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Article

Flavonoids and Triterpenes from the Nest of the Stingless Bee *Trigona spinipes*

Marinalva O. Freitas, Flávio A. F. Ponte, Mary Anne S. Lima and Edilberto R. Silveira*

Curso de Pós-Graduação em Química Orgânica, Departamento de Química Orgânica e Inorgânica, Centro de Ciências, Universidade Federal do Ceará, CP 12 200, 60451-970 Fortaleza-CE, Brazil

No nordeste do Brasil, a abelha-sem-ferrão *Trigona spinipes* perfura o tronco de espécimens cultivados de *Eucalyptus citriodora* para a produção de exudatos. A investigação química do extrato etanólico do ninho de *T. spinipes* permitiu o isolamento dos triterpenos cicloartanos ácido magniferólico e ácido 3β-hidroxi-24-metilenocicloartan-26-óico, além dos flavonóides 3'-metil-quercetina, sakuranetina, éter 7-metil campferol, tricetina e éter 7-metil aromadendrina como compostos majoritários. O isolamento de sakuranetina, éter 7-metil campferol e éter 7-metil aromadendrina do ninho de *T. spinipes* e do exudato de *Eucalyptus citriodora*, sugere esta espécie como origem botânica dos constituintes do ninho destas abelhas-sem-ferrão no nordeste do Brasil. A caracterização estrutural dos compostos isolados foi realizada utilizando-se métodos espectrométricos e comparação com dados da literatura.

In the Northeast of Brazil the stingless bee *Trigona spinipes* Fabricius injures the tree bark of cultivated *Eucalyptus citriodora* specimens in order to make them exudate. The chemical investigation of the ethanol extract of an entire nest of *T. spinipes* allowed the isolation of the cycloartane triterpene magniferolic acid and 3β-hydroxy-24-methylenecicloartan-26-oic acid, besides the flavonoids 3'-methyl quercetin, sakuranetin, kaempferol 7-methyl ether, tricetin and aromadendrin 7-methyl ether as the main compounds. The isolation of sakuranetin, kaempferol 7-methyl ether, and aromadendrin 7-methyl ether from both *Trigonas spinipes´* nest and the exudate from *Eucalyptus*, may suggest this species as a botanical origin of the nest constituents of these stingless bee in the Northeast of Brazil. The structural characterization of the isolated compounds was accomplished by spectrometric means and comparison with the literature data.

Keywords: *Trigona spinipes* nest, cycloartane triterpenes, flavonoids, *Eucalyptus citriodora* exudate

Introduction

More than 500 species stingless bees of subfamily Meliponinae are of pantropical distribution, however, the great diversity of species from the tribe Trigonini and all from the trib e Meliponini are found in the Neotropics.[1]

Indigenous stingless bee species from South America collect resinous material from plants and mix it with beewax and soil to form "*geopropolis"* or *"divine elixir*", terms suggested for the Meliponinae honeys.[2,3] In a general way, they build more complex nests than *Apis mellifera*, although there are a great variety of forms, size and place of construction. The majority of species use closed cavities to build their nests, but some species of the genus *Trigona* build completely exposed aerial nests with an entrance normally build of wax and mud, and a passage way generally built with geopropolis ending at the storage pots.[4]

Until now, chemical reports are concentrated to the honey bee *Apis mellifera* and only a few information is known about the chemistry of stingless bees, in spite of the estimated number of over 500 Neotropical species.[5] Beekeeping of stingless bees or "meliponiculture" is well practiced in Guatemala, Mexico and Venezuela and have been subject of an increasing interest for future research activities, especially because geopropolis seems to possess biological potentialities similar to that of honey bee propolis.[2,3] The medicinal uses of geopropolis in folk medicine include properties related to digestive, respiratory, female fertility, skin and visual disorders. Pollen and cerumen of the nests are also used in therapy and the larvae of *Melipone* and *Trigona* species are used in local diets.[5,6] In addition, the knowledge of the botanical origin of propolis

*e-mail: edil@ufc.br

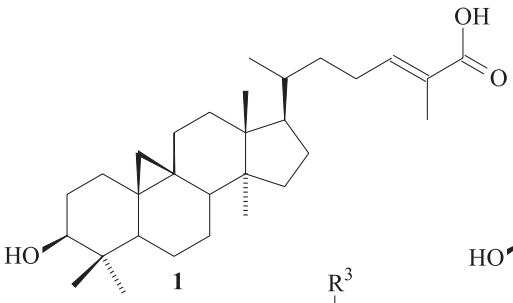
and geopropolis is important to beekeepers to assure that bees have specific plants in their flight range, because the determination of the chemical types of their honey products based on the source plant is important for the biological activity associated with its chemistry.

Previous analysis of geopropolis from the indigenous stingless bees *Firesomellita varia, Melipona favosa, Melipona compressipes, Scaptotrigona depilis* and *Paratrigona anduzei* in tropical Venezuela, revealed a phenolic profile characterized by the occurrence of polyprenylated benzophenones.[7] In the chemical investigation of geopropolis of the Brazilian stingless bees *Melipona compressipes*, *Melipona quadrifasciata anthidioides* and *Tetragona clavipes,* Bankova *et al*. showed by GC-MS analysis that the chemical composition of three samples was completely different among them.[8] Diterpenes acids were found in all samples of geopropolis, and their amounts were significant in *M. quadrifasciata anthidioides* and *T. clavipes*. On the other hand, the pentacyclic triterpene β-amyrin was identified as the main component in *T. clavipes,* the flavonoid pinobanksin in *M. compressipes* and aromatic aldehydes in *Melipona quadrifasciata anthidioides*, respectively. Surprisingly, the prenylated benzophenones characteristic of geopropolis from Venezuela were absent in geopropolis from Brazil, including the one from *Melipona compressipes* that was analyzed in both tropical areas. These findings indicated that the chemical composition of geopropolis from stingless bees from Brazil varied with the location of the bee species, and also with the available flora as the most important factor for the composition.

In the Northeast of Brazil, the indigenous stingless bee *Trigona spinipes* is known as "arapuá", "irapuá" or "bee-of-dog". They possess black coloration measuring around 5 to 6.5 mm in length. In our previous studies of the exudates from a *Eucalyptus citriodora* specimen, it was observed that *T. spinipes* usually visit the plant injuring its bark in order to make it exudates. In this work we report the isolation of cycloartane triterpenes and flavonoids as the main constituents of an entire nest of *T. spinipes,* in order to suggest that *Eucalyptus citriodora* is one of the botanical sources of the chemical constituents of this kind of stingless bees from Northeast of Brazil.

Results and Discussion

In our chemical investigation of the nest of the stingless bee *Trigona spinipes* from Northeast of Brazil, we have isolated and characterized by NMR the mixture of the cycloartane triterpenes, magniferolic acid (**1**) and 3β-hydroxy-24-methylenecycloartan-26-oic acid (**2**), besides the flavonoids 3'-methyl quercetin (**3**), sakuranetin (**4**), kaempferol 7-methyl ether (**5**), tricetin (**6**) and aromadendrin 7-methyl ether (**7**) as the main compounds. Although flavonoids are widespread in the propolis constitution of temperate zones, until now only the flavanone pinobanksin has been found in geopropolis of *M. favosa* from Venezuela and *M. compressipes* in Brazil.[7,8] Pentacyclic triterpenes alcohols have been isolated from geopropolis of *Tetragona clavipes*,[8] and those with a cycloartane-type skeleton have been found only in Egyptian and Brazilian propolis from honey bees from southeastern region.[9,10] However, this is the first report on the occurrence of magniferolic acid (**1**) and



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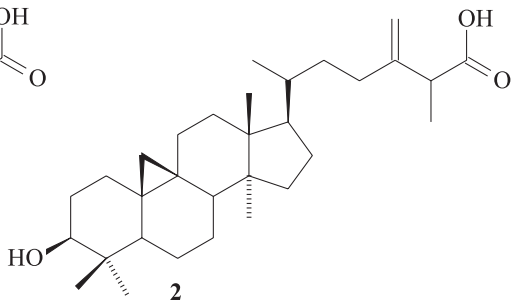

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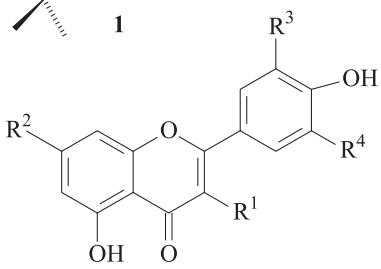
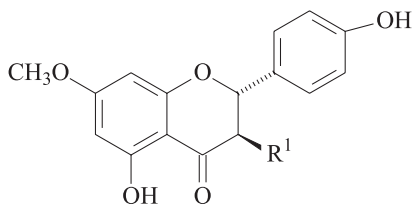


3 R^1 = OH, R^2 = OH, R^3 = OCH_3, R^4 = H

5 R^1 = OH, R^2 = OCH_3, R^3 = H, R^4 = H

6 R^1 = H, R^2 = OH, R^3 = OH, R^4 = OH



4 R^1 = H

7 R^1 = OH

3β-hydroxy-24-methylenecycloartan-26-oic acid (**2**) from stingless bees sources.

As well documented in the literature, the main sources of propolis and geopropolis in South America, West Asia and North America are resin exudates from trees. Bud exudates of *Populus* species are the main source of flavonoids of propolis from temperate zones. In Venezuela, where *Populus* are not native plants, stingless bees and honey bees visit *Clusia* specimens in order to collect a resin excreted at the bases of their flowers stamens.[11] As a consequence, the chemical composition of both tropical propolis and geopropolis is particularly characterized by the presence of polyprenylated benzophenones, in accordance to the chemical constituents identified from *Clusia* flowers.[8] In the Brazilian southeast, the profiles of flavonoids and other phenolic compounds were quite similar when samples of propolis and the exudate of *Baccharis dracuncufolia* were compared, revealing this species as the botanical origin for propolis production in this region.[12]

It was observed that *Trigona spinipes* bees from Northeast of Brazil visit specimens of *Eucalyptus citriodora* to collect the "kino", a dark resin exudate excreted after the bark is injured. In a previous chemical investigation of the kino from *E. citriodora* we have isolated the flavonoids sakuranetin, narigenin, aromadendrin 7-methyl ether, aromadendrin, kaempferol 7-methyl ether, gallic acid, the glycosides 1-*O*-cinnamoyl-6-*O*-(*p*-coumaroyl)-β-D-glucopyranoside, 7-methylaromadendrin-4'-*O*-(6''-*trans*-*p*-coumaroyl)-β-D-glucopyranoside, 6-O-(*p*-coumaroyl)-D-glucopyranoside and the tannin 1-*O*-2,*O*-digalloyl-6-*O*-(*p*-coumaroyl)-β-D-glucopyranoside.[13,14] The isolation of sakuranetin (**4**), kaempferol 7-methyl ether (**5**), and aromadendrin 7-methyl ether (**7**) in both the nest and *E. citriodora* exudates, clearly evidenced their chemical relationship.

The above findings suggest that *T. spinipes* from the Northeast of Brazil does store biologically active compounds in their nests, and the kino of *E. citriodora* is one of its botanical sources. However, it is evident that further studies on the chemical constituents of stingless bees from different geographic areas in the Northeast region are needed in order to confirm the chemical relationship between this class of bees and the flora available to them as a source of resin.

Experimental

General

Melting points were obtained on a Mettler FP82HT apparatus and are uncorrected. IR spectra were recorded using a Perkin Elmer 1000 FT-IR spectrophotometer. The mass spectra were obtained on a Hewlett-Packard 5971 mass spectrometer by electron impact ionization (70 eV). ^{1}H and ^{13}C NMR where recorded on a Bruker Avance DRX-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C). Silica gel 60 (Merck, kiesegel 60 F254, 0.20 mm) were used for analytical TLC. Silica gel 60 (Merck, 230-240 mesh) was used for column chromatography. All compounds were visualized on TLC by spraying with vanillin/perchloric acid/EtOH followed by heating.

Trigona spinipes nest

The nest of the stingless bees *Trigona spinipes* Fabricius was collected in Fortaleza, state of Ceará, Northeast of Brazil. The bees were identified by Dr. Daniel Santiago Pereira of Universidade Federal Rural do Semi-Árido (UFERSA).

Extraction and isolation

14.7 kg of the *T. spinipes* nest were pulverized and extracted with EtOH at room temperature (3 × 8 L). The solvent was removed under reduced pressure to give a brown viscous extract (2.24 kg). An aliquote of the EtOH extract (29.0 g) was dissolved in a mixture of MeOH:H_2O (1:1 v/v) and submitted to liquid-liquid partition with hexane, $CHCl_3$ and EtOAc to give the correspondent four fractions after solvent evaporation. Chromatography on a Si gel column of the hexane fraction (18.6 g) by elution with hexane, $CHCl_3$, EtOAc and MeOH as binary mixtures with increasing polarity yielded 23 sub-fractions. The sub-fraction 14 yielded a solid residue that was recrystallized from MeOH to give a mixture of magniferolic acid (**1**) [15] and 3β-hydroxy-24-methylenecycloartan-26-oic acid (**2**) (14.0 mg).[16] The $CHCl_3$ fraction (8.17 g) was subjected to Silica gel column chromatography by elution with hexane, $CHCl_3$, EtOAc and MeOH to give 4 fractions. Successive flash chromatography of the sub-fraction EtOAc (3.8 g) using $CHCl_3$:EtOAc as a binary mixture with increasing polarity yielded 33 fractions. The fractions 4-6 (602.0 mg), 7-10 (185.0 mg) and 26-29 (275.0 mg) were further purified by treatment over Sephadex LH-20 by elution with MeOH. Fraction 4-6 yielded compounds 3'-methyl quercetin (**3**) (4.0 mg) and sakuranetin (**4**) (8.0 mg),[17] fraction 7-10 gave compounds tricetin (**6**) [17] (7.0 mg) and aromadendrin 7-methyl ether (**7**)[17] (4.0 mg), and the fraction 26-29 yielded compound kaempferol 7-methyl ether (**5**) (6.0 mg),[17] respectively. Structural characterization of all compounds was established on the basis of spectroscopic methods (supplemental material), particularly 1D and 2D NMR, and comparison with data from literature.

Acknowledgments

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Supplementary Information

Suplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

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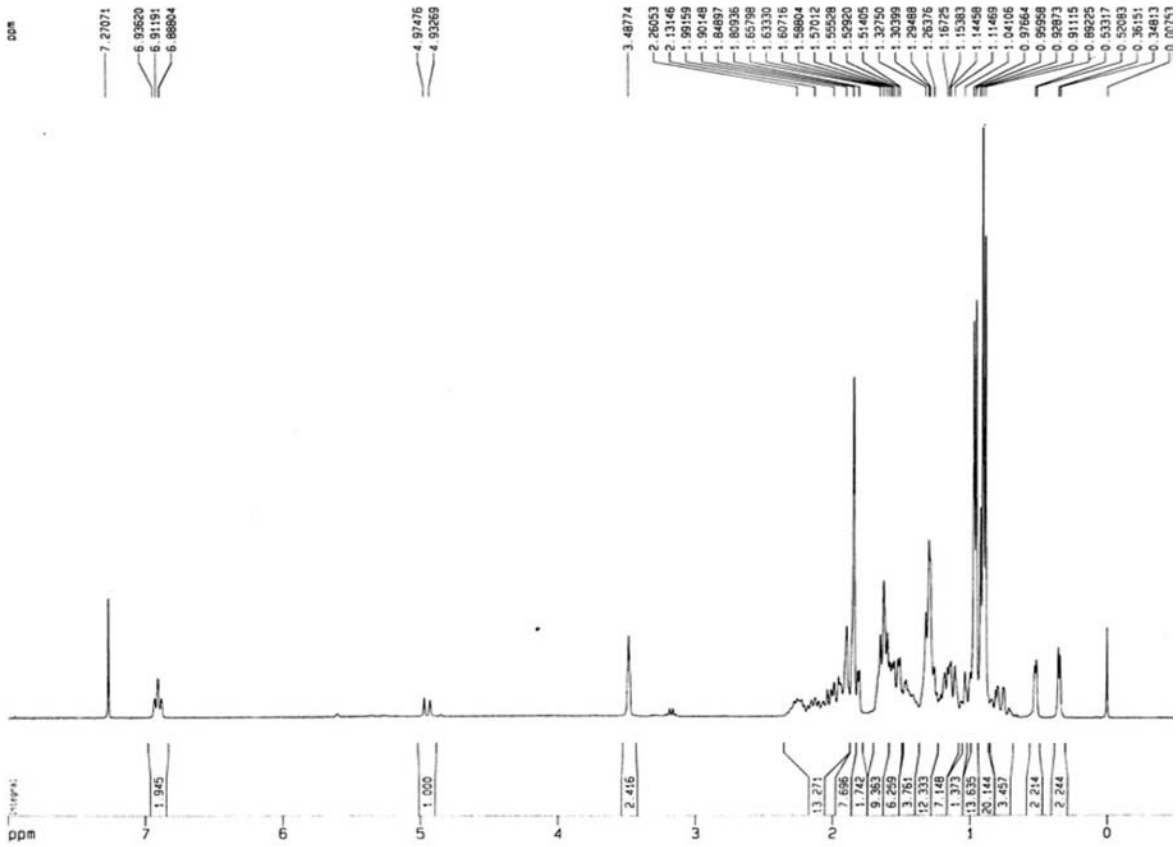

Figure S1. ^{1}H NMR spectrum (500 MHz, MeOD) of magniferolic acid (**1**) and 3β-hydroxy-24-methylenecicloartan-26-oic acid (**2**)

*e-mail: edil@ufc.br

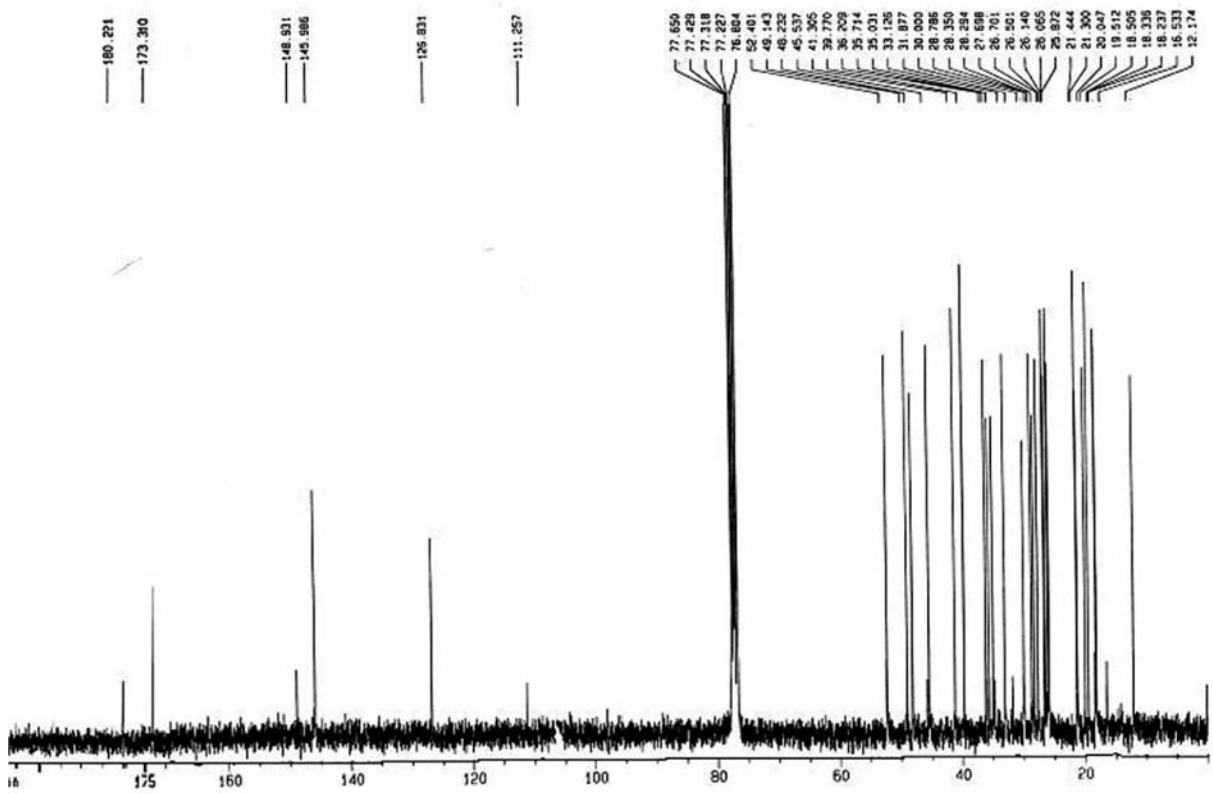

Figure S2. ^{13}C-BB NMR spectrum (125 MHz, MeOD) of magniferolic acid (**1**) and 3β-hydroxy-24-methylenecicloartan-26-oic acid (**2**)

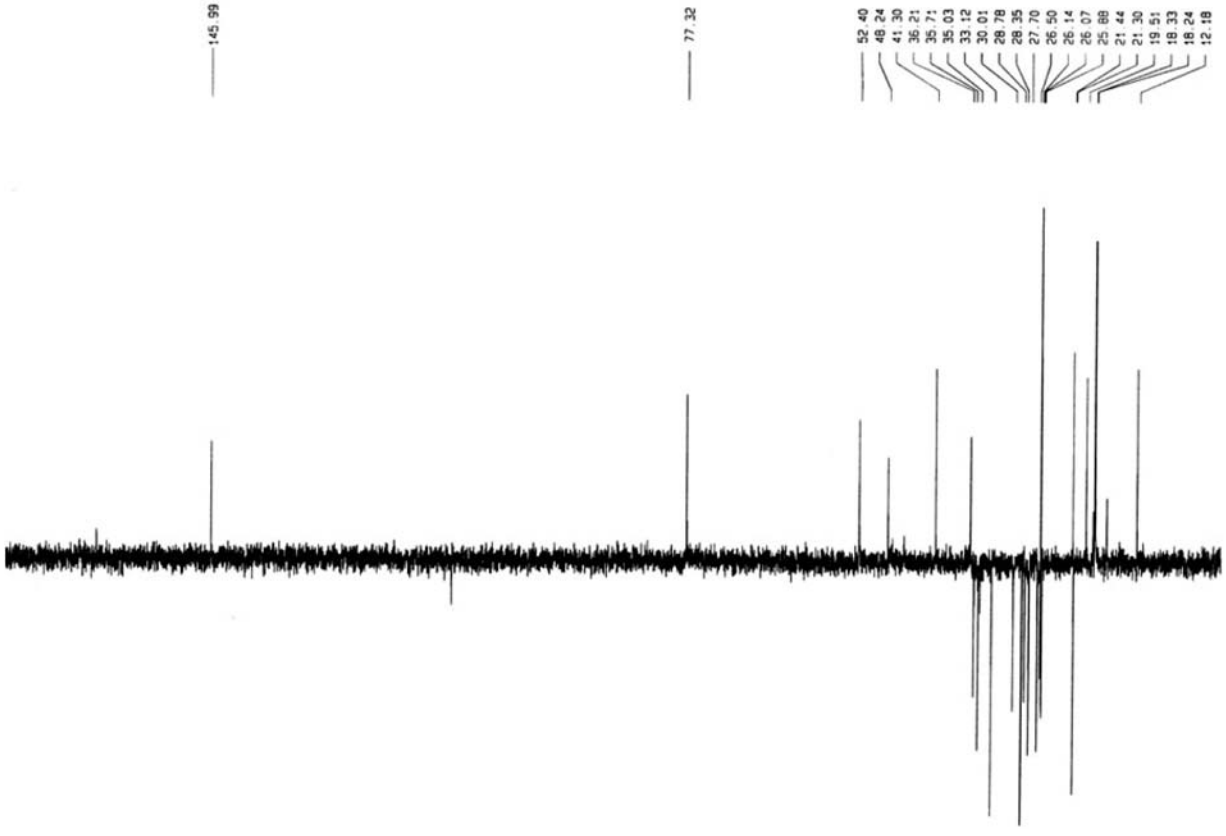

Figure S3. ^{13}C-DEPT 135° NMR spectrum (125 MHz, MeOD) of magniferolic acid (**1**) and 3β-hydroxy-24-methylenecicloartan-26-oic acid (**2**)

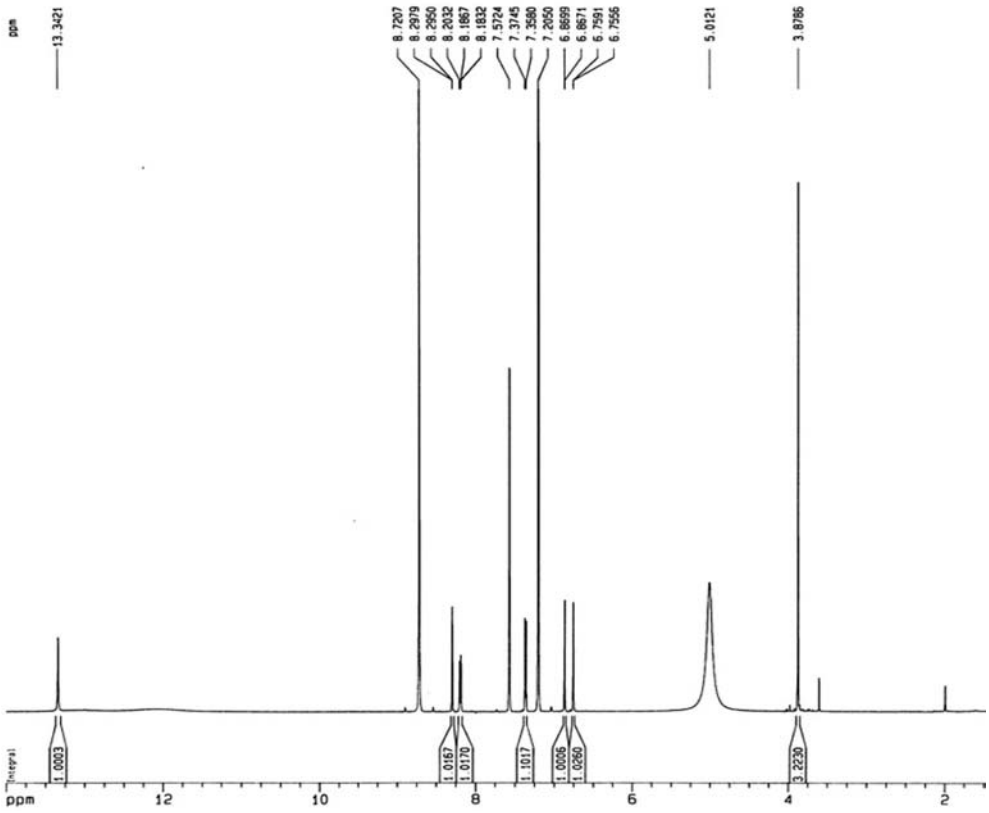

Figure S4. ^{1}H NMR spectrum (500 MHz, C_5H_5N) of 3´-methyl-quercetin (**3**)

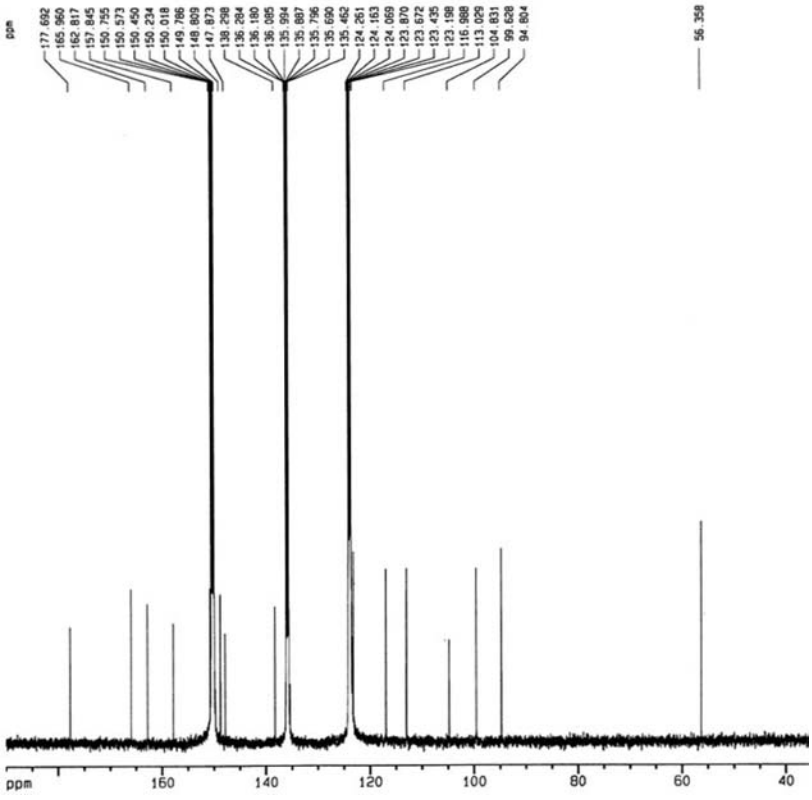

Figure S5. ^{13}C -BB NMR spectrum (125 MHz, C_5H_5N) of 3´-methyl-quercetin (**3**)

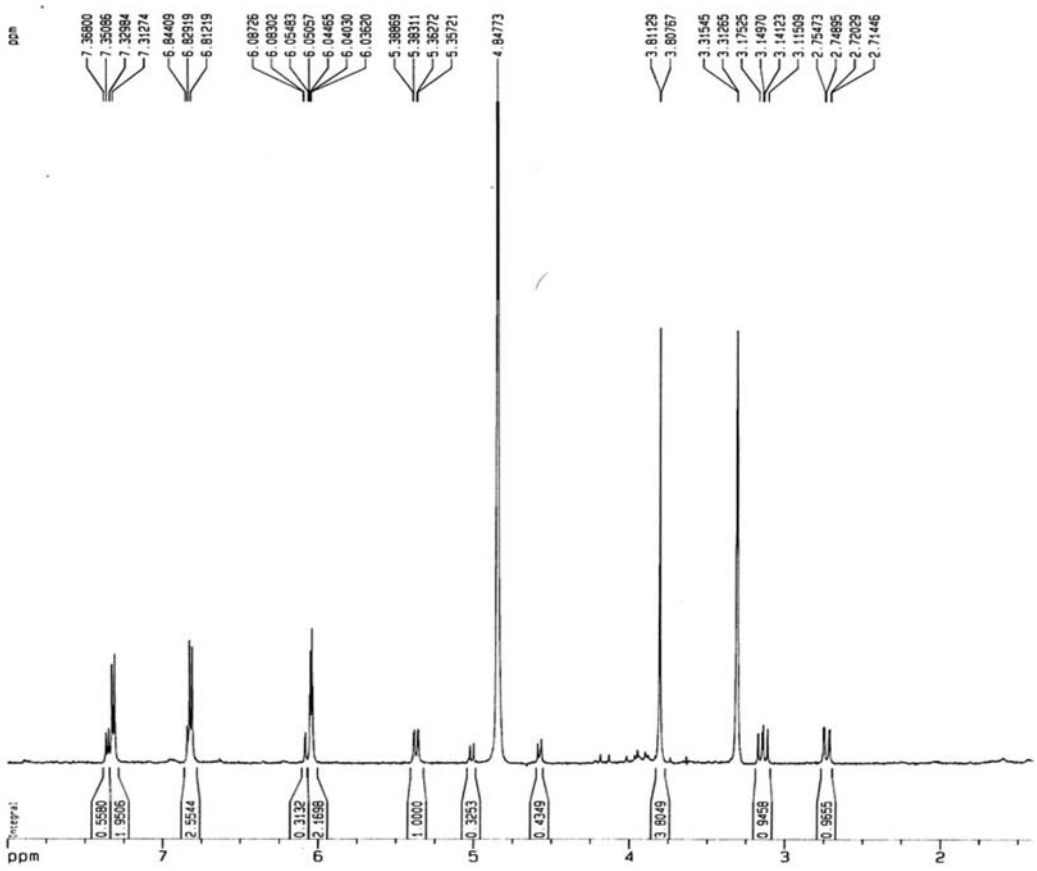

Figure S6. [1]H NMR spectrum (500 MHz, MeOD) of sakuranetin (**4**)

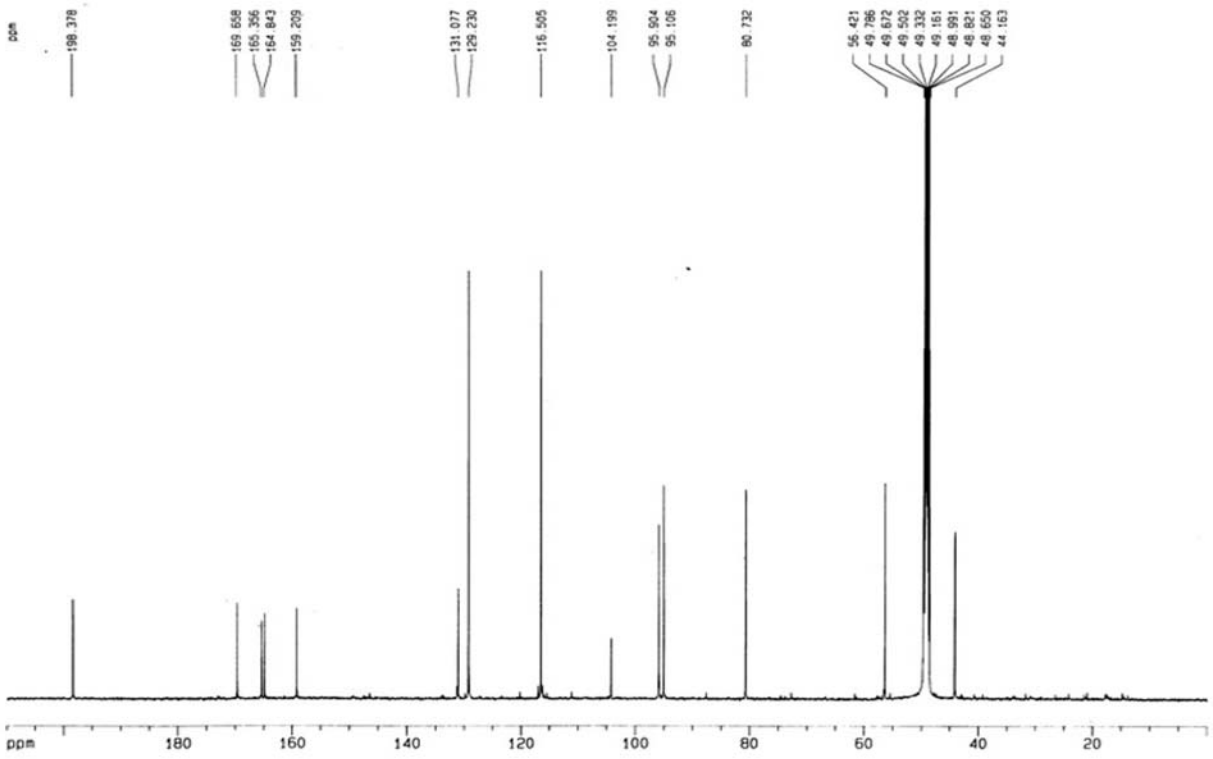

Figure S7. [13]C -BB NMR spectrum (500 MHz, MeOD) of sakuranetin (**4**)

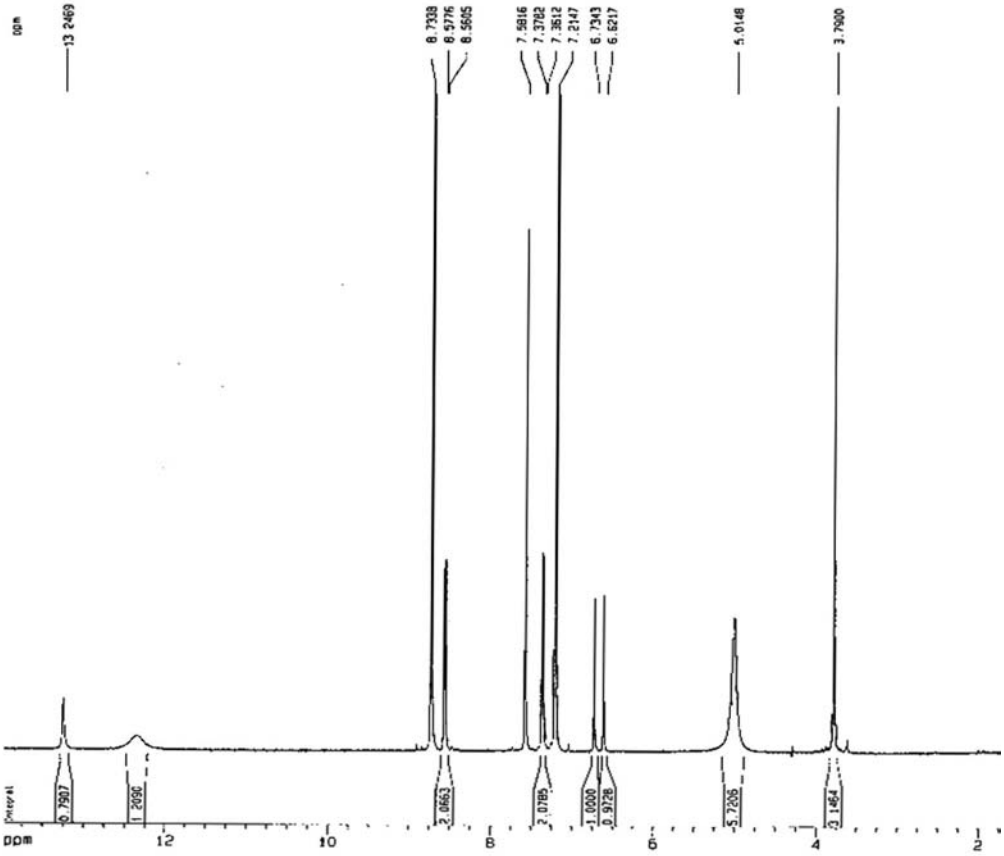

Figure S8. ^{1}H NMR spectrum (500 MHz, C_5H_5N) of kaempferol 7-methyl eter (**5**)

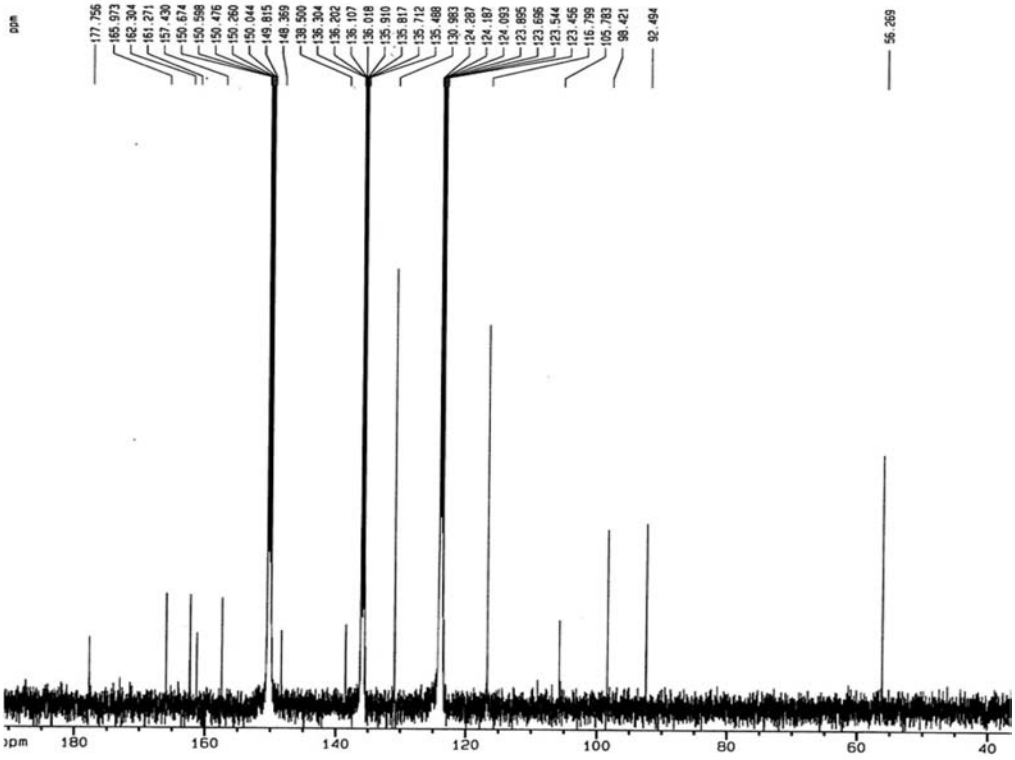

Figure S9. ^{13}C -BB NMR spectrum (125 MHz, C_5H_5N) of kaempferol 7-methyl eter (**5**)

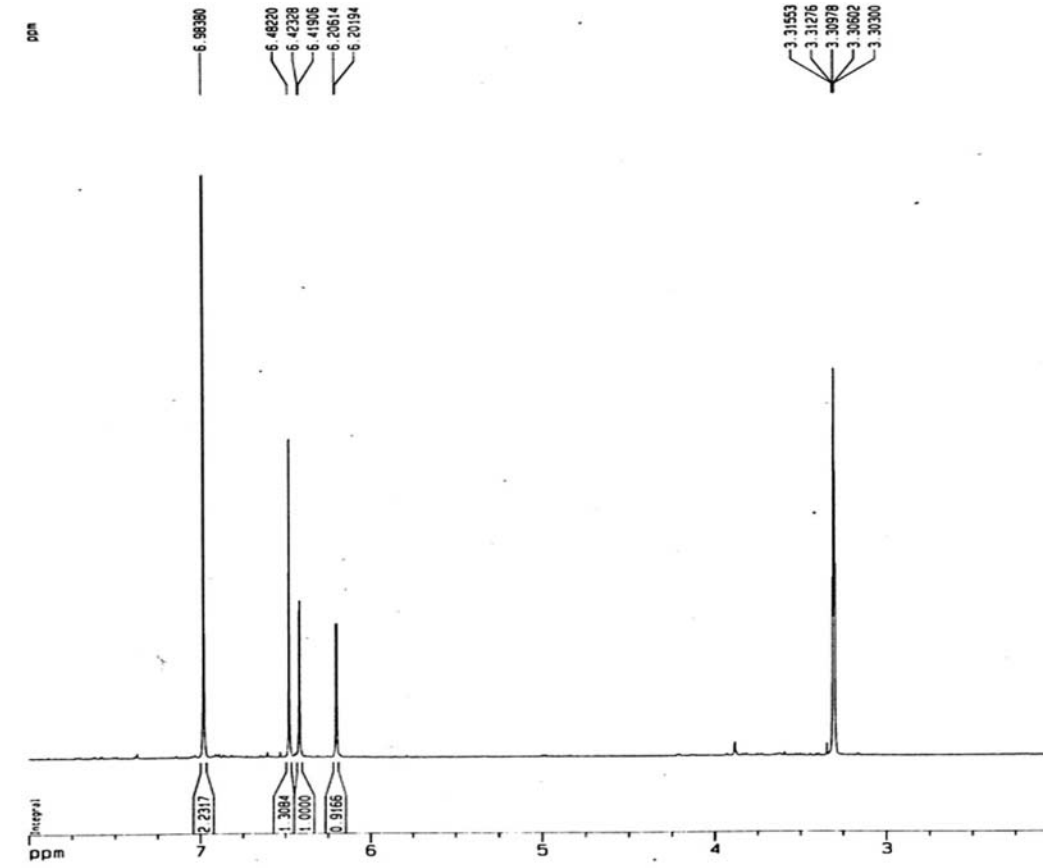

Figure S10. [1]H NMR spectrum (500 MHz, MeOD) of tricetin (**6**)

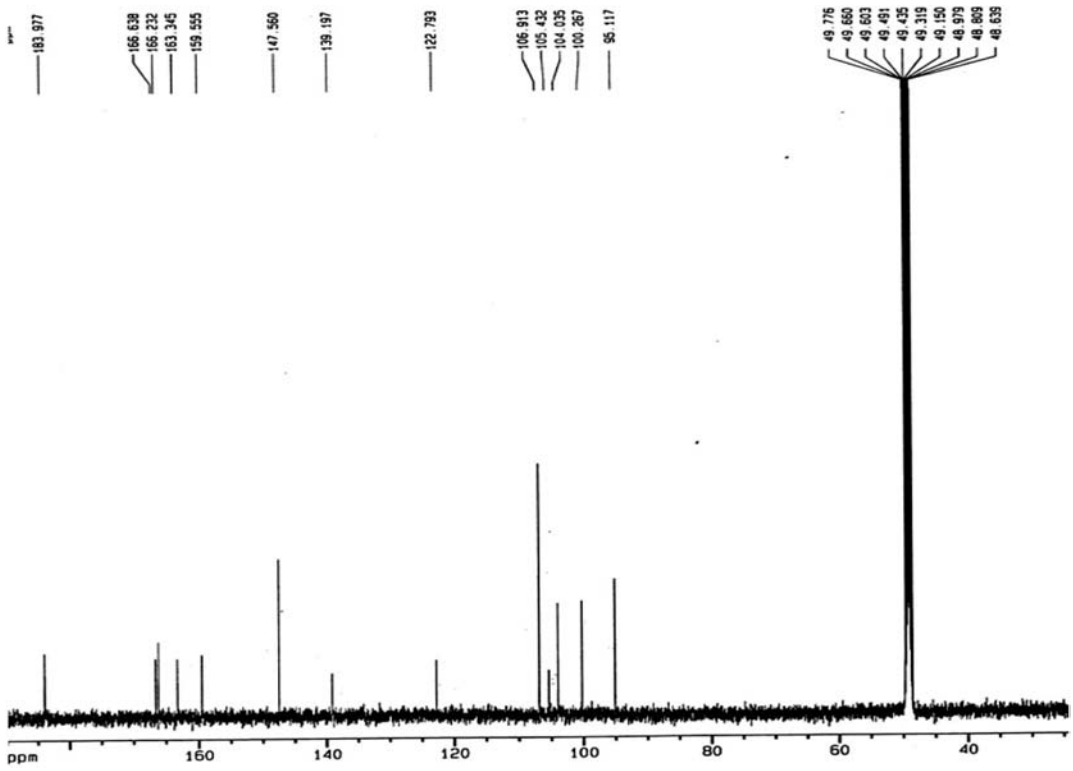

Figure S11. [13]C -BB NMR spectrum (500 MHz, MeOD) of tricetin (**6**)

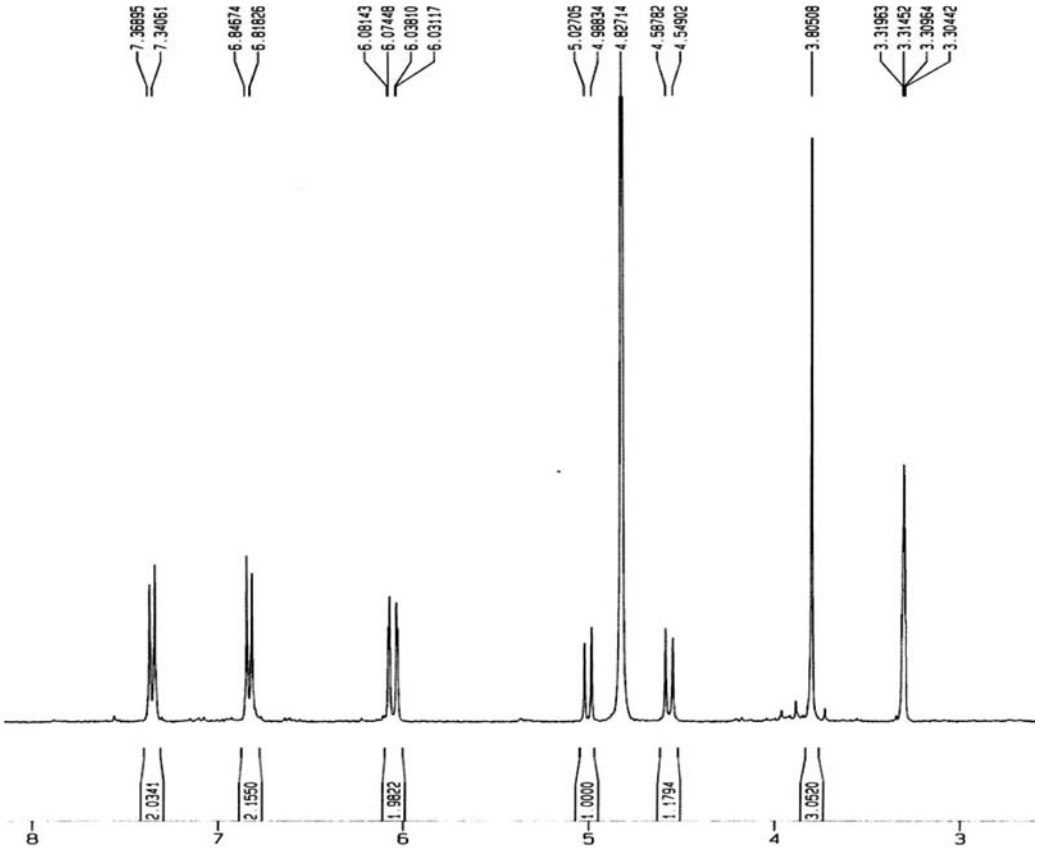

Figure S12. [1]H NMR spectrum (500 MHz, MeOD) of aromadendrin 7-methyl eter (**7**)

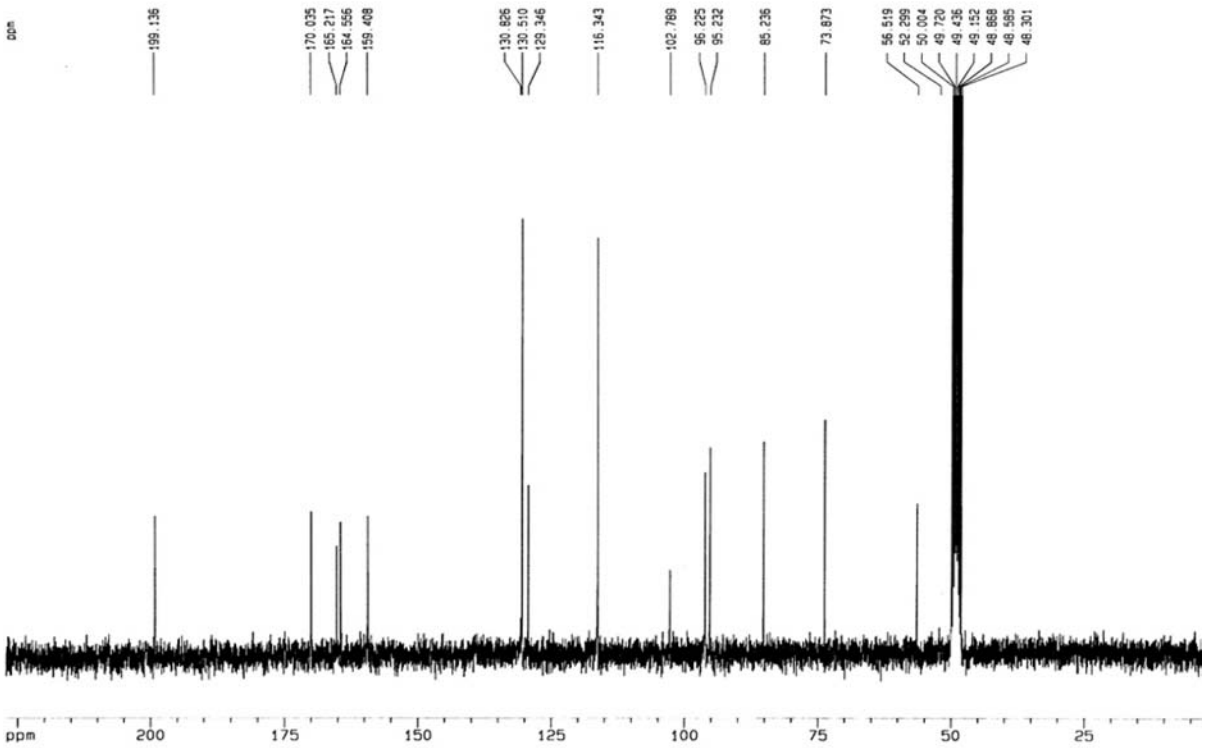

Figure S13. [13]C -BB NMR spectrum (500 MHz, MeOD) of aromadendrin 7-methyl eter (**7**)