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ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITY OF THE FLAVONOID FROM THE ROOTS OF *CUDRANIA TRICUSPIDATA*

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ABSTRACT

New flavonoid (1) was isolated from ethanol extract of the roots of *Cudrania tricuspidata* by preparative thin-layer chromatography technique. The roots alcoholic extract of *Cudrania tricuspidata* dose dependently inhibited the growth of *Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae*. The structure of compound **1** was elucidated on the basis of MS, IR, UV, 1H NMR and 13C NMR spectroscopic data, including 2D NMR experiments to be 5,7,4'-Trihydroxy-6-prenylflavanone.

KEYWORDS: Isolation, Structural Elucidation, Phytochemical, Flavonoids, *Cudrania tricuspidata*.

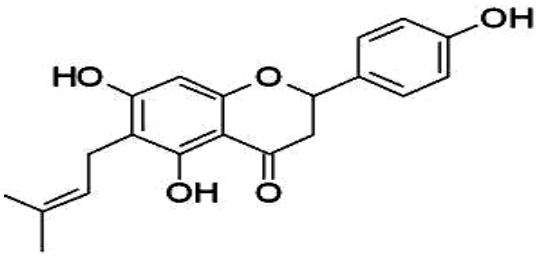
INTRODUCTION

Flavonoids are phenolic substances isolated from a wide range of vascular plants, and more than 8150 different flavonoids have been reported. [1] Flavonoids are located inside the cells or on the surface of various plant organs and have various functions in plants. [2] They act in plants as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellents, and for light screening. [3]

Many studies have shown that flavonoids exhibit biological and pharmacological activities, including antioxidant, cytotoxic, anticancer, antiviral, antibacterial, cardioprotective, hepatoprotective, neuroprotective, antimalarial, antileishmanial, antitrypanosomal and antiamebial properties, [4-8] These biological and pharmacological properties are usually

attributed to their free radical scavenging efficacies, metal complexion capabilities, and their ability to bind to proteins with a high degree of specificity. [9]

Cudrania tricuspidata (carr.) Bur. (Moraceae) is a small thorny tree native to East Asia and distributed mainly in the north part of Iraq. The cortex and root bark of this plant has been used for the treatment of gonorrhea, jaundice, hepatitis, neuritis, and inflammation. [10] Several prenylated xanthones and flavonoids. [11-15] isolated from the root of the genus *Cudrania*, were found to have cytotoxic16-19, antifungal20, antioxidant21, antiatherosclerotic. [22] anti-inflammatory [22] and hepatoprotective. [23, 24] activities and display monoamine oxidase inhibitory effects. [25, 26] In our search for structurally and biological interesting compounds from plants found in Kurdistan-Iraq, new prenylated flavonoid (**1**), was isolated from the roots of *Cudrania tricuspidata*. We report here the isolation and structural elucidation of this compounds (1) and the inhibitory activity of the plant extract against some clinical isolated bacteria (*Escherichia coli, Staphylococcus aureus* and *Klebsiella pneumoniae*). The structure of compound (1) was characterized by MS, IR, UV, 1H and 13C NMR spectroscopy, including 2D NMR experiments.



Compound 1

EXPERIMENTAL

General Experimental Procedures: All melting points were determined on a Yanaco micromelting point apparatus and are uncorrected. UV spectra (Shimadzu UV-1203) were recorded in MeOH, whereas IR spectra (Nicolet 510P FT-IR) were obtained as a KBr disk film. 1H NMR (Bruker AM-500, 500 MHz) and 13C NMR (Bruker AC-200, 75 MHz) spectra were acquired in MeOH-*d4* with TMS as internal standard, whereas EIMS (Shimadzu QP-5000/Gc-17A/DI-50) and HREIMS (VG-ZAB-VSEQ) were recorded at 70 eV (ionizing potential) using a direct inlet system. To monitor the preparative separations, analytical thin-layer chromatography (TLC) was performed at room temperature on pre-coated 0.25 mm thick silica gel 60 F254 glass plates (20 x 20 cm). Chromatograms were visualized after

drying (i) by UV light and (ii) by a phenol specific spray reagent, FeCl3 (3% in dry ethanol). All other chemicals and reagents were analytical grade.

MATERIALS AND METHODS

Plant Material: Roots of *Cudrania tricuspidata* were collected from Rania (Kursistan-Iraq), in February 2014. The plant was identified by the Botany Department, Sulaimania University, and voucher specimens were deposited in the herbarium of that Department. The plant sample was air-dried and ground into uniform powder using a Thomas-Willey milling machine.

PHYTOCHEMICAL SCREENING

Chemical tests were carried out on the aqueous and alcoholic extracts and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara27, Trease and Evans28 and Harborne. [29]

Preparation of Aqueous Extract

The aqueous extract was prepared by extracting 100 g of dried powdered sample with 500 ml of distilled water for 12 h. The extracts were filtered.

Preparation of Alcoholic Extract

The alcoholic extract was prepared by extracting 100 g of dried powdered sample with 500 ml of (95%) ethanol for 5 days at room temperature. The extract was filtered and the ethanol was removed by evaporation under reduced pressure at relatively low temperature (<35oC) to give solids.

Test for Tannins

About 0.5 g of the dried powdered sample of *Cudrania tricuspidata* was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% FeCl3 solution were added and a blue-black coloration observed in the extract indicated the presence of tannins.

Test for Steroids

Two ml of acetic anhydride were added to 0.5 g ethanolic extract of *Cudrania tricuspidata* with 2 ml concentrated H2S04. The color changed from violet to green indicating the presence of steroids.

Test for Flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample. Five ml of 20% NH3 solution were added to a portion of the aqueous filtrate of the plant extract followed by addition of concentrated H2S04. A yellow coloration was observed indicated the presence of flavonoids.

Few drops of 1% aluminium solution were added to a portion of the aqueous filtrate of the plant extract. A dark yellow coloration was observed indicating the presence of flavonoids.
A portion of the powdered plant sample was in heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of 20% NH3 solution. A yellow coloration was observed indicating a positive test for flavonoids.

Test for Alkoloids

Five ml of 2N hydrochloric acid was added to 0.5 g ethanolic extract of the sample and the solution was heated with stirring in a water bath for 10 minutes. The cooled solution was filtered and a few drops of Dragendorff's reagent (0.85 g of bismuth nitrate was dissolved in 10 mL acetic acid and 40 mL of water was added + 8.0 g of potassium iodide was dissolved in 20 mL water) were added to a portion of this solutions. A formation of a reddish-brown precipitates were considered as a positive test for alkaloids. Alkaloids were absent in *Cudrania tricuspidata*.

Test for Terpenoids

Five ml of the extract was mixed with 2 ml of chloroform, and concentrated H2S04 (3 ml) was carefully added to form a layer. A reddish brown coloration of the interface was formed in *Cudrania tricuspidata* to show positive results for the presence of terpenoids. **Test for Saponins.** About 2 g of the dried powdered sample of *Cudrania tricuspidata* was boiled in 20 ml of water in a test tube and then filtered. Ten ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion. Sponins were absent in *Cudrania tricuspidata*.

BIOLOGICAL ACTIVITIES OF FLAVONOIDS

Micro-Organism: The following bacteria clinical isolates were obtained from stock culture of pathological strains, preserved at the Microbiology Laboratory, Faculty of Science,

University of Raparin, Rania, Kurdistan-Iraq: *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae,* and *Candida albicans*. **Antimicrobial Activity.** The disc diffusion method as described by national committee for clinical laboratory standards, was used to determine the growth inhibition of bacteria by the plant extract. Discs containing different concentrations (100 and 200 mg/ml) of dissolved extract were prepared using sterile Whatmann filter paper No. 1, (6 mm in diameters). The discs were dried at 50 oC. Overnight cultures of each of the bacterial isolates was diluted with sterile normal saline to give inoculums size of 106 cfu/ml. Nutrient agar medium was prepared, sterilized, cooled and poured into sterile Petri dishes to a depth of 4 mm (about 25 ml per plate) to solidify. Pure cultures of the test organisms were used to inoculate the Petri dishes. This was done by spreading the inocula on the surface of the prepared nutrient agar plate using sterile cotton swabs which have been dipped in the diluted suspension of the organism. The discs were then aseptically placed evenly on the surface of the inoculation and gently pressed down to ensure contact using a pair of forceps. The plates were finally incubated at **37 oC** for 18–24 h. Amoxcillin (25 mg) was included in each of the inoculated plates as positive control. Plates prepared using the same procedures without extract or antibiotic were equally set as negative control. The plates were examined after 24 h for clear zone of inhibition. Antibacterial activity by the extract was measured and recorded using a pair of calipers and compared to the standard antibiotics as in this study.

EXTRACTION AND ISOLATION

Extraction of Flavonoids from the Roos of *C. tricuspidata:* Air-dried barks of *C. tricuspidata* (1kg) were powdered and extracted with 95% ethanol (5L) at room temperature for 6 days. The extract was filtered, and the solvent was removed under reduced pressure at relatively low temperature (<35oC) to leave a dark brown solid (84 g). **Isolation of Flavonoids from the Roos of *C. tricuspidata***

Thin-Layer Chromatography of the Crude Products: Silica gel 60F254 and water were mixed to form a slurry which was spread over clean glass plates. These plates were used without activation. Small amount of the crude product of *Cudrania tricuspidata* was dissolved in 95% ethanol and applied as concentrated spots on silica gel plates. Many solvent systems have been employed for the separation of flavonoids using TLC. However, the solvent system that achieved the best separation was *n*-BuOH–HOAc–H2O, (3:1:1). Only onespot was detected by spraying with 3% solution of ferric chloride FeCl3 in ethanol.

Preparative Thin-Layer Chromatography: Small amount of the crude product of *Cudrania tricuspidata* was dissolved in the minimum amount of ethanol and applied on (20 x 20 cm) silica gel plates as a narrow strip. The plates were developed with the solvent system *n*-BuOH–HOAc–H2O, (3:1:1) and the chromatograms were located under UV light. The sole major band was scratched and the product extracted from silica gel with ethanol. After filtration, the solvent was removed in *vacuo* to leave a solid (**compound 1**).

5,7,4'-Trihydroxy-6-prenylflavanone: yellow powder; UV (LC-PDA) λmax 292 nm; IR (film) 3412, 2922, 1631, 1550, 1513, 1469, 1437 and 1271 cm-1.; NMR (360 MHz, MeOH-*d*4); HRESIMS [M - H]- 339.1226 *m*/*z* calcd for C20H20O5 (1.8 ppm); ESIMSMS product ions *m*/*z* (% base peak) A fragment 219, B fragment 119, other product ions 175

RESULTS AND DISCUSSION

The results of the phytochemical analysis show that, flavonoids, tannins, alkaloids and terpenoids are present in the extract. Steroids and saponins were not detected (Table 1). The extract produced a dose dependent zone of inhibition in all the organisms tested except for *C. albicans* where the extract did not show any activity. However, the effects observed were less than those produced by the standard agent (Amoxicillin) (Table 1).

Table 1: Phytochemistry and Antimicrobial screening of leaves alcoholic extract of *Cudrania tricuspidata.*

Tests	**Tannins**	**Alkaloids**	**Flavonoids**	**Steroids**	**Terpenoids**	**Saponins**
Result	+	+	+	-	+	-
Zone of Inhibition (mm)						
Extraction Conc.(mg/mL)		Staphylococcus aureus		Klebsiella pneumoniae		Escherichia coli
200		14		15		15
100		12		10		12
Amoxicillin (25)		17		18		19

Compound **1** was isolated as a faint yellow powder from ethanolic extract of the roots of *Cudrania tricuspidata*. The ethanolic extract of the plant was subjected to preparative TLCon silica gel and the TLC plates were developed with the solvent system CH3Cl3-MeOH, (96:4) to obtain compound **1**. The structure of compound **1** was elucidated on the basis of MS, IR, UV, 1H NMR and 13C NMR spectroscopic data, including 2D NMR experiments.

The IR spectrum of compound **1** (Fig. 1) showed characteristic absorption bands at ν (KBr) 3412 (OH), 2922 (CH-stretching), 1631 (C=O), 1550, 1513, 1469 and 1437 (C=C, Ar), 1271 (C-O) cm-1. The presence of carbonyl at 1631 cm-1 indicated that compound **1** belongs to:

flavones, flavonols, isoflavone, flavanone dihydroflavonols, chalcones, dihydrochalcones or aurones. The various flavonoid classes can be recognized from each other by their UV spectra. Isoflavones, flavanones and dihydroflavonols all give similar UV spectra as a result of their having little or no conjugation between the A-and B-rings. They are all readily distinguished from flavones and flavonols by their UV spectra, which typically exhibit an intense Band II absorption with only a shoulder or low intensity peak representing band I30. The Band II absorption of isoflavones usually occurs in the region 245 – 270 nm. Both flavanones and dihydrofavonols have their major absorption peak (Band II) in the range 270 – 295 nm and are therefore clearly distinguished from the spectra of isoflavones (which have their Band II peaks between 245 and 270 nm)31.

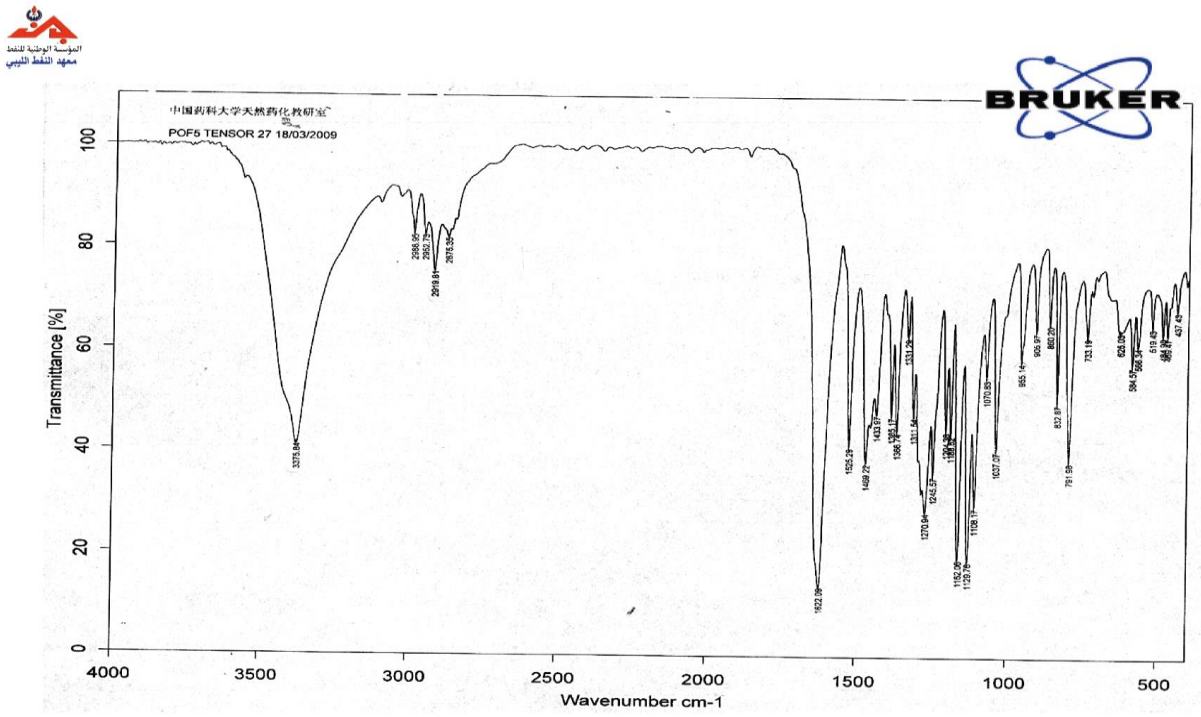


Figure 80: IR (KBr, disc) spectrum of 5.

The UV spectrum of compound **1** in MeOH (Fig. 2) showed characteristic intense absorption band at 292 (Band II) with only a shoulder or low intensity peak representing Band I, indicating the presence of a flavanone or dihydroflavonol skelton31.

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UV-Visible

Spectrum

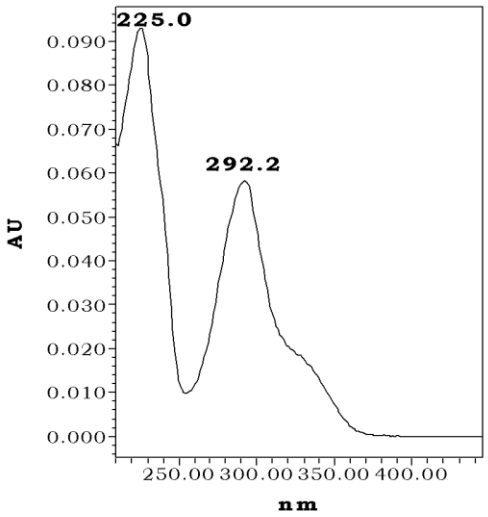
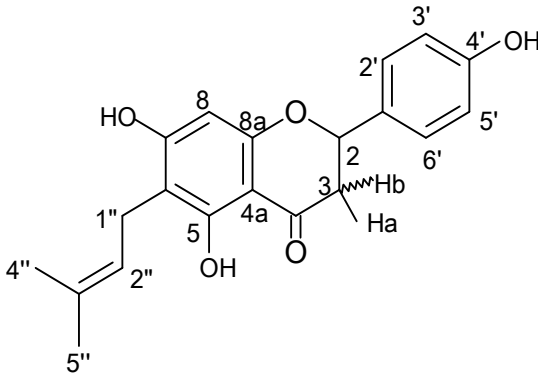


Figure 81: UV spectrum of compound 1.

The 1H NMR spectrum (Fig. 3) of compound **1** showed a number of signals characteristic of flavanone and isopreny moieties32-34. The flavanone skelton of **1** was identified by the signal for the C-2 proton appeared as quartet (two doublets *J*cis = 3.1 Hz, *J*trans = 12.8 Hz) at δ 5.29 ppm as a result of the coupling of the C-2 proton with the two protons at C-331. The C-3 protons couple with each other (*J* = 17.0 Hz) in addition to their spin-spin intraction with the C-2 proton, thus giving rise to two overlapping quartets at δ 3.10 (H-3a, *J* = 17.6 and 12.8.5 Hz) and 2.67 (H-3b, *J* = 17.0 and 3.1 Hz). Two of the signals of each quartet, however, are weak and are often not observed (Fig. 3). Two 2H doublets resonating at δ 7.31 (*J*2',3'/6',5' = 8.5 Hz) and 6.81 (*J*3',2'/5',6' = 8.5 Hz) are due to the aromatic H-2',6' and H-3',5', respectively, of the B-ring and their coupling constant showed that they are *ortho*-coupled. A singlet resonating at δ 5.93 (s, 1H) was assigned to H-8. The presence of a prenyl (isopentenyl) unit was indicated by typical chemical shifts and *J* values35. The H-2″ olefinic proton signal of isopentenyl unit was observed as a triplet at δ 5.18 (1H, t, *J* = 7.2 Hz), while the two methyl signals typically appeared as singlets at δ 1.75 and 1.65 (each 3H, s). The signal corresponding to the methylene CH2-1″ protons appeared as one broad isochronic (2H) doublet at δ 3.20 with *J* = 7.2 Hz.

1H	ppm	Mult (J)	Integ
H-2,6	7.31	d (8.5)	2H
H-3,5	6.81	d (8.5)	2H
H-8	5.93	s	1H
H-2	5.29	dd(12.8, 3.1)	1H
H-2"	5.18	t (7.2)	1H
CH_2-1"	3.20	br-d	2H

H-3a	3.10	dd (17.0, 12.8)	1H
H-3b	2.67	dd (17.0, 3.1)	1H
CH_3-4"	1.75	s	3H
CH_3-5"	1.65	s	3H



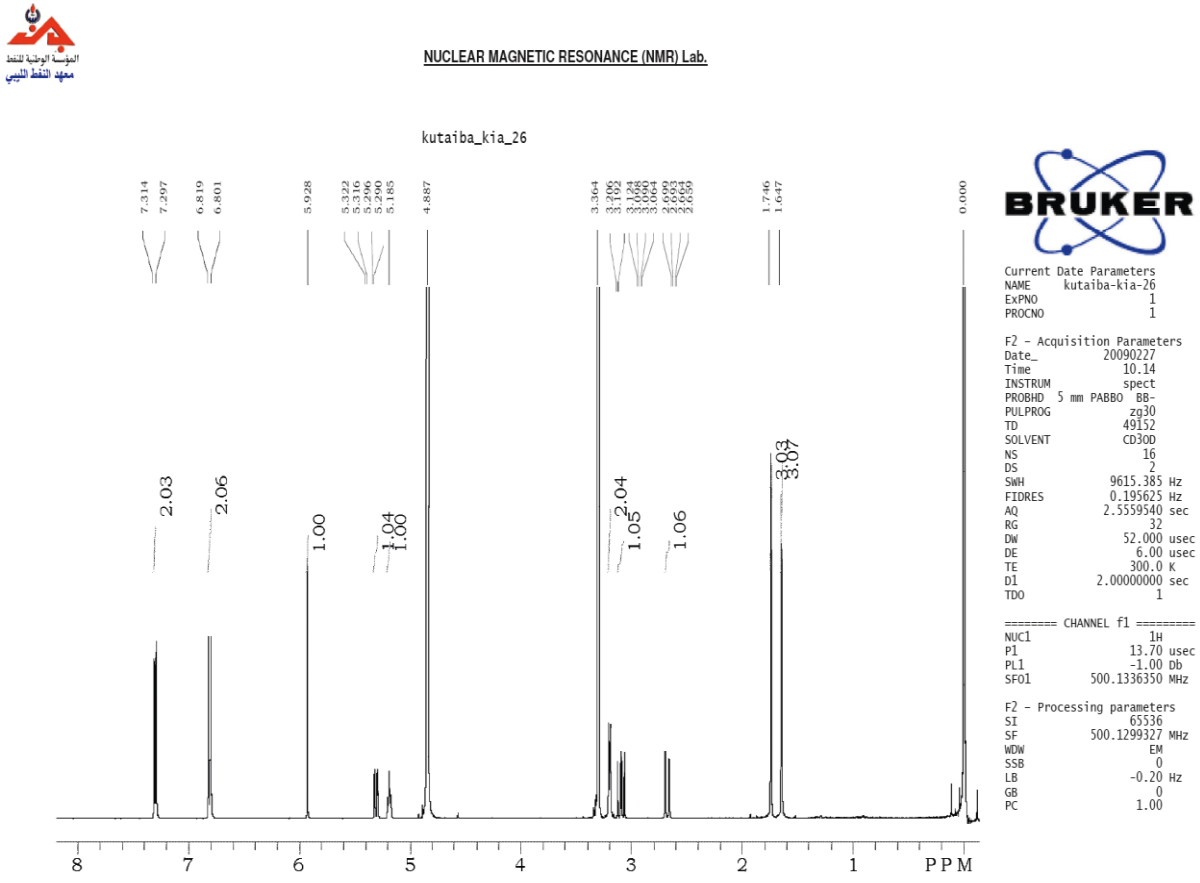
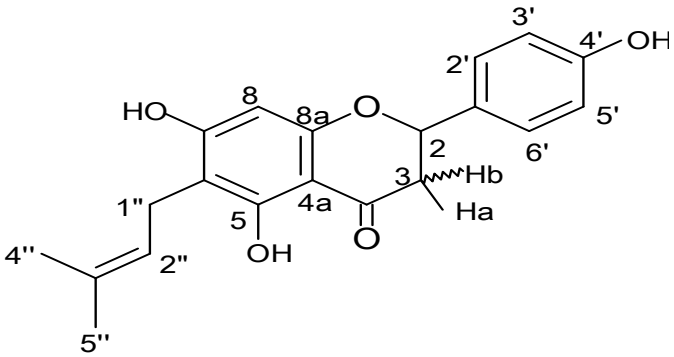


Figure 3: 1H NMR spectrum of compound 1 in MeOH-*d*4.

The ^{13}C-NMR spectrum (Fig. 4) of compound **1** showed resonances for twenty carbon atoms with two methyl, two methylene, seven methine and nine quaternary carbons in the molecule. The flavanone skeleton of **1** was further supported by the presence of two characteristics

methine carbon at δ_C 80.3 (C-2), a methylene carbon at δ_C 44.02 (C-3) and a carbonyl carbon at δ_C 198.1 (C-4), 143.5 (β-C). The downfield chemical shift of the C=O ketonic carbon indicated the presence of a hydroxyl group at the adjacent C-5 position in A-ring [32]. The other signals at δ_C 106.0 (C-4a), 166.2 (C-8a), 109.1 (C-8), 163.2 (C-7), 96.4 (C-6), 158.94 (C-5), 22.52 (C-1"), 124.0 (C-2"), 131.6 (C-3"), 25.9 (C-4") and 17.9 (C-5") further supported the presence of a flavanone skeleton and isoprenyl group substituted A-ring. Similarly the carbons of the aromatic B-ring resonated at δ_C 131.4 (C-1'), 128.9 (C-2',6'), 116.3 (C-3',5') and 158.9 (C-4'), indicating a C-4' substituted B-ring. The complete ^{13}C-NMR chemical shift data for **1**, when compared with the reported data, indicated that the aglycone was a flavanone having isoprenyl as a side chain[36]. The complete ^{13}C-NMR and multiplicity data of compound **1** are presented in Figure 4.

13C	ppm	HMQC	HMBC
C=O	198.1		H-α; H-β
C-8a	166.2		H-1"
C-7	163.2		H-5', H-1"
C-5	161.6		OCH_3-6'; H-5'
C-4'	158.9		H-3,5; H-2,6
C-3"	131.6		H-4",5"
C-1'	131.4		H- α; H-3,5
C-2',6'	128.9	x	H-β; **H-2,6**
C-2"	124.0	x	H1"; Me-4",5"
C-3',5'	116.3	x	H-2,6; **H-3,5**
C-8	109.1		H-5', H-1"
C-4a	106.0		H-5'
C-6	96.4	x	
C-2	80.3		
MeOH	49.0		
C-3	44.0		
C-4"	25.9	x	CH_3-5"
C-1"	22.5	x	
C-5"	17.9	x	CH_3-4"
TMS	0.0		



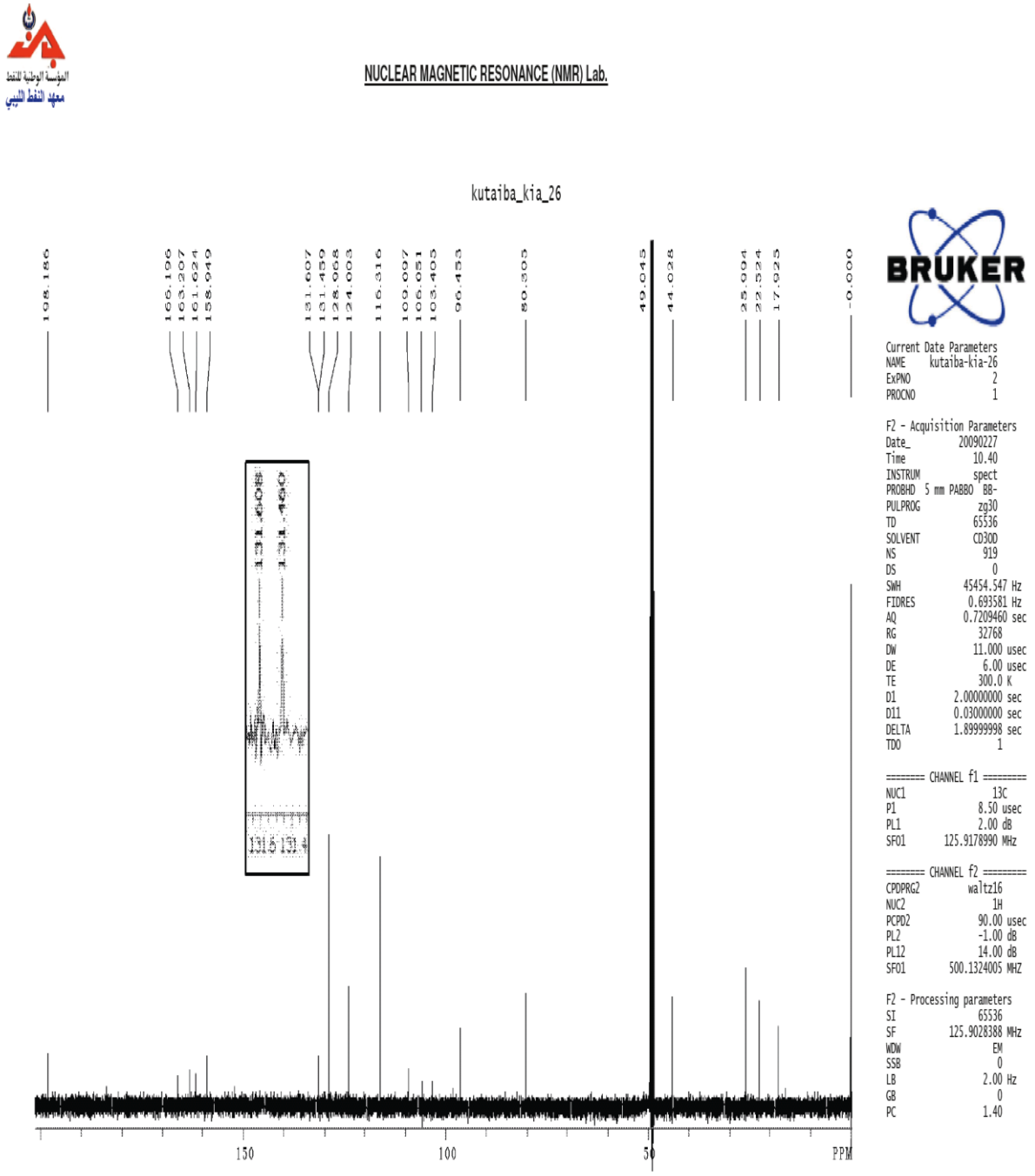


Figure 4: ^{13}C NMR spectrum of compound 1 in MeOH-d_4.

The HMQC technique was used to establish the direct one-bond ^{1}H-^{13}C connectivities. The protons of rings A, B and C i.e. H-6 (δ_H 5.93), H-2 (δ_H 5.29), H-3a,b (δ_H 3.10, 2.67), H-2',6' (δ_H 7.31) and H-3',5' (δ_H 6.81) showed one-bond correlations with C-6 (δ_C 96.45), C-2 (δ_C 80.3), C-3 (δ_C 44.0), C-2,6 (δ_C 128.96) and C-3,5 (δ_C 116.3), respectively (Fig. 5). Similarly protons signals of the isopentenyl moiety, i.e. H-1" (δ_H 3.20), H-2" (δ_H 5.18), H-4" (δ_H 1.75) and H-5" (δ_H 1.65) also showed direct connectivities with C-1" (δ_C 22.5), C-2" (δ_C 124.0), C-4" (δ_C 25.99) and C-5" (δ_C 17.9), respectively.

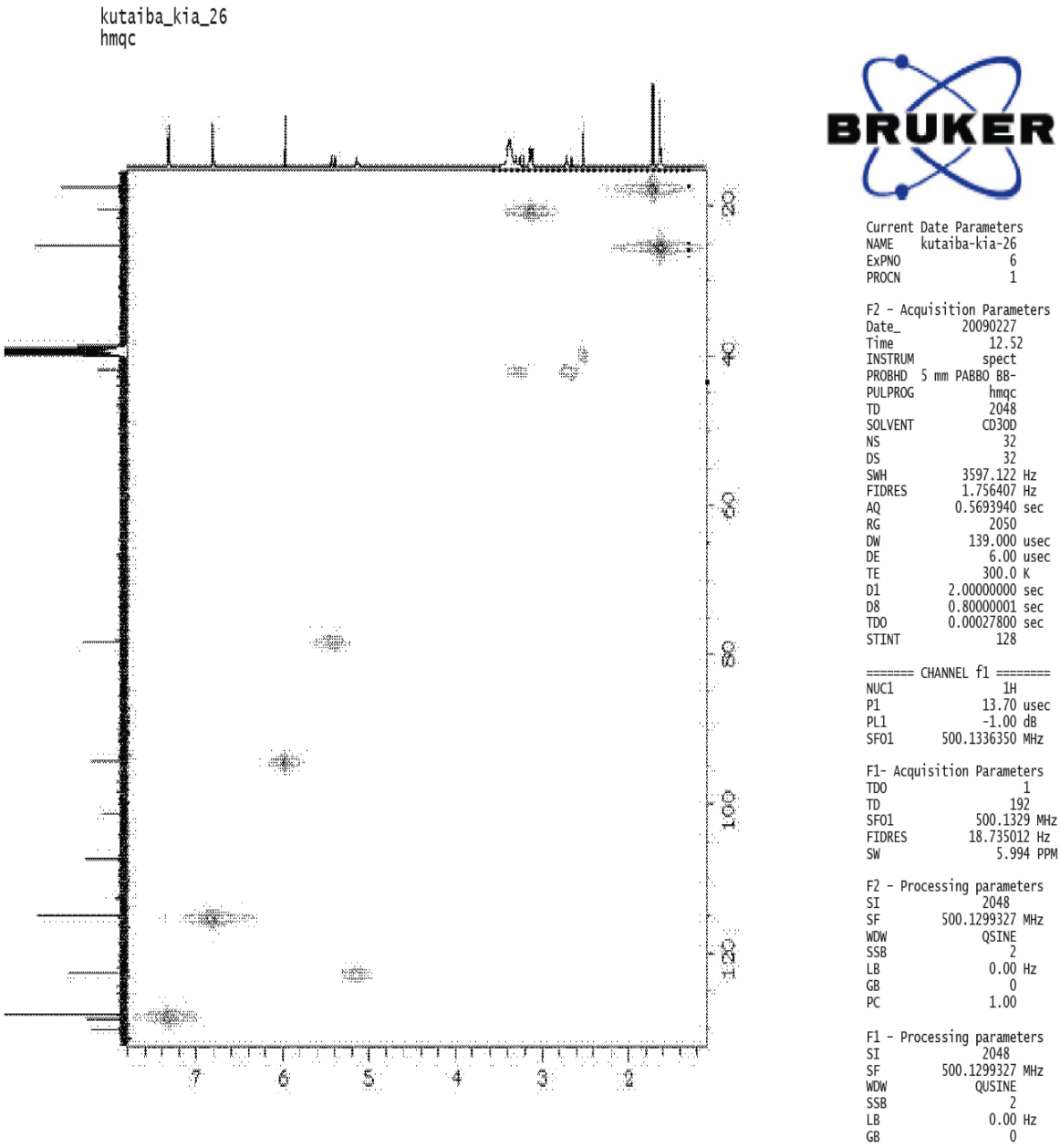


Figure 5: ^{1}H-^{13}C HMQC NMR spectrum of 1 in MeOH-d_4.

^{1}H-^{1}H COSY NMR spectrum of compounds **1** showed coupling between the doublets of H-2',6' (δ_H 7.31), H-3',5' (δ_H 6.81) of aromatic B-ring (Fig. 6). Interaction of the C-1" methylene protons (δ_H 3.20) with the C-2" methine proton (δ_H 5.20) was observed. The latter (H-2") also showed coupling with the C-4" methyl protons (δ_H 1.76), which was in turn coupled with the C-5" methyl protons (δ_H 1.65). The C-1" methylene protons showed correlation with the C-4" methyl protons (δ_H 1.76), which was in turn coupled with the C-5" methyl protons (δ_H 1.65).

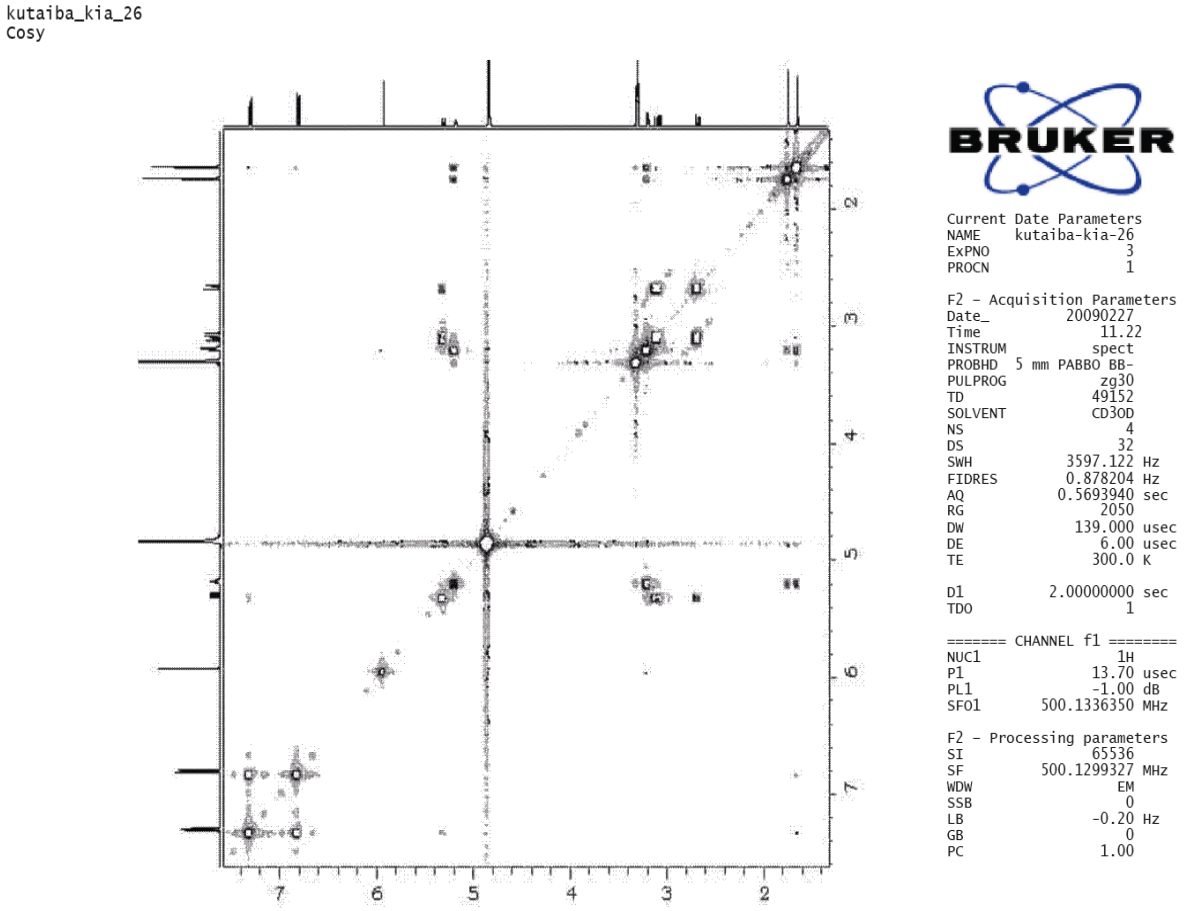


Figure 6: ^{1}H-^{1}H COSY NMR spectrum of compounds 1 in MeOH-d_4.

Structure **1** was finally assembled with the help on Heteronuclear Multiple Bond Connectivity (HMBC) experiment (Fig. 7). The H-6 (δ_H 5.93) showed two-bond coupling with C-7 (δ_C 163.2) and C-5 (δ_C 158.9), three-bond coupling with C-8 (δ_C 109.1) and C-4a (δ_C 106.0). The H-2',6' (δ_H 7.31) of aromatic B-ring B showed coupling with C-2',6' (δ_C 128.9), C-3',5' (δ_C 116.3), C-4' (δ_C 158.9) and C-2 (δ_C 80.3), while H-3',5' signal (δ_H 6.81) was found to be coupled with C-3',5' (δ_C 116.3), C-4' (δ_C 158.9) and C-2',6' (δ_C 128.9). The H-3a (δ_H 3.10) showed coupling with C-2 (δ_C 80.44), C-1' (δ_C 131.4), C-2',6' (δ_C 128.9), C=O (δ_C 198.1), C-4a (δ_C 106.0) and C-8a (δ_C 166.1), while H-3b (δ_H 2.67) signal was found to be coupled with C-3 (δ_C 44.0). The H-2 (δ_H 5.29) showed coupling with C-1' (δ_C 131.6). The attachment of the isopentenyl moiety at C-8 of ring A was established by the HMBC interaction of methylene H-1" (δ_H 3.20) with C-8 (δ_C 109.4) of aglycone. The H-1" (δ_H 3.20) showed two-bond couplings with C-8 (δ_C 109.4) and C-2" (δ_C 124.0), and three-bond couplings with C-8a (δ_C 166.1), C-7 (δ_C 163.2) and C-3" (δ_C 131.6). The Me-4" (δ_H 1.75) showed couplings with C-3" (δ_C 131.6) and C-1" (δ_C 22.5), while Me-5" (δ_H 1.65) signal was found to be coupled with C-3" (δ_C 131.6) and C-1" (δ_C 22.5).

Some observations in the HMBC spectrum included the following:

- There were no correlations observed with C-3.
- A C-8/H-8 crosspeak appears as a "doublet" in the F1 direction.

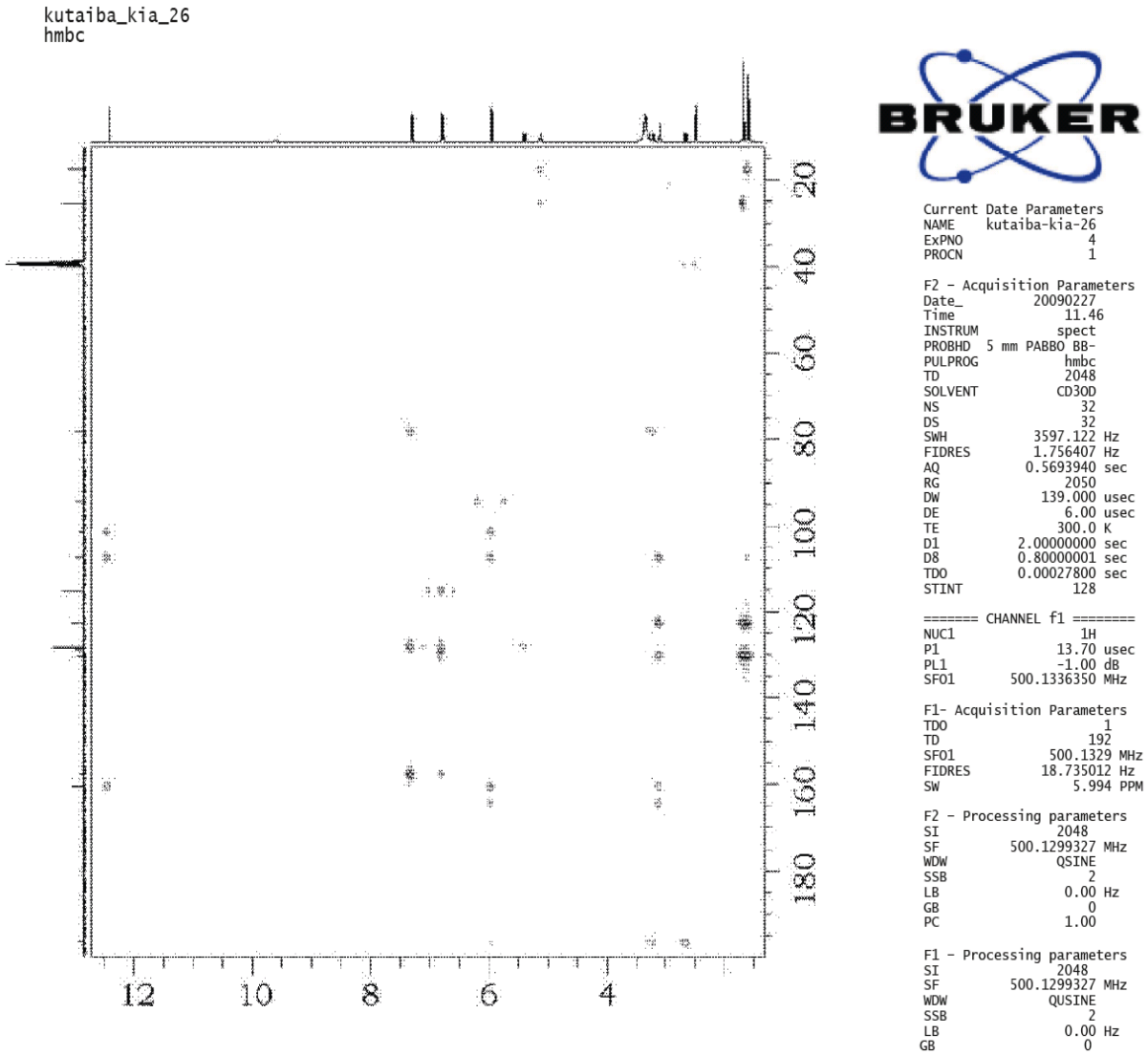


Figure 7: ^{1}H-^{13}C HMBC NMR spectrum of 1 in MeOH-d_4

The high-resolution mass spectrum of **1** (Fig. 8) showed a $[M-1]^-$ ion at 339.1226 corresponding to a molecular formula of $C_{20}H_{20}O_5$. The electrospray ionization mass spectrum (ESI-MS) of **1** showed several fragments characteristic of an isoprenelyted flavanone skeleton. The major peaks at *m/z* 219 (A fragment) and 119 (B fragment) were due to the cleavage of ring C through a retro-Diels Alder mechanism (Fig. 9), and indicated the presence of an isoprenelyl unit and two hydroxy groups on ring A and a hydroxyl group on Ring Boft heagly cone. [37, 38]

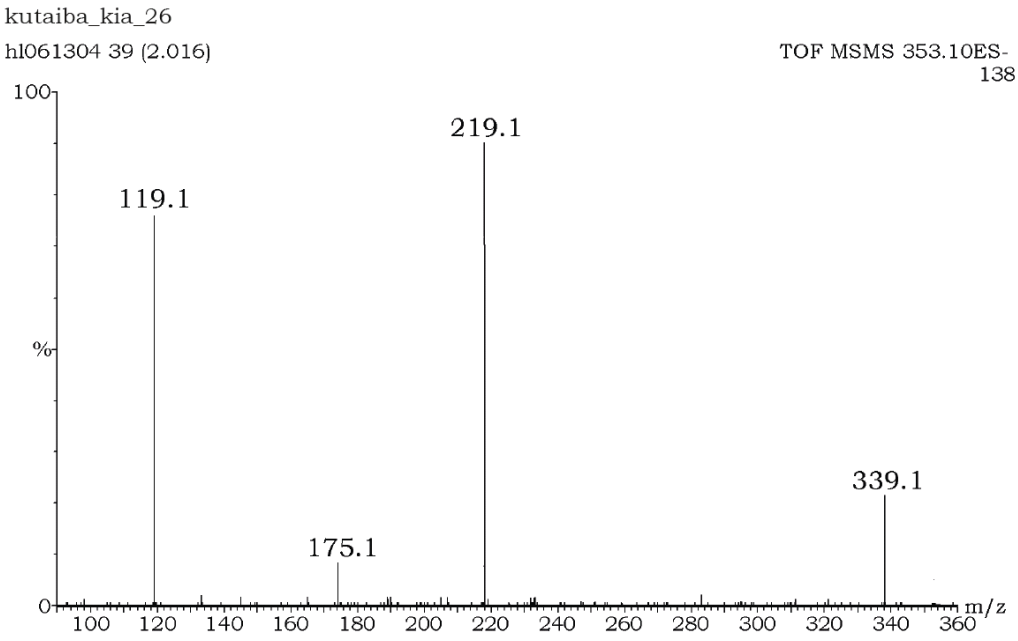


Figure 8: ESI-MS^2 spectrum of compounds 1.

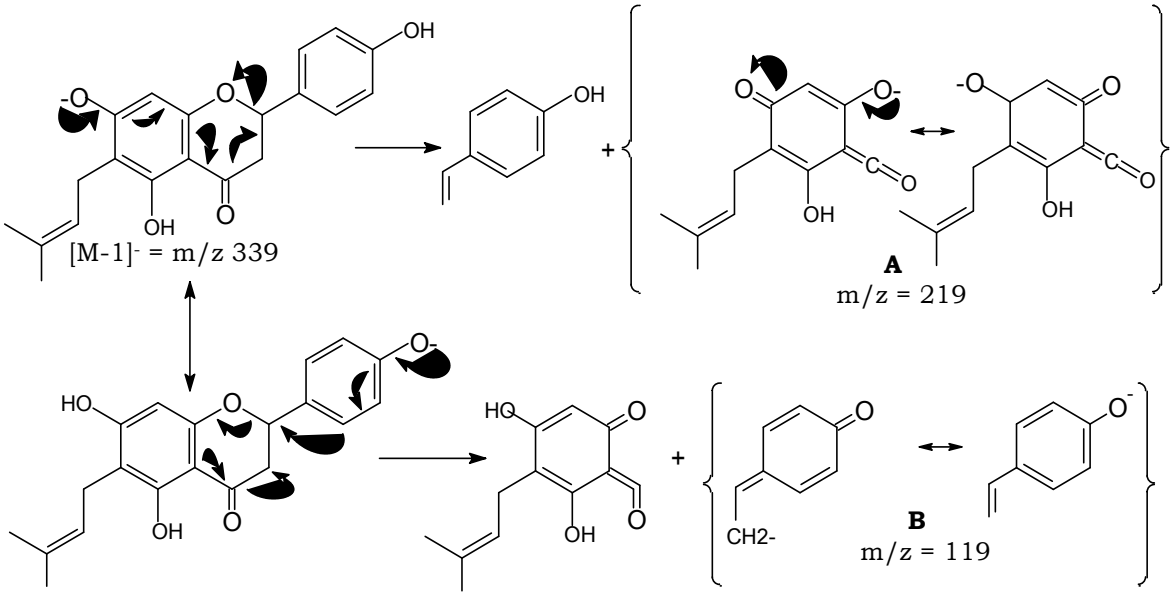


Figure 9: Fragmentation pathways to A and B fragments of compound 5

On the basis of the above spectral evidences, the structure of compound **1** was deduced to be 6-prenylnaringenin.

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