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Artigo

OCCURRENCE OF BIFLAVONES IN LEAVES OF *Caesalpinia pyramidalis* SPECIMENS

Marcus V. Bahia
Centro de Formação de Professores, Universidade Federal do Recôncavo da Bahia, 45300-000 Amargosa – BA, Brasil
Juceni P. David
Faculdade de Farmácia, Universidade Federal da Bahia, 40170-290 Salvador - BA, Brasil
Jorge M. David*
Instituto de Química, Universidade Federal da Bahia, 40170-290 Salvador - BA, Brasil

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The chloroform partition of methanol extract of leaves of *Caesalpinia pyramidalis* was submitted to different chromatographic procedures which afforded besides agathisflavone and taxifolin, the minor biflavones loniflavone, amentoflavone, 5'-hydroxyamentoflavone and podocarpusflavone A. The structures of the compounds were established on the basis of NMR and MS data analysis. Besides, the content of biflavones of different specimens of *C. pyramidalis,* which are collected in different habitats of the Brazilian semi-arid region, was determinated by LC-APCI-MS analysis. These analysis demonstrated that only the specimens harvested in Bahia state showed collectively the presence of agathisflavone, amentoflavone, sequoiaflavone and podocarpusflavone A.

Keywords: *Caesalpinia pyramidalis;* biflavones; flavonoid analysis.

INTRODUCTION

Caesalpinia pyramidalis Tul. is a tree belonging to the family Leguminosae-Caesalpinoideae, which is endemic of Brazilian northeastern, especially in the caatinga. It is popularly known as "catingueira" or "pau-de-rato" and its leaves are used in the preparation of infusions and decocts, which are used by the local population as diuretic, antidispeptic, stomach aches and for fever.[1]

Species of this genus are known to present different biological activities. For instance, the extract of seeds of *C. bonducella* showed antimicrobial activities[2] and they are also employed in the treatment of diabetes.[3] Extracts of *C. volkensii* and *C. pluviosa* presents antimalarial activity[4] and *C. pulcherrima* antiviral activity.[5] The extract of *C. pyramidalis* is responsible for antibacterial,[6] larvicidal and moluscicidal[7] activities.

The presence of diterpenes and flavonoids besides other phenolics is characteristic of this genus as well as the subfamily.[8] From the previous studies regarding with *C. pyramidalis* were isolated phenylpropanoids, biflavonoids, lignan, flavonoids besides gallic acid.[9]

In the present study it was reported the isolation and characterization of the minor biflavonoids loniflavone (**1**), amentoflavone (**2**), 5 -hydroxyamentoflavone (**3**) and podocarpusflavone A (**5**), besides agathisflavone (**4**) and taxifolin (**6**) from the leaves of *C. pyramidalis* (Figure 1). It was also evaluated by LC-MS the composition of biflavonoids present in leaves of specimens of *C. pyramidalis* collected in different habitats of Brazilian semi-arid region.

EXPERIMENTAL

General procedures

The NMR spectra were obtained in Varian Gemini 2000, Inova 500 and Bruker AMX500 spectrometers operating at 300 and 500 MHz (^{1}H) and, 75 and 125 MHz (^{13}C), employing CD_3OD, C_5D_5N and $(CD_3)_2CO$ as solvents and TMS as internal standard. The MS and the LC-MS analysis were carried out in a Shimadzu chromatographer mod.

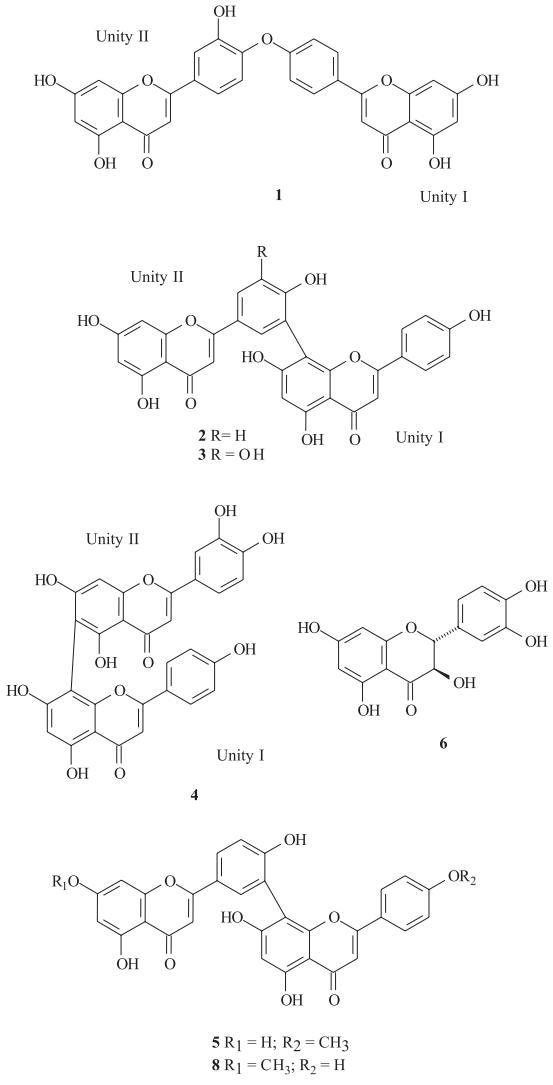

Figure 1. *Biflavonoids isolated from leaves of Caesalpinia pyramidalis*

*e-mail: jmdavid@ufba.br

Table 1. Biflavonoid variation (%) found in diverse specimens of *C. pyramidalis*

Specimen	Agathisflavone	Amentoflavone	Sequoiaflavone	Podocarpusflavone A
Feira de Santana*	0.5	-	-	-
Feira de Santana* (tree age: 14 years old)	0.05	-	-	-
Feira de Santana*(tree age: 10 years old)	0.001	-	-	-
Ipirá*	0.01	-	-	-
Riachão do Jacuípe*	-	0.03	0.001	-
Valente*	1.0	0.001	-	0.005
Serra Talhada**	-	-	-	-
Sertânia**	-	-	-	-
Santa Luzia***				

*Bahia State, **Pernambuco State, ***Paraíba State

LCMS-2310, with autosampler 5 µL loop. The detection of biflavonoids was obtained in positive and negative APCI mode. The chromatograms were obtained using a VP-ODS (RP18 - 5 µm; 3.9 x 150 mm) column and as mobile phase the isocratic system of MeOH:H_2O (75:25), with flow rate of 0.2 mL min^{-1}. The pure biflavonoids used as standards were kindly provided by Prof. M. G. de Carvalho (UFRRJ). In the conventional chromatographic methods were used for CC silica gel 60 (63-200 µm) and Sephadex LH-20 and, it was used silica gel TLC plates to monitor the chromatographic fractions which were revealed employing iodine fumes and UV light (254/366 nm).

Plant material

Botanical material of *Caesalpinia pyramidalis* were collected in the surroundings of Valente (BA), Feira de Santana (BA), Riachão do Jacuípe (BA), Ipirá (BA), Serra Talhada (PE), Sertânia (PE) and Santa Luzia (PB). The specimens were identified by Prof. Dr. L. P. de Queiróz (Universidade Estadual de Feira de Santana) and Prof[a]. Dr[a]. M. de F. Agra (Laboratório de Tecnologia Farmacêutica UFPB). The vouchers were deposited at Herbário Alexandre Leal da Costa of Instituto de Biologia of Universidade Federal da Bahia under number 240291.

Extraction and isolation

The dried and grounded leaves (680 g) were submitted to extraction with MeOH for 48 h. The methanolic extract was sequentially submitted to partition between hexane and MeOH:H_2O (9:1) furnishing the hydroalchoolic and hexanic phases (7.5 g). In sequence, it was added H_2O in the hydromethanolic solution in order to obtain a solution of MeOH/H_2O (6:4). Thus, this hydroalchoolic solution was submitted to a partition between $CHCl_3$ furnishing the $CHCl_3$ phase (23.9 g).

The $CHCl_3$ phase was submitted to a silica gel CC using mixtures of $CHCl_3$:EtOAc in increasing polarities. The fractions eluted with $CHCl_3$:EtOAc (6:4) were grouped (936.4 mg) after TLC analysis and submitted to a Sephadex LH-20 CC using a mixture of CH_2Cl_2:$(CH_3)_2CO$ (1:4) with permitted to obtain enriched phenol fractions (254.4 mg). This mixture was submitted to a silica gel 60 CC and eluted with $CHCl_3$:MeOH (95:5 to 8:2) which permitted to obtain the compounds **1** (7.9 mg), **2** (6.3 mg), **3** (9.3 mg), **4** (100.0 mg), **5** (15.5 mg) and **6** (9.5 mg).

Determination of biflavonoids in specimens of *C. pyramidalis*

The leaves of *C. pyramidalis* (100 g) collected in different places were submitted to extraction with hexane and $CHCl_3$. The $CHCl_3$ phase was submitted to CC over silica gel 60 and mixtures of $CHCl_3$:EtOAc (Table 1). The biflavonoid enriched fractions were eluted with $CHCl_3$: EtOAc (1:1). These fractions were submitted to a LC-MS analysis in order to determinate the content of biflavonoids.

Loniflavone (**1**). Amorphous yellow power. m. p. 242-244 °C. MS (APCI) *m/z*= 537 [M - H]$^-$.^{1}H NMR [500 MHz, $(CD_3)_2CO$]: Unity I: δ 6.71 (*s*, 1H, H-3); δ 12.92 (*s*, 1H, H-5); δ 6.26 (*d*, 1H, *J* = 2.0 Hz, H-6); δ 6.54 (*d*, 1H, *J* = 2.0 Hz, H-8); δ 8.06 (*d*, 2H, *J* = 9.0 Hz, H-2'/H-6'); δ 7.14 (*d*, 2H, *J* = 9.0 and 2.0 Hz, H-3'/H-5'); Unity II: δ 6.70 (*s*, 1H, H-3); δ 12.93 (*s*, 1H, H-5); δ 6.27 (*d*, 1H, *J* = 2.0 Hz, H-6); δ 6.55 (*d*, 1H, *J* = 2.0 Hz, H-8); δ 7.89 (*d*, 1H, *J* = 2.0 Hz, H-2'); δ 7.28 (*d*, 1H, *J* = 8.0 Hz, H-5'); δ 7.91 (*dd*, 1H, *J* = 8.0 and 2.0 Hz, H-6').

Amentoflavone (**2**). Amorphous yellow power. m. p. 254-256 °C. MS (APCI) *m/z*= 538. ^{1}H NMR (300 MHz, CD_3OD): Unity I: δ 6.56 (*s*, 1H, H-3); δ 6.22 (*s*, 1H, H-6); δ 7.66 (*d*, 2H, *J* = 8.7 Hz, H-2'/H-5'); δ 6.58 (*d*, 2H, *J* = 8.7 Hz, H-3'/H-5'); Unity II: δ 6.49 (*s*, 1H, H-3); δ 5.99 (*d*, 1H, *J* = 2.0 Hz, H-6); δ 6.05 (*d*, 1H, *J* = 2.0 Hz, H-8); δ 8.26 (*d*, 1H, *J* = 2.3 Hz, H-2'); δ 7.09 (*d*, 1H, *J* = 8.7 Hz, H-5'); δ 7.88 (*dd*, 1H, *J* = 8.7 and 2.3 Hz, H-6'). ^{13}C NMR (75 MHz, CD_3OD): Unity I: δ 165.5 (C, C-2); δ 102.7 (CH, C-3); δ 182.8 (C, C-4); δ 162.4 (C, C-5); δ 100.1 (CH, C-6); δ 164.2 (C, C-7); δ 103.8 (C, C-8); δ 156.6 (C, C-9); δ 108.5 (C, C-10); δ 121.9 (C, C-1'); δ 129.3 (CH, C-2'/C-6'); δ 115.1 (CH, C-3'/C-5'); δ 162.0 (C, C-4'); Unity II: δ 165.6 (C, C-2); δ 102.9 (CH, C-3); δ 183.8 (C, C-4); δ 163.5 (C, C-5); δ 99.9 (CH, C-6); δ 163.5 (C, C-7); δ 94.8 (CH, C-8); δ 156.6 (C, C-9); δ 108.5 (C, C-10); δ 122.2 (C, C-1'); δ 132.3 (CH, C-2'); 123.1 (C, C-3'); δ 161.9 (C, C-4'); δ 117.4 (CH, C-5'); δ 128.7 (CH, C-6').

5'-Hydroxyamentoflavone (**3**). Amorphous yellow power. m. p. 288-290 °C. MS (APCI) *m/z*= 554. ^{1}H NMR (500 MHz, C_5D_5N): Unity I: δ 6.94 (*s*, 1H, H-3); δ 6.75 (*d*, 1H, *J* = 1.8 Hz, H-6); δ 6.84 (*d*, 1H, *J* = 1.8 Hz, H-8); δ 8.56 (*d*, 1H, *J* = 2.5 Hz, H-2'); δ 7.96 (d, 1H, *J* = 2.5 Hz, H-6'); Unity II: δ 6.93 (*s*, 1H, H-3); δ 6.89 (*s*, 1H, H-6); δ 7.89 (*d*, 2H, *J* = 8.5 Hz, H2'/H6'); δ 7.47 (*d*, 2H, *J* = 8.5 Hz, H3'/H5').

Taxifolin (**6**). Yellow cristals. m. p. 242 °C. ^{1}H NMR (500 MHz, CD_3OD): δ 4.92 (*d*, 1H, *J* = 11.0 Hz, H-2); δ 4.50 (*d*, 1H, *J* = 11.0 Hz, H-3); δ 5.91 (*d*, 1H, *J* = 2.0 Hz, H-6); δ 5.89 (*d*, 1H, *J* = 2.0 Hz, H-8); δ 6.86 (*dd*, 1H, *J* = 2.0 and 8.0 Hz, H-6'); δ 6.81 (*d*, 1H, *J* = 8.0 Hz, H-5'); δ 6.97 (*d*, 1H, *J* = 2.0 Hz, H-2'). RMN ^{13}C (75 MHz, CD_3OD): δ 85.09 (CH, C-2); δ 73.64 (CH, C-3); δ 198.37 (C, C-4); δ 164.27 (C, C-5); δ 97.29 (CH, C-6); δ 168.72 (C, C-7); δ 96.27 (CH, C-8); δ 164.47 (C, C-9); δ 101.79 (C, C-10); δ 129.82 (C, C-1'); δ 115.85 (CH, C-2'); δ 116.05 (CH, C-5'); δ 147.11 (C, C-4'); δ 146.28 (C, C-3'); δ 120.88 (CH, C-6').

RESULTS AND DISCUSSION

The structure of compound **1** was elucidated by MS and NMR data analysis. The pseudo-molecular ion observed at *m/z* 537 [M-H]

recorded in negative MS-APCI mode together with ^{1}H and ^{13}C NMR data, including DEPT, permitted to propose the molecular formula $C_{30}H_{18}O_{10}$ and consequently determinate the biflavonoid nature of compound **1**. It was observed two singlets at δ 12.93 and 12.92 in the ^{1}H NMR spectra, whose disappeared in presence of D_2O, they were indicative of presence of two flavonoid units bearing hydroxyl groups at the C-5 with hydrogens bonding at carbonyl groups (C-4). The presence of two other singlets (δ 6.71 and 6.70) in the same spectra was characteristic of H-3 of flavones. It was also possible to verify the presence of a 1,4-disubstitued B ring for one unity (I) due the doublets observed at δ 8.06 and 7.14 (J = 9.0 and 2.0 Hz). For the other B ring unity (II) the spectra showed peaks of an AMX system at δ 7.91 (J = 8 and 2 Hz), 7.89 (J =2 Hz) and 7.28 (J =8 Hz). The presence of four doublets for A ring (J = 2 Hz each) indicated the linkages between the two unities of flavones were not placed in these rings. The ^{13}C NMR spectra data (Experimental Section) and the ^{1}H-^{1}H gCOSY showed hydrogen coupling systems and corroborated compound **1** was a biflavone.

The analysis of the HSQC spectra of **1** permitted to verify correlations which were important to distinguish the carbons and the respective hydrogens. So, this spectra was important in the identification of the aromatic carbons of 1,4-dissubstitued system of B ring of Unity I. In the same way it contributed to recognize the resonances of aromatic carbons of B ring of Unity II.

Comparison of NMR data (Table 2) of compound **1** with loniflavone and ochnaflavone[10] (**7**) was important to propose that the linkage between both units occurred by B rings. The HMBC correlations indicated **1** was a C-4'-O-C-4' biflavone through the observed correlations of H-6' (δ 7.91) and C-4' (δ 142.8), H-2'/H-5' and C-3' and C-4' (Figure 2). These findings permitted to identify **1** as being loniflavone. This is the second occurrence of this biflavone, it was previously isolated in *Lonicera japonica* (Caprifoliaceae).[11]

Table 2. ^{13}C NMR data of **1**, loniflavone and ochnaflavone [125 MHz, $(D_3C)_2CO$, δ (ppm)]

C		δ ^{13}C	
Unity I/Unity II	**1**	Loniflavone[11]	Ochnaflavone[10]
2/2	163.63/162.98	163.4/163.1	163.1/163.5
3/3	104.80/104.49	105.0/104.8	105.1/104.8
4/4	182.71/182.68	182.5/182.5	183.1/182.9
5/5	161.61/161.61	161.1/161.1	163.4/103.2
6/6	99.50/99.50	99.9/99.9	99.9/96.9
7/7	164.77/164.72	166.1/166.1	165.0/167.4
8/8	94.51/94.49	94.7/94.7	94.8/95.9
9/9	158.46/158.40	157.4/157.4	158.8/166.3
10/10	104.99/105.03	105.0/104.7	105.4/103.2
1'/1'	125.84/124.09	125.8/125.1	126.0/132.3
2'/2'	128.85/121.35	125.8/121.4	129.1/121.5
3'/3'	117.09/153.62	116.5/154.7	117.5/142.8
4'/4'	161.61/142.85	161.0/144.4	162.1/150.5
5'/5'	117.09/118.63	116.5/118.8	117.5/118.4
6'/6'	128.85/125.56	128.4/124.8	129.1/125.5

Compound **3** was identified by MS and NMR data analysis and comparison with literature. The molecular ion observed at *m/z* 554 in the APCI-MS together with ^{1}H and ^{13}C NMR data permitted to propose the molecular formula $C_{30}H_{18}O_{11}$ and determinate **3** was a biflavonoid. This compound was identify as 5'-hydroxyamentoflavone by comparison with amentoflavone[12] spectral data and with previously data published to this compound. This compound was formerly isolated from *Bartramia ithyphylla* (Bartramiaceae)[13] and

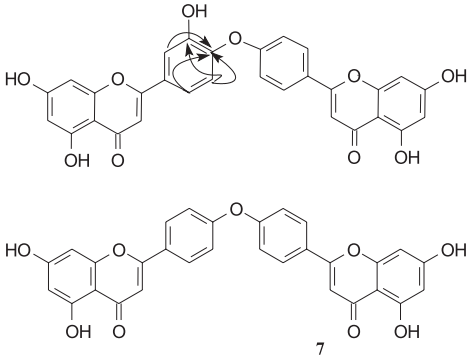

Figure 2. *Key correlations observed in the HMBC of 1 and structure of compound* ***7***

Rhytidiadelphus squarrosus (Hylocomiaceae)[14] but it is the first occurrence in Leguminosae.

Amentoflavone (**2**), agathisflavone (**4**), podocarpusflavone A (**5**) and taxifolin (**6**)[15] were identify by spectrometric data analysis and direct comparison with literature.

Since, in previous studies with leaves of *C. pyramidalis* it was isolated agathisflavone and other minor biflavonoids, it was developed a method to determinate the content of these compounds in different specimens of *C. pyramidalis* whose were collected in diverse neighborhoods of Brazilian "caatinga" region. For determination of the biflavones was utilized LC-APCI-MS analysis employing detection in negative mode and, agathisflavone, amentoflavone, sequoiaflavone (**8**)[16] and podocarpusflavone A were used as standards. So, the $CHCl_3$ extracts of leaves of different specimens of *C. pyramidalis* were submitted to a CC and the phenolic enriched fractions were injected in the HPLC. Table 1 summarizes the results obtained in these analysis in percentage relation of $CHCl_3$ extracts. In the samples harvested in Santa Luzia-PB, Serra Talhada-PE and Sertânia-PE was not detected the presence of any biflavones in the fractions. In all the specimens collected in Bahia State it was detected the occurrence of these compounds. However in some of them the presence of minor biflavonoids was widely changed. Nevertheless in all samples agathisflavone (**4**) was the biflavonoid present in major concentration (0.01-1%) and in one specimen (Riachão do Jacuípe) it was not detected. These preliminary findings indicate the occurrence of biflavonoids in *C. pyramidalis* can be dependent of habitat (e.g climate, soil composition), physical characteristics (age of tree) or botanical variations. Beside this, the presence of biflavonoids in Leguminosae is still rare. They are restricted only in selected species such as *Ormocarpum kirkii,*[17] *Diphysa robinioides,*[18] *Lupinus albus*[19] and *Dioclea lasiophylla*.[20] This is the first occurrence of **1**, **2**, **5** and **6** in the Leguminosae family.

SUPPLEMENTARY MATERIAL

Supplementary information as chromatograms of fractions of different specimes of *C. pyramidalis* and NMR spectra of biflavonoids are available free of charge as PDF file at http://quimicanova.sbq.org.br.

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Marcus V. Bahia
Centro de Formação de Professores, Universidade Federal do Recôncavo da Bahia, 45300-000 Amargosa – BA, Brasil
Juceni P. David
Faculdade de Farmácia, Universidade Federal da Bahia, 40170-290 Salvador - BA, Brasil
Jorge M. David*
Instituto de Química, Universidade Federal da Bahia, 40170-290 Salvador - BA, Brasil

Figure 1S. *Especimens of Caesalpinia pyramidalis (photos by J. M. David)*

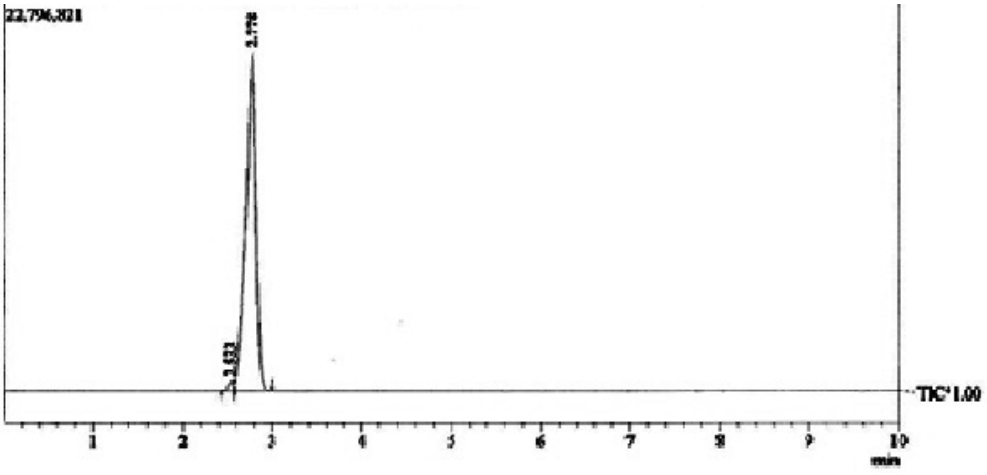

Figure 2S. *Chromatogram of the agathisflavone used as standard*

*e-mail: jmdavid@ufba.br

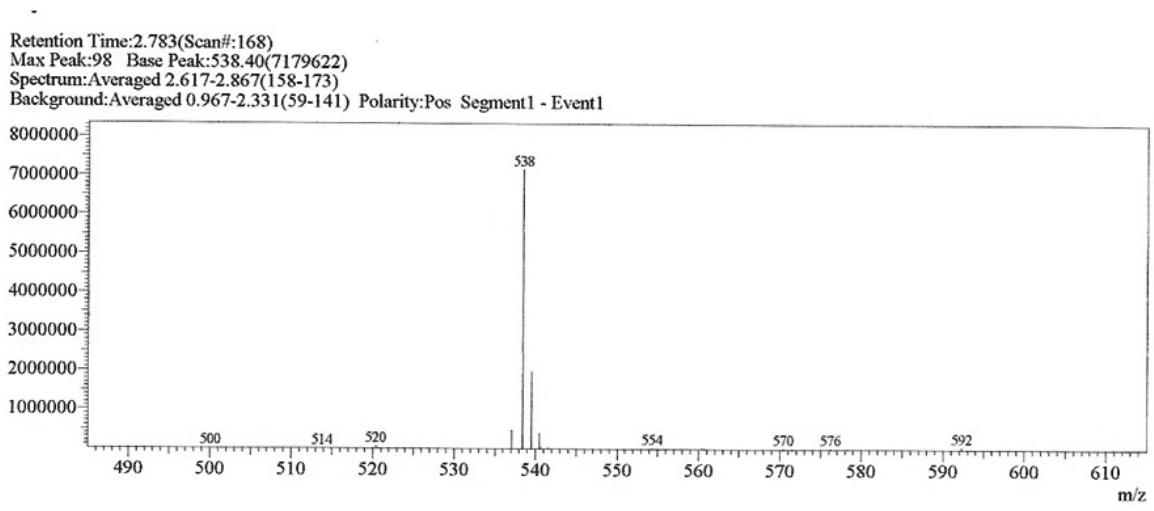

***Figure 3S**. APCI-Mass Spectrum of the agathisflavone standard*

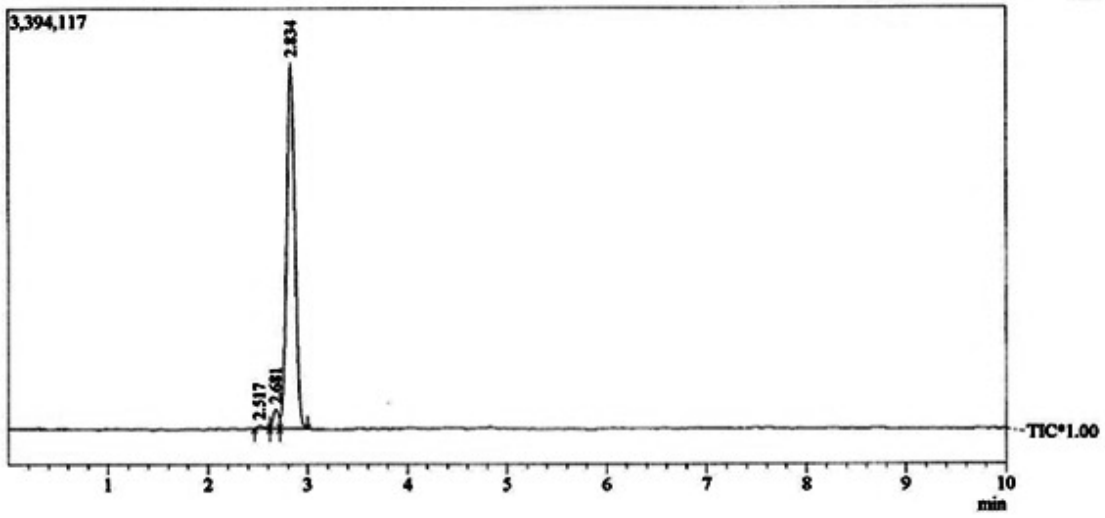

***Figure 4S.** Chromatogram of the amentoflavone standard*

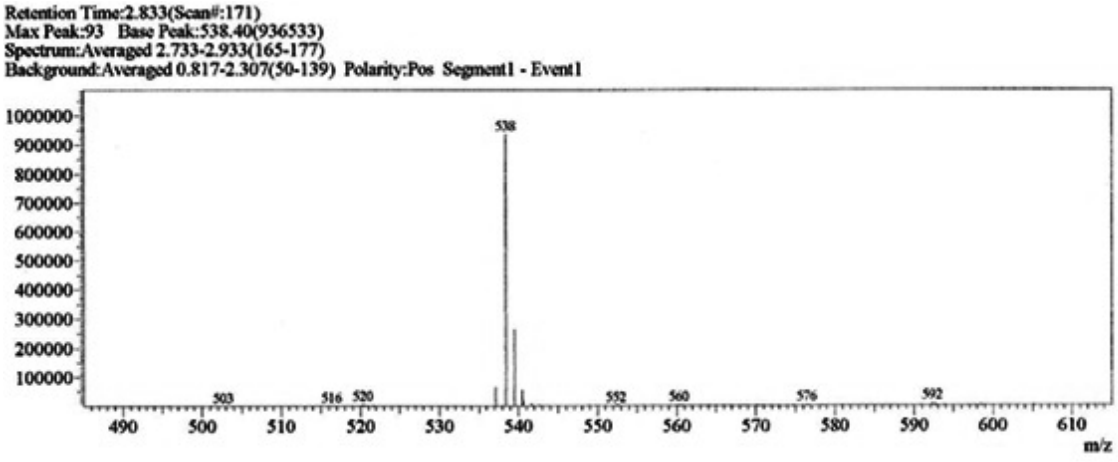

***Figure 5S.** APCI-Mass Spectrum of the amentoflavone standard*

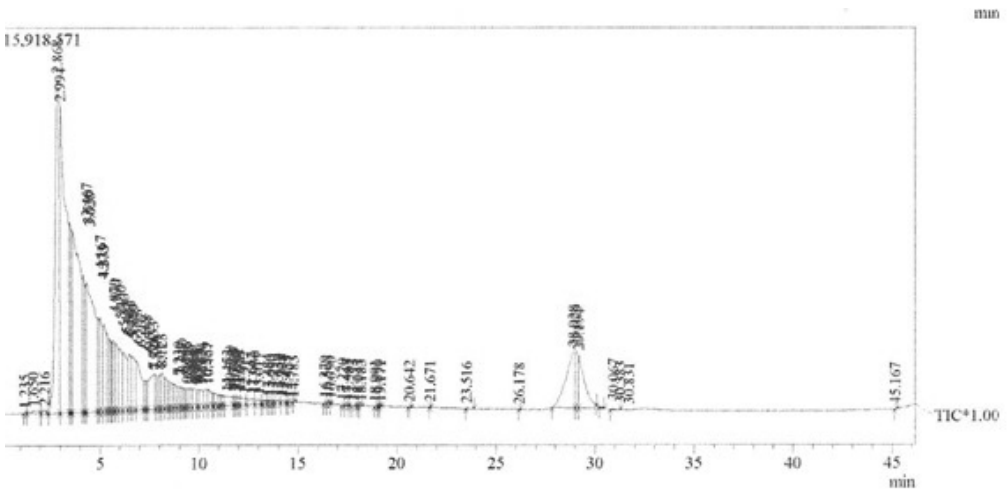

Figure 6S. *HPLC chromatogram of the specimen collected at the neighborhood of Serra Talhada - PE*

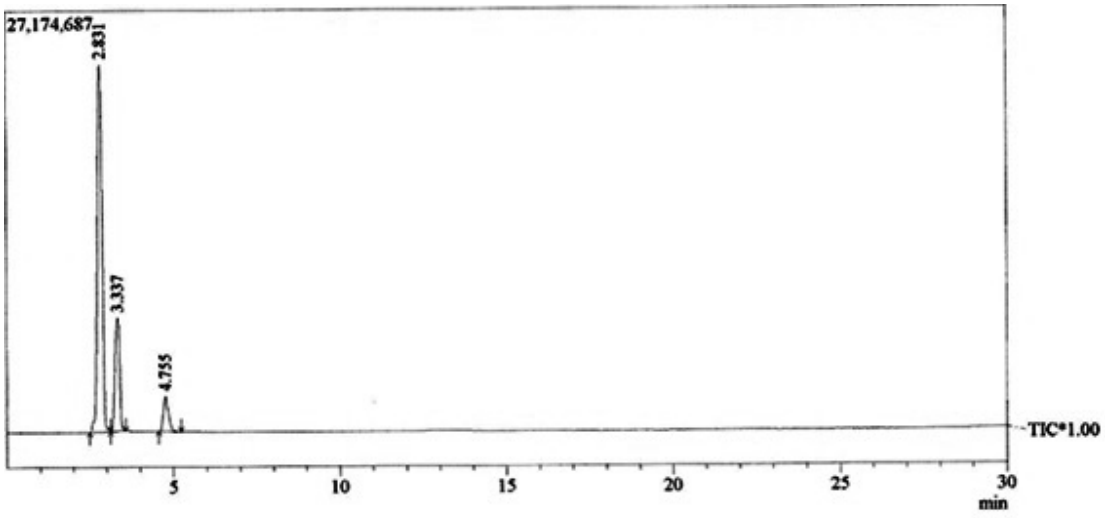

Figure 7S. *HPLC chromatogram of the specimen collected at the neighborhood of Riachão do Jacuípe - BA*

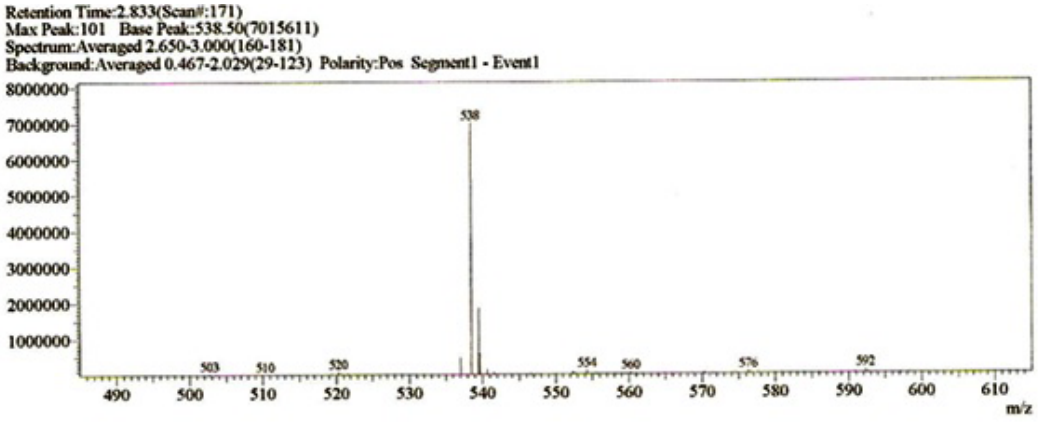

Figure 8S. *APCI - Mass Spectrum of the specimen collected at neighborhood of Jacuípe - BA*

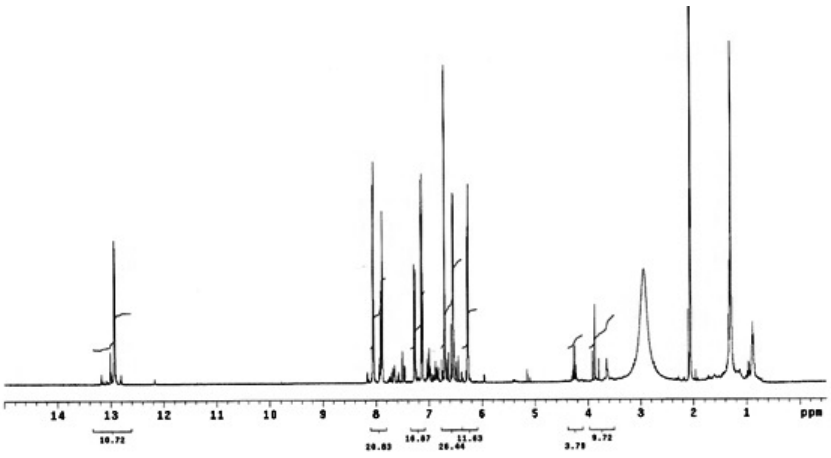

Figure 9S. *^{1}H NMR spectra of compound* ***1*** *[500 MHz, $(D_3C)_2CO$]*

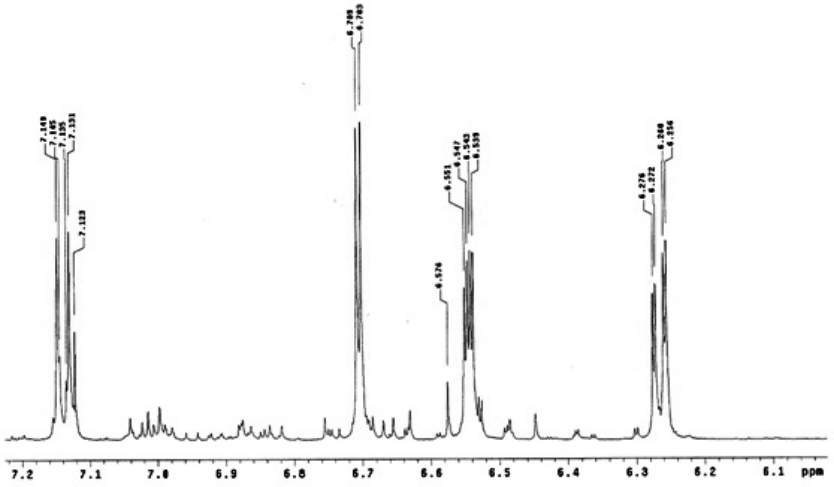

Figure 10S. *Expansion of ^{1}H NMR spectra of compound* ***1*** *[500 MHz, $(D_3C)_2CO$]*

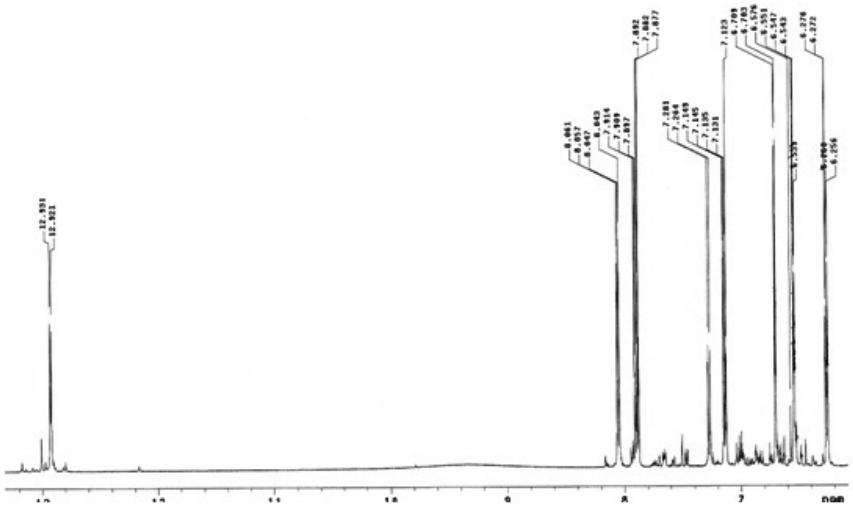

Figure 11S. *Expansion of ^{1}H NMR spectra of compound* ***1*** *[500 MHz, $(D_3C)_2CO$]*

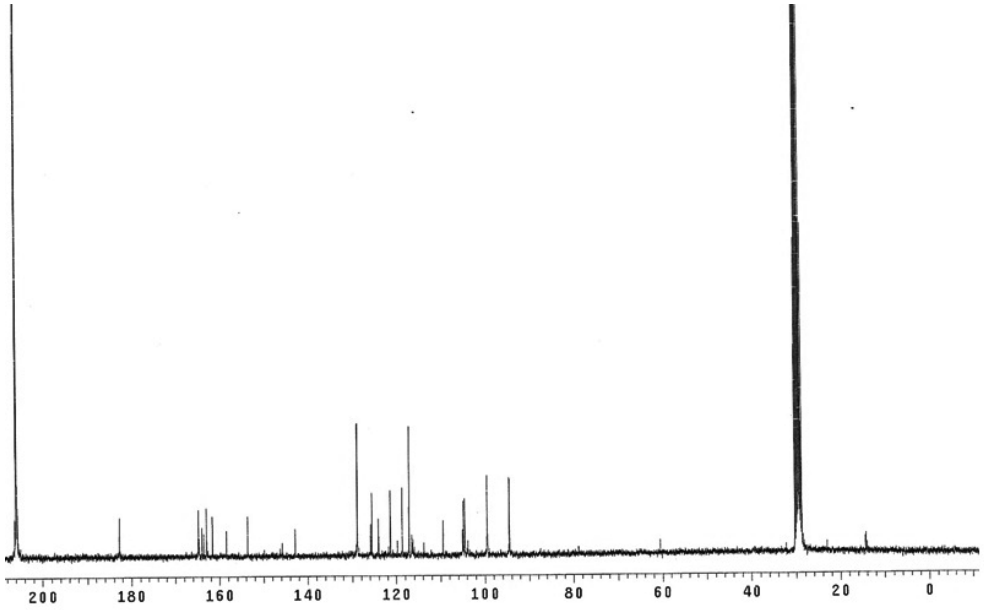

Figure 12S. *^{13}C NMR spectra of compound **1** [75 MHz, $(D_3C)_2CO$]*

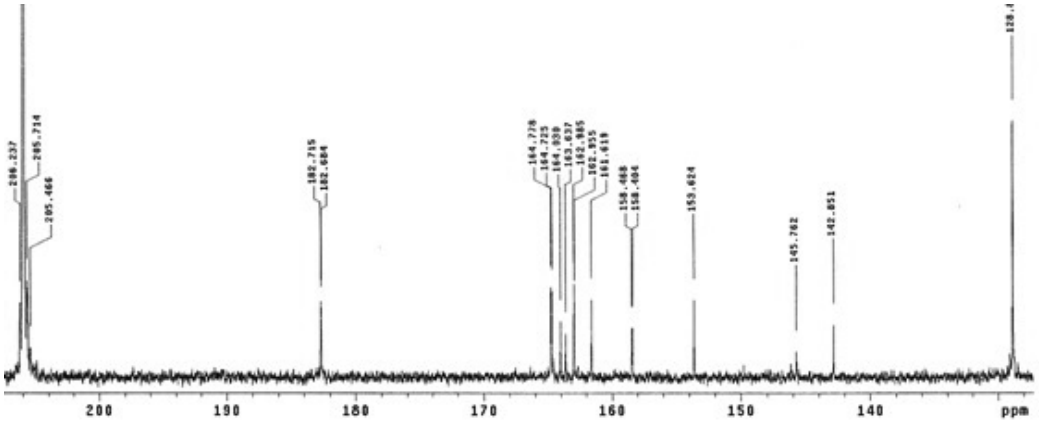

Figure 13S. *Expantion of ^{13}C NMR spectra of compound **1** [75 MHz, $(D_3C)_2CO$]*

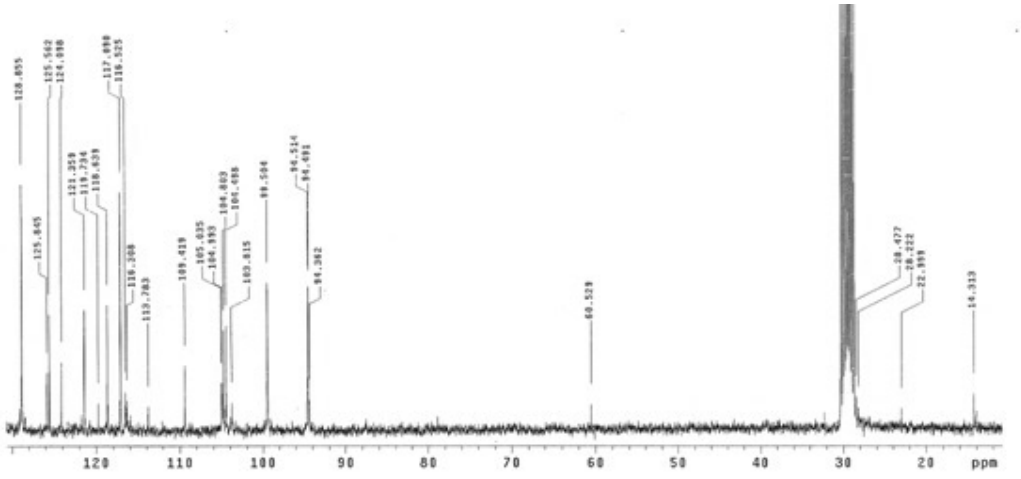

Figure 14S. *Expantion of ^{13}C NMR spectra of compound **1** [75 MHz, $(D_3C)_2CO$]*

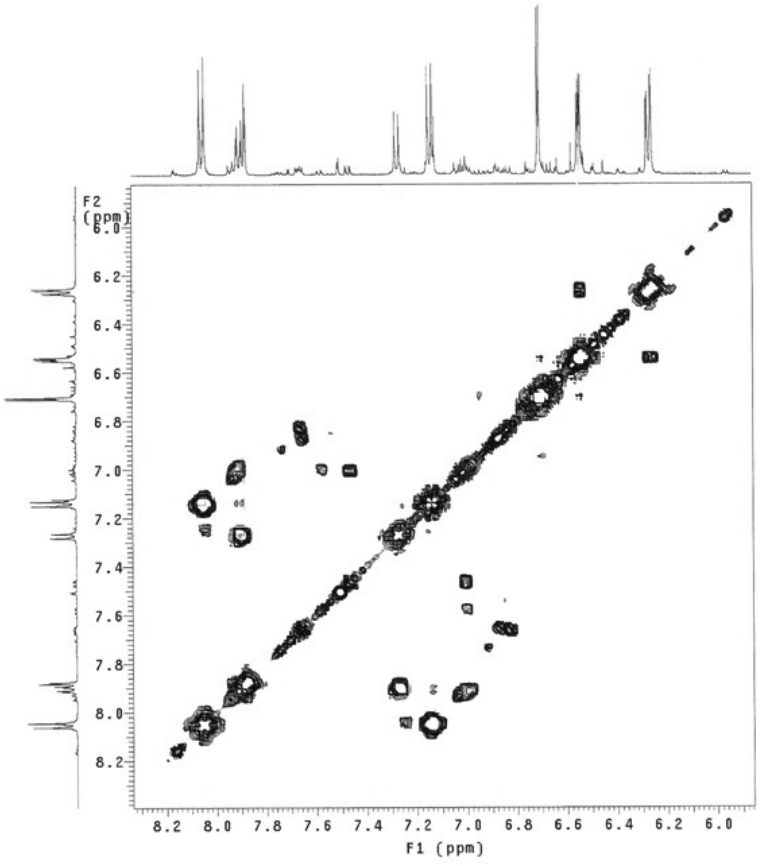

Figure 15S. *gCOSY ^{1}H-^{1}H spectra of compound* ***1*** *[500 MHz, $(D_3C)_2CO$]*

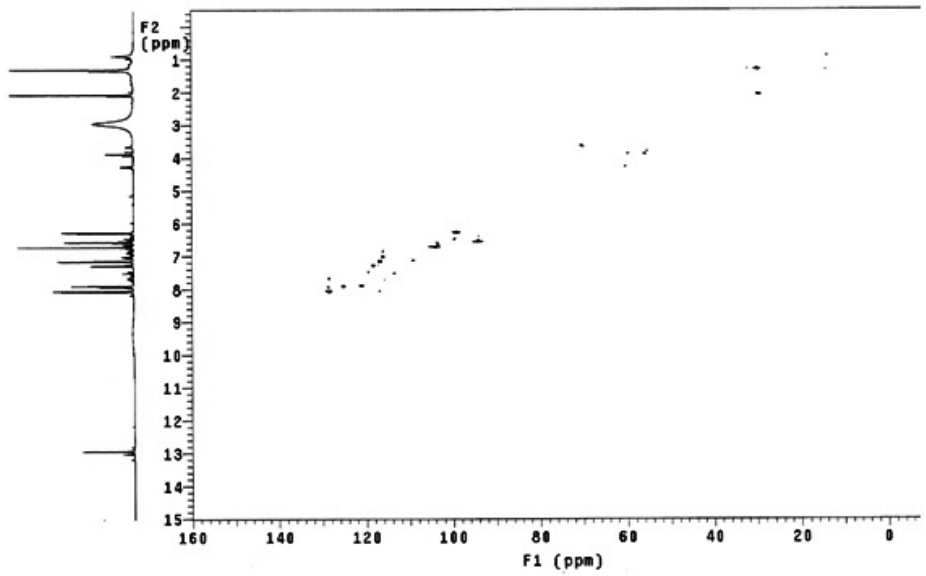

Figure 16S. *HSQC spectra of compound* ***1*** *[500 MHz for ^{1}H and 125 MHz for ^{13}C, $(D_3C)_2CO$]*

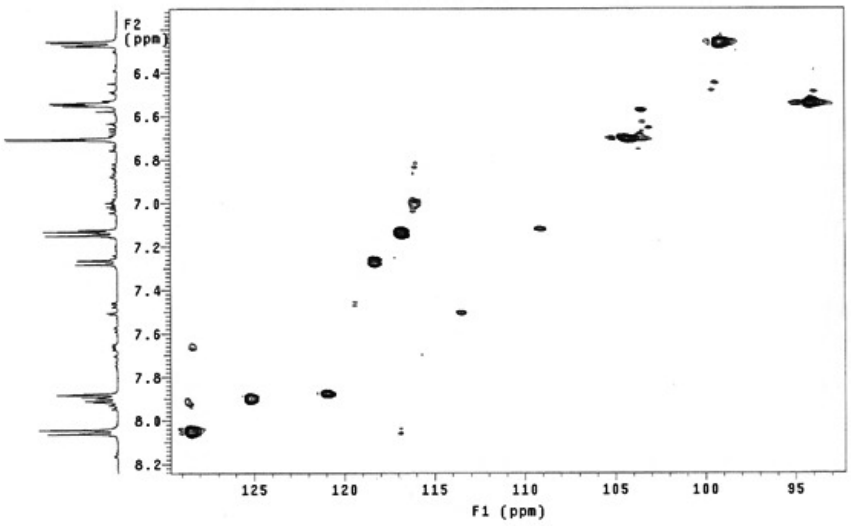

Figure 17S. *Expansion of HSQC spectra of compound* ***1*** *[500 MHz for ^{1}H and 125 MHz for ^{13}C, $(D_3C)_2CO$]*

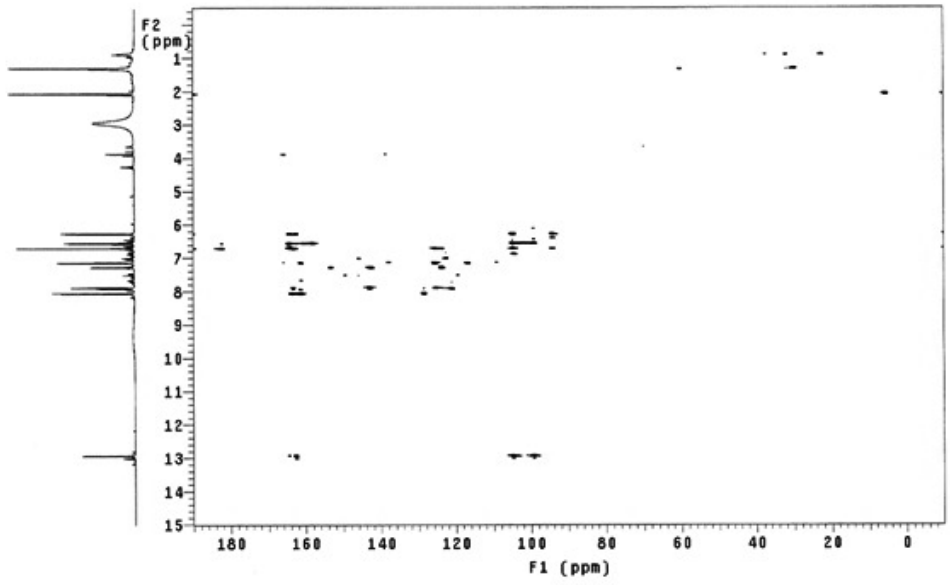

Figure 18S. *HMBC spectra of compound* ***1*** *[500 MHz for ^{1}H and 125 MHz for ^{13}C, $(D_3C)_2CO$]*

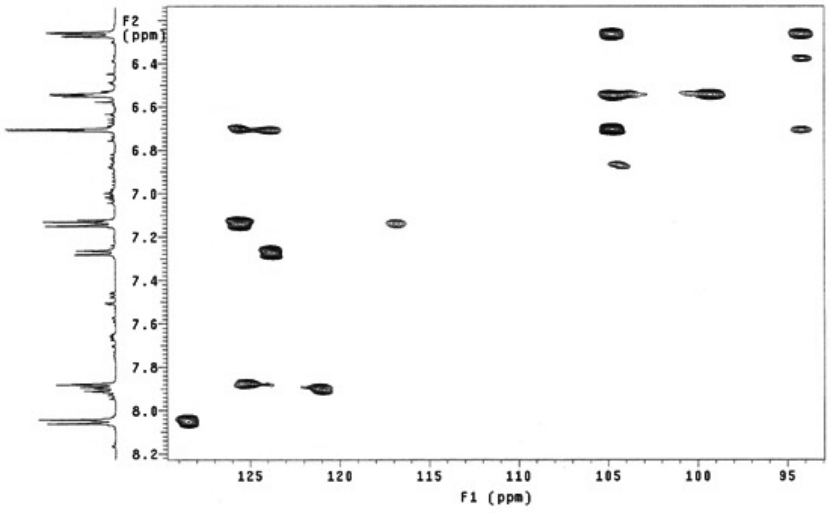

Figure 19S. *Expansion of HMBC spectra of compound* ***1*** *[500 MHz for* ^{1}H *and 125 MHz for* ^{13}C*,* $(D_3C)_2CO$*]*

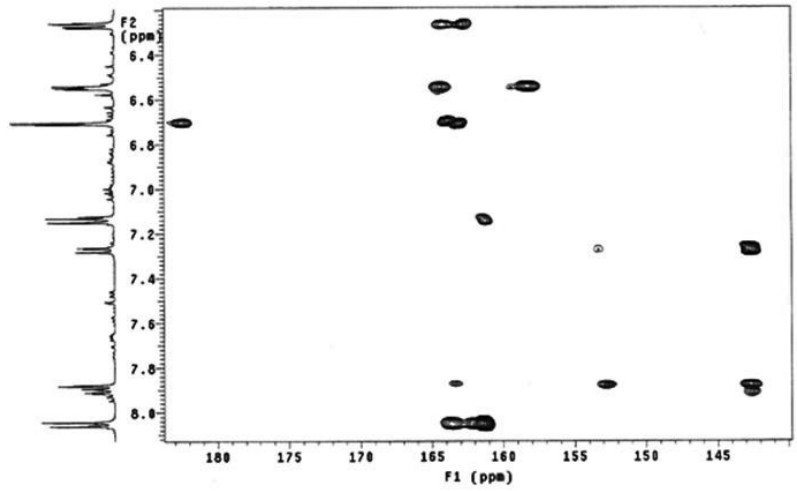

Figure 20S. *Expansion of HMBC spectra of compound* ***1*** *[500 MHz for* ^{1}H *and 125 MHz for* ^{13}C*,* $(D_3C)_2CO$*]*

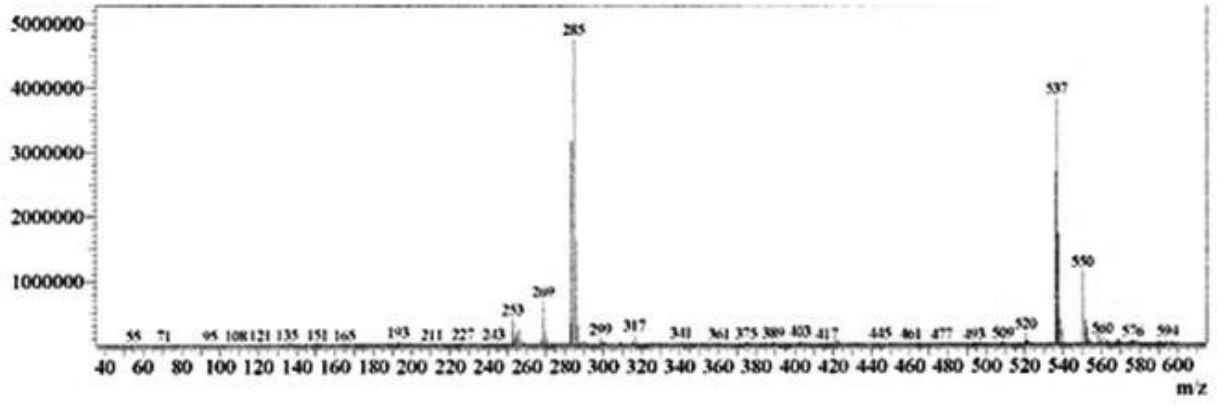

Figure 21S. *Negative APCIMS of compound* ***1***