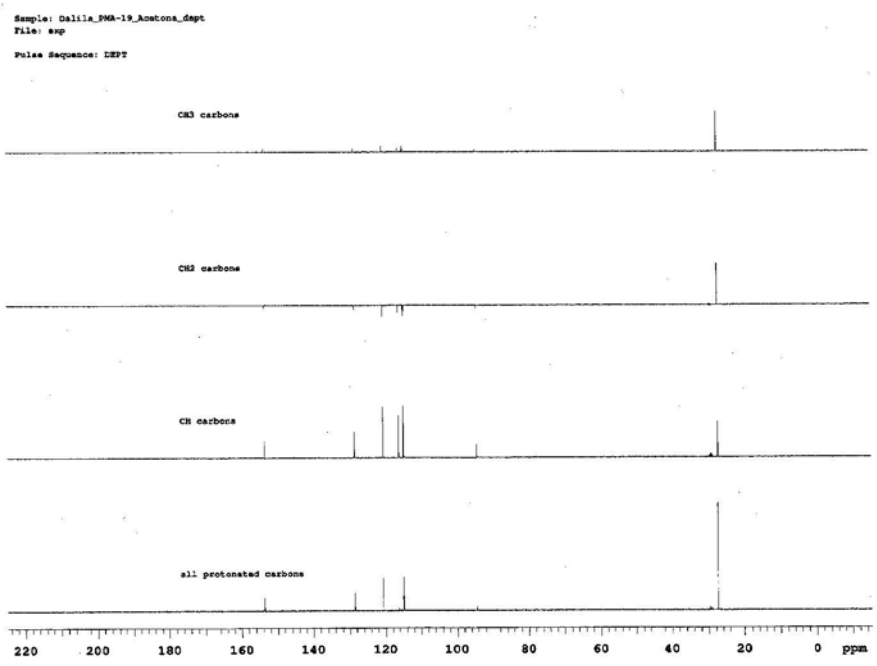


Figure S32. DEPT spectrum (100MHz, C_3D_6O) of isoflavone **4**.

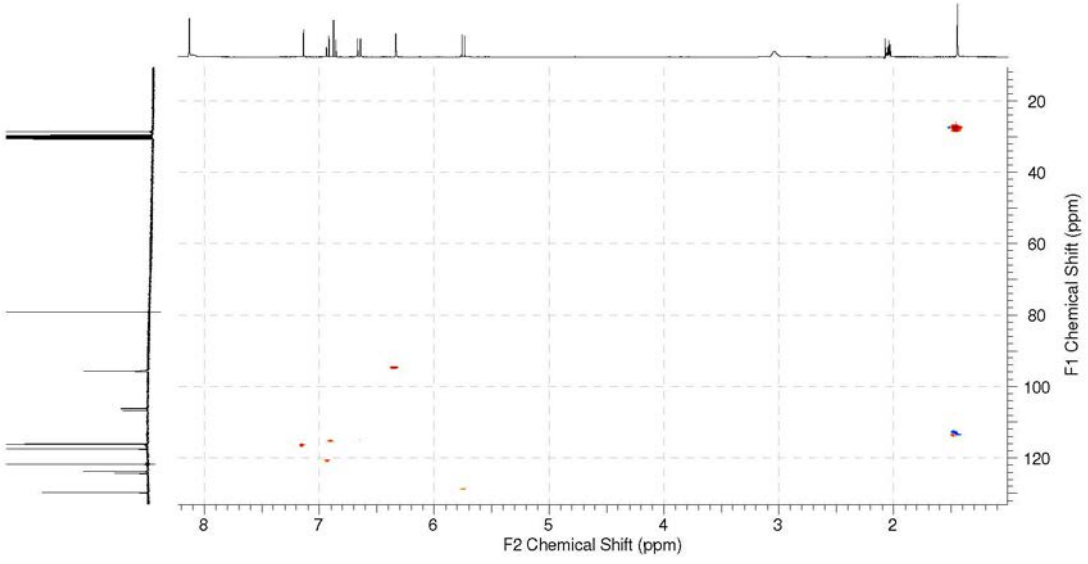


Figure S33. HMQC spectrum (400MHz, C_3D_6O) of isoflavone **4**.

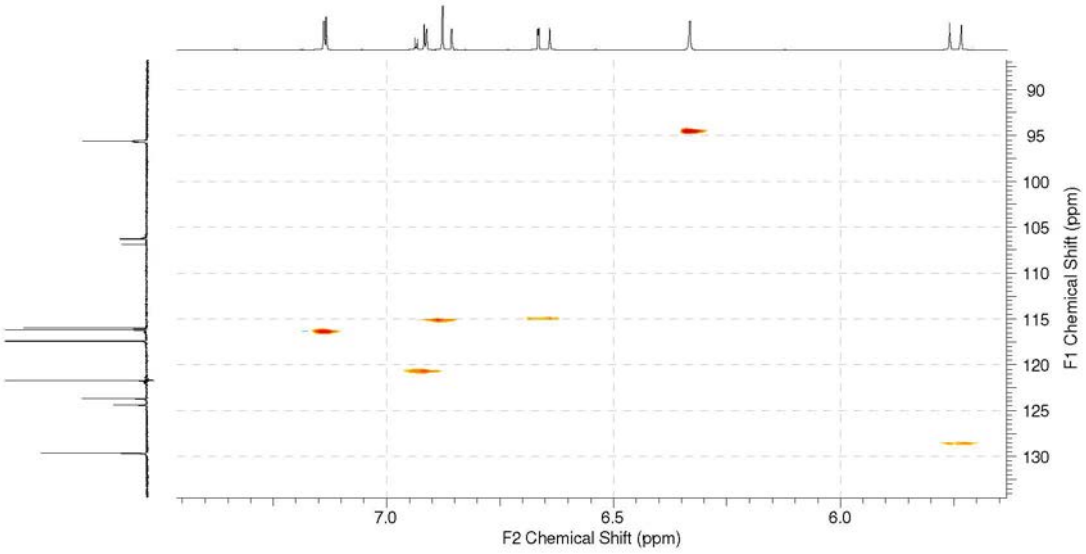


Figure S34. Expanded HMQC spectrum (400MHz, C_3D_6O) of isoflavone **4**.

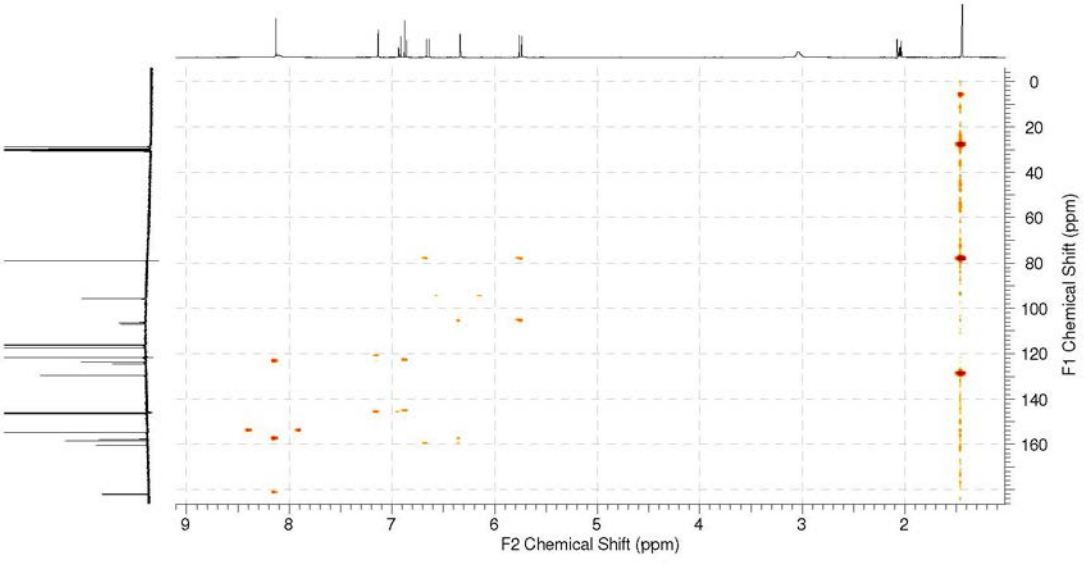


Figure S35. HMBC spectrum (400MHz, C_3D_6O) of isoflavone **4**.

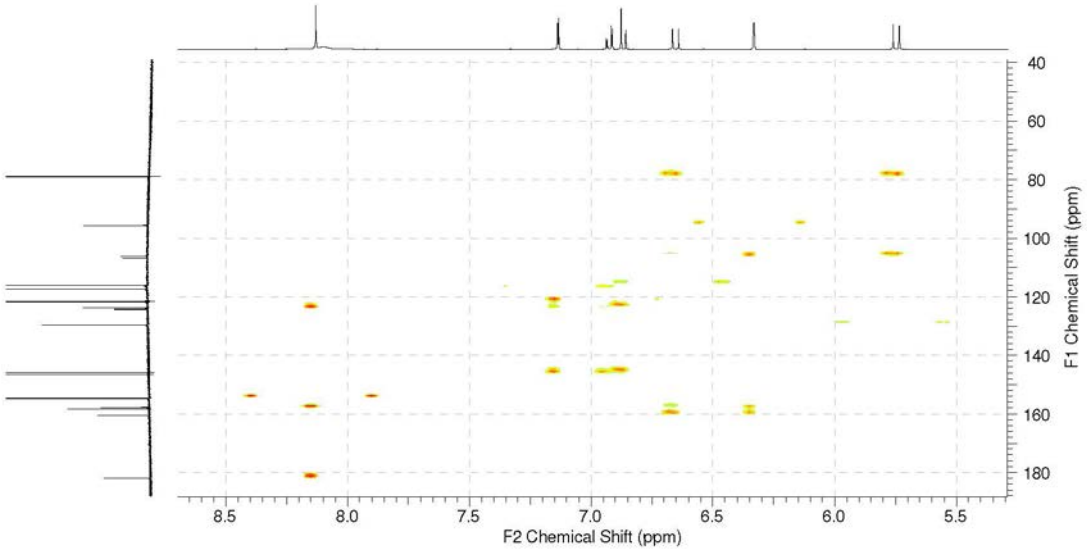


Figure S36. Expanded HMBC spectrum (400MHz, C_3D_6O) of isoflavone **4**.

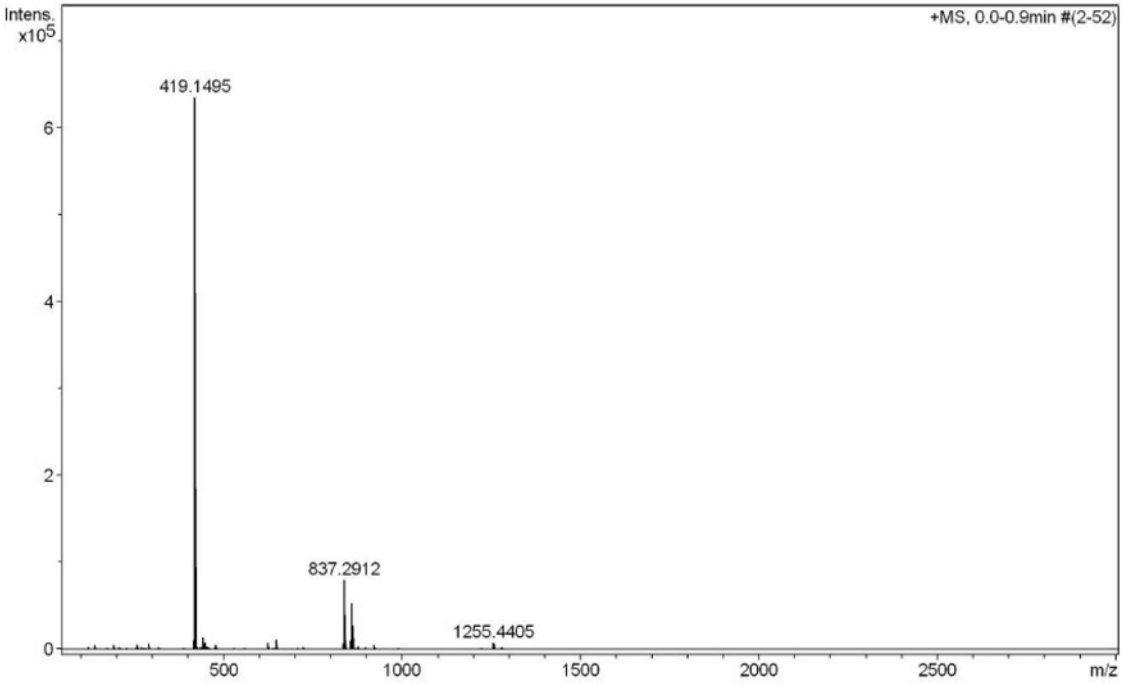


Figure S37. MS spectrum of isoflavone **1** (Part 1).

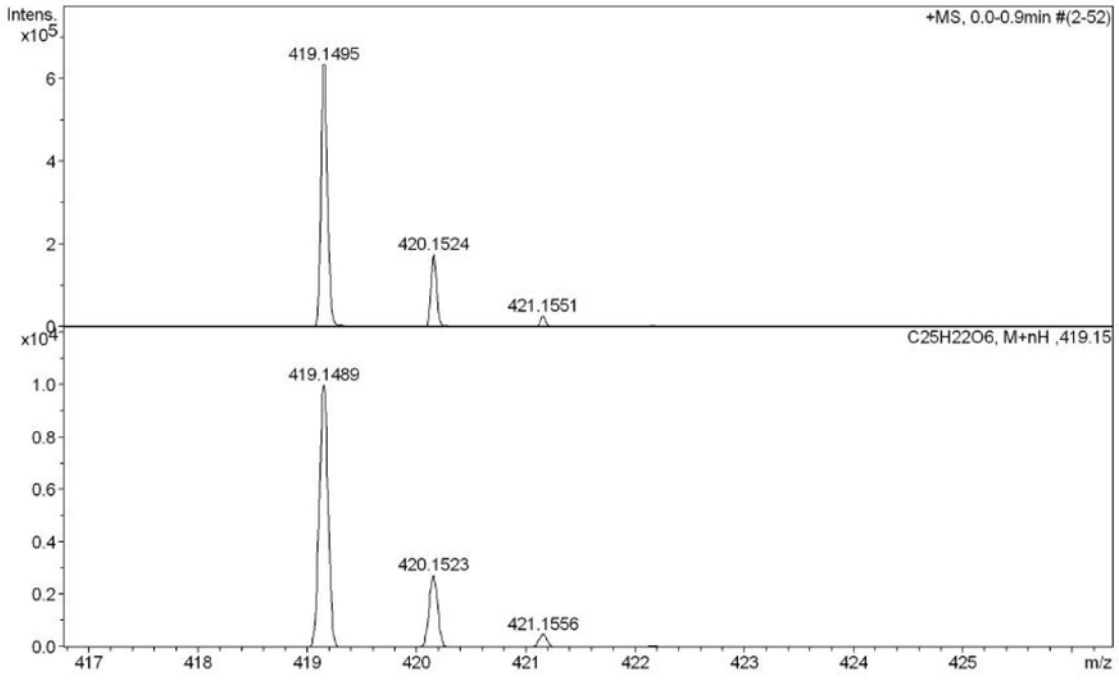


Figure S38. MS spectrum of isoflavone **1** (Part 2).

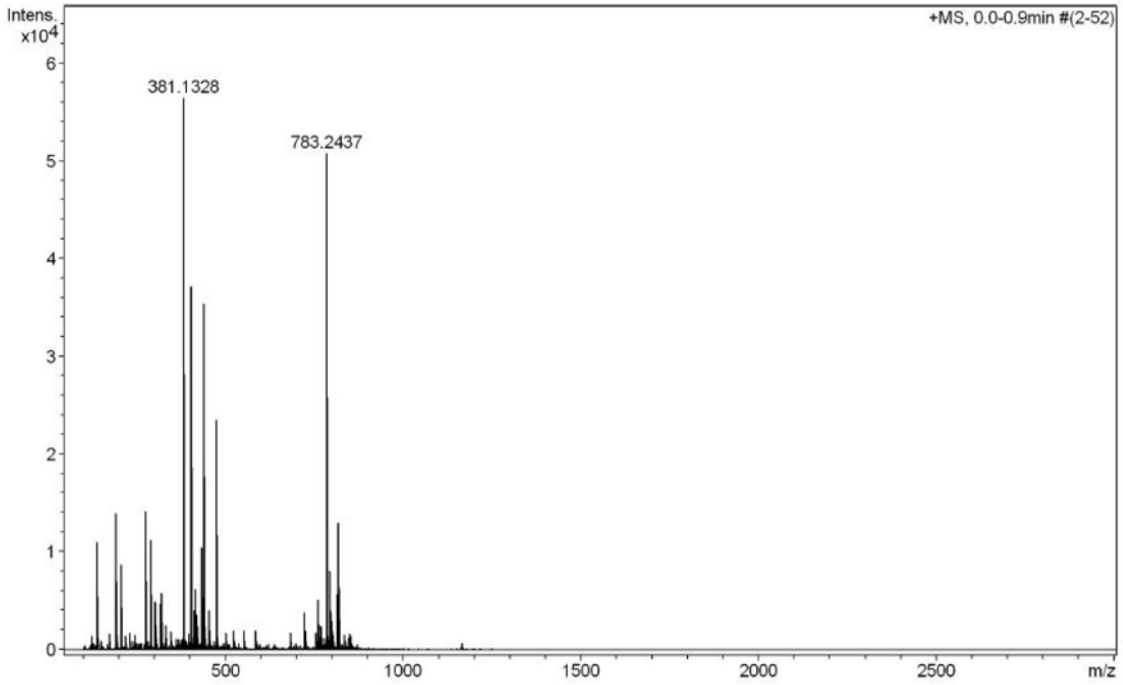


Figure S39. MS spectrum of isoflavone **2** (Part 1).

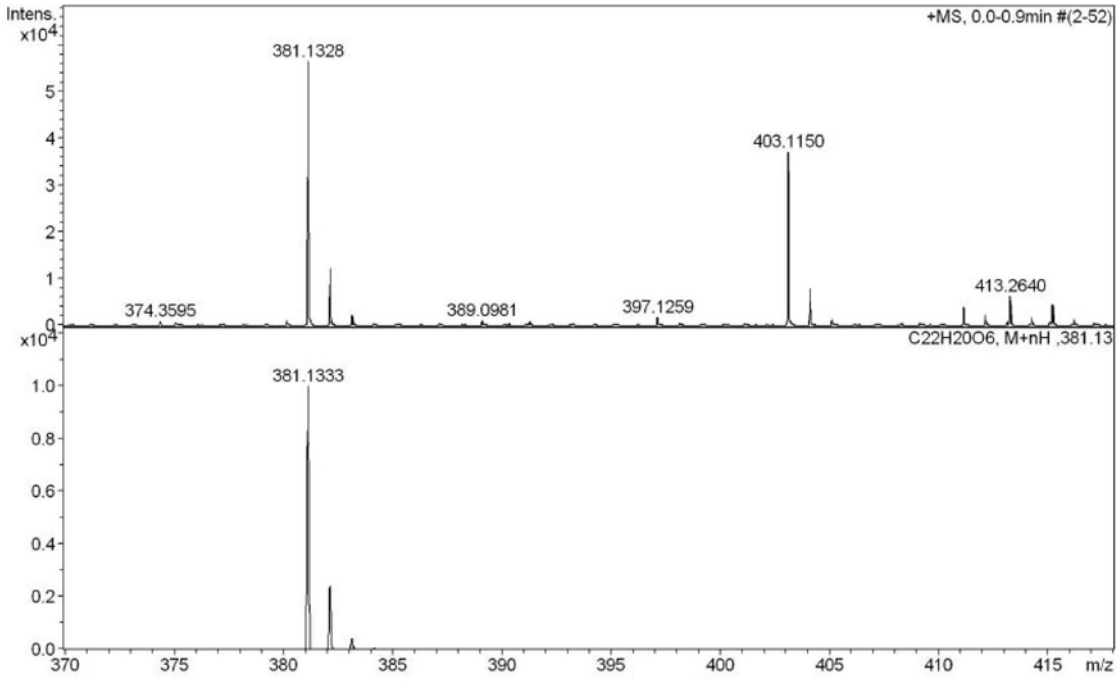


Figure S40. MS spectrum of isoflavone **2** (Part 2).

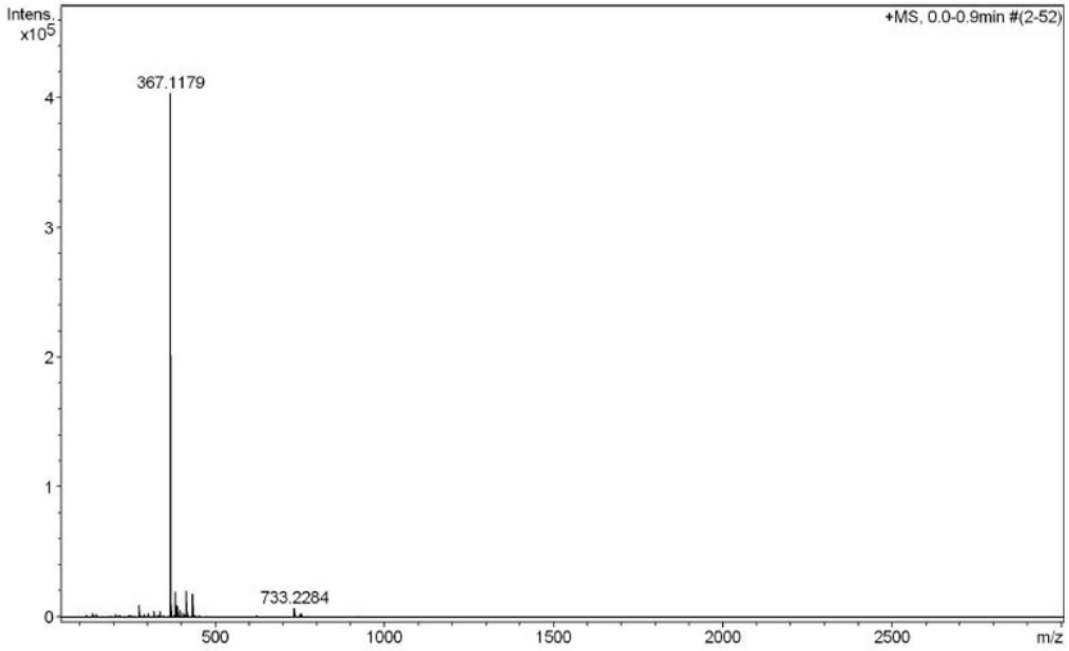


Figure S41. MS spectrum of isoflavone **3** (Part 1).

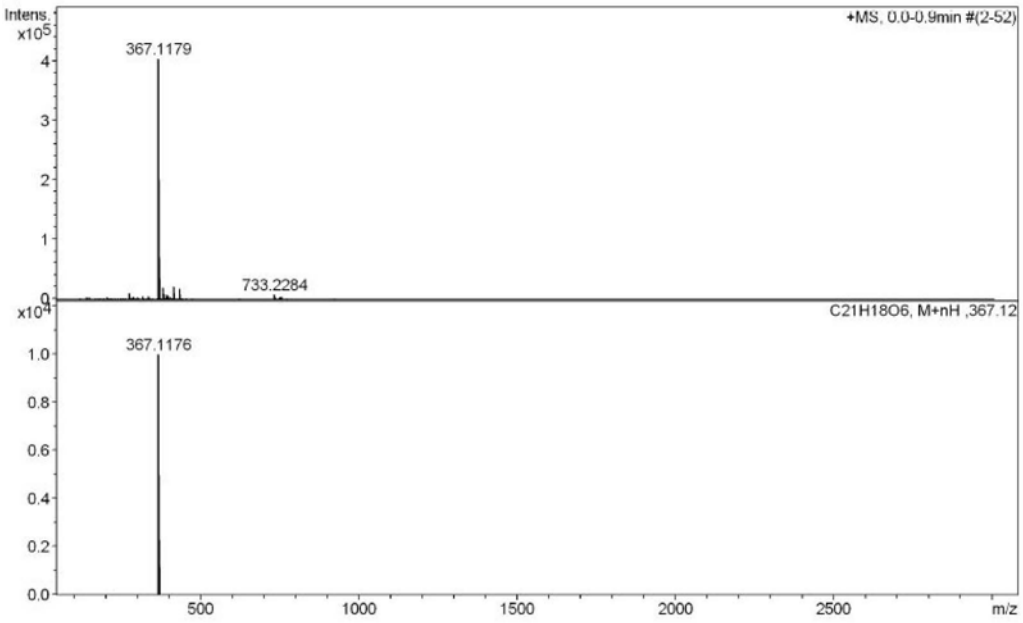


Figure S42. MS spectrum of isoflavone **3** (Part 2).

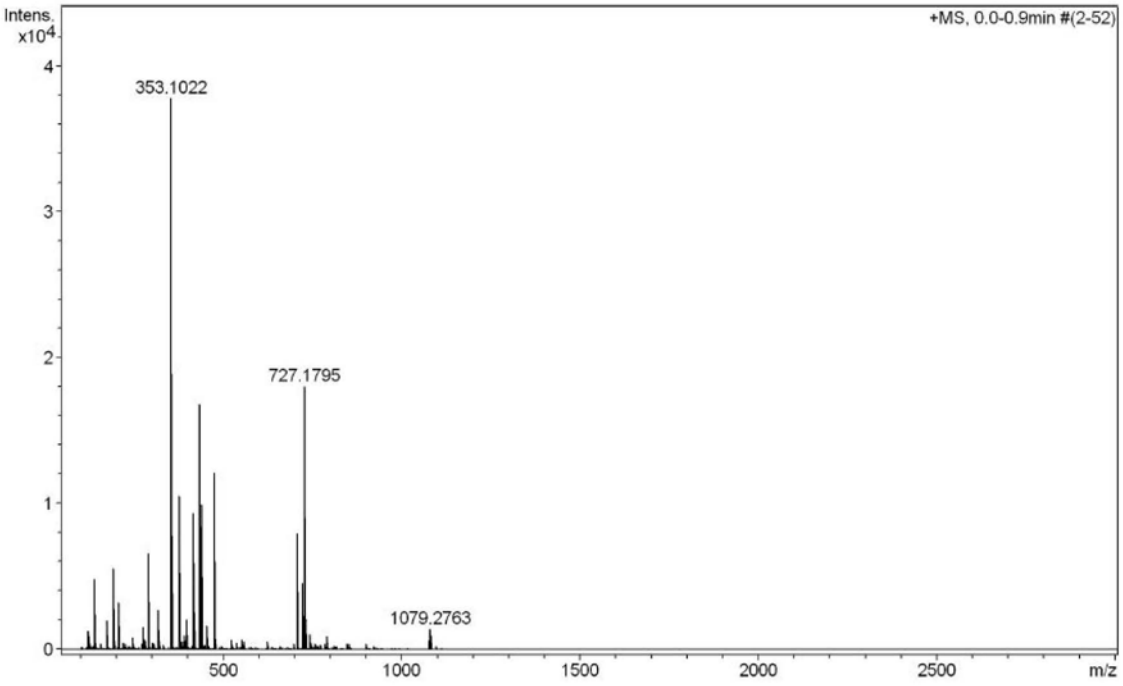


Figure S43. MS spectrum of isoflavone **4** (Part 1).

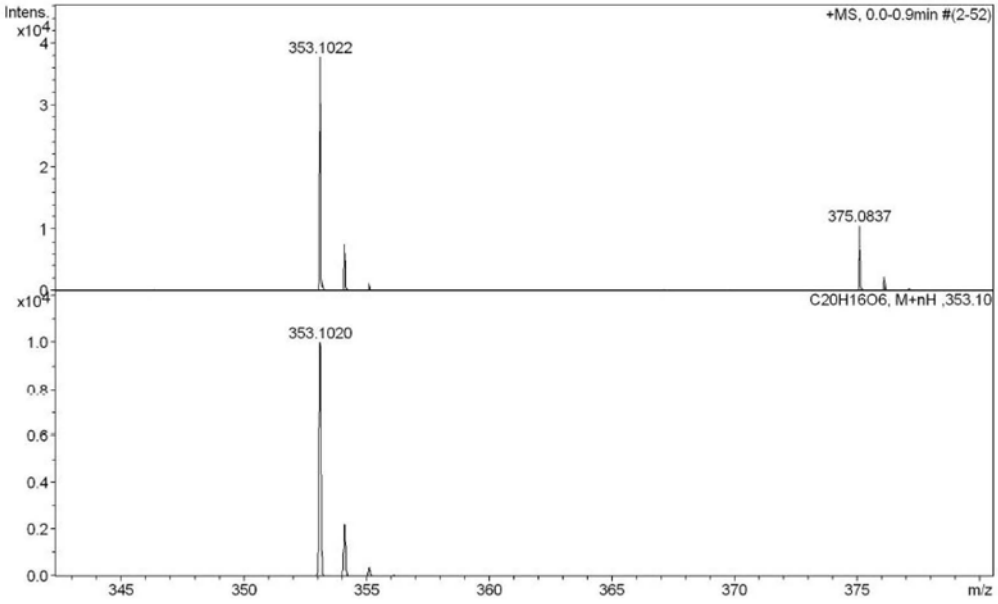


Figure S44. MS spectrum of isoflavone **4** (Part 2).