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ISOLATION, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITY OF THE FLAVONOID FROM THE LEAVES OF *JUNIPERUS PHOENICEA*

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ABSTRACT

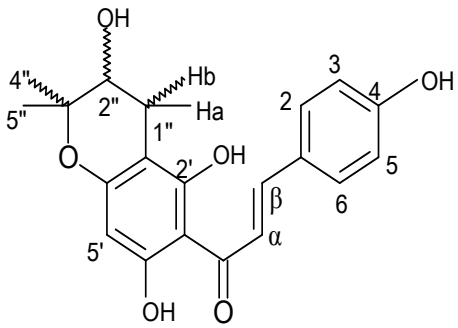
New flavonoid (1) was isolated from ethanol extract of the leaves of Juniperus phoenicea by preparative thin-layer chromatography technique. The leaves alcoholic extract of J. phoenicea dose dependently inhibited the growth of Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae. The structure 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 1-(3,4-dihydro-3,5,7-trihydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-3-(4-hydroxyphenyl)- -propen-1-one.

KEYWORDS: Isolation, Structural Elucidation, Phytochemical, Flavonoids, Juniperus phoenicea.

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] Juniperus phoenicea is an evergreen coniferous shrub or small tree occurring a throughout the Mediterranean region, from Morocco and Portugal east to Turkey and Egypt, and also on Madeira and the Canary Islands, and on the mountains of western Saudi Arabia near the Red Sea and grows up to 10 m in height. Juniper berries have long been used as flavoring agents in foods. Oil of Juniper is used for catgut ligatures, diuretic, carminative, steam inhalant in the management of bronchitis and to control arthritis. Juniper has been used in phytotherapy and cosmetics in the eastern Mediterranean region. [10] Reported therapeutic uses of juniper include juniper baths for the treatment of neurasthenic neurosis [11] and management of scalp psoriasis. [12] In our search for structurally and biological interesting compounds from plants found in Rania (Kurdistan-Iraq), new flavonoids (1), was isolated from the leaves of Juniperus phoenicea. 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) bya phenol specific spray reagent, FeCl3 (3% in dry ethanol). All other chemicals and reagents were analytical grade.

MATERIALS AND METHODS

Plant Material: Leaves of Juniperus phoenicea were collected from Rania (Kurdistan-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 airdried 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 Sofowara,[13] Trease and Evans[14] and Harborne. [15]

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 Juniperus phoenicea 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 Juniperus phoenicea 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 reddishbrown precipitates were considered as a positive test for alkaloids. Alkaloids were absent inJuniperus phoenicea.

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 Juniperus phoenicea to show positive results for the presence of terpenoids.

Test for Saponins. About 2 g of the dried powdered sample of Juniperus phoenicea 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 Juniperus phoenicea.

Biological Activities of Flavonoids

Micro-Organism. The following bacteria clinical isolates were obtained from stock culture of pathological strains, preserved at the Microbiology Laboratory, 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 at50oC. 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 37oC 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 Leaves of J. Phoenicea. Air-dried leaves of J. phoenicea (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 green solid (104 g).

Isolation of Flavonoids from the Leaves of J. Phoenicea

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 Juniperus phoenicea 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 EtOAc–HCOOH–HOAc–H2O, (100:11:11:26). Only one spot was detected by spraying with 3% solution of ferric chloride FeCl3 in ethanol.

Preparative Thin-Layer Chromatography. Small amount of the crude product of Juniperus phoenicea 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 EtOAc–HCOOH–HOAc–H2O, (100:11:11:26) 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). (2E)-1-(3,4-dihydro-3,5,7-trihydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-3-(4-hydroxyphenyl)-2-propen-1-one (1): yellow orange solid;; UV (LC-PDA) λmax 372 nm; IR (film) 3300, 2976, 2923, 1605, 1507, 1348, 1226, 1166, 1132, 830 cm-1; NMR (360 MHz, MeOH-d4); HRESIMS [M - H]-355.1188 m/z calcd for C20H20O6 (1.8 ppm); ESIMSMS product ions m/z (% base peak) A fragment 235 (8), B fragment 119 (40), other product ions 297, 283, 163.

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 Juniperus phoenicea

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 yellow-orange powder from ethanolic extract of the leaves of Juniperus phoenicea. The ethanolic extract of the plant was subjected to preparative TLC on silica gel and the TLC plates were developed with the solvent system EtOAc–

HCOOH–HOAc–H2O, (100:11:11:26) 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 υ (KBr) 3422 (OH), 2955 (CH-stretching), 1639 (C=O), 1516, 1472 and 1439 (C=C, Ar), 1242 (C-O) cm-1. The presence of conjugated carbonyl at 1625 cm-1indicated that compound 1 belongs to: flavones, flavonols, chalcones or aurones.

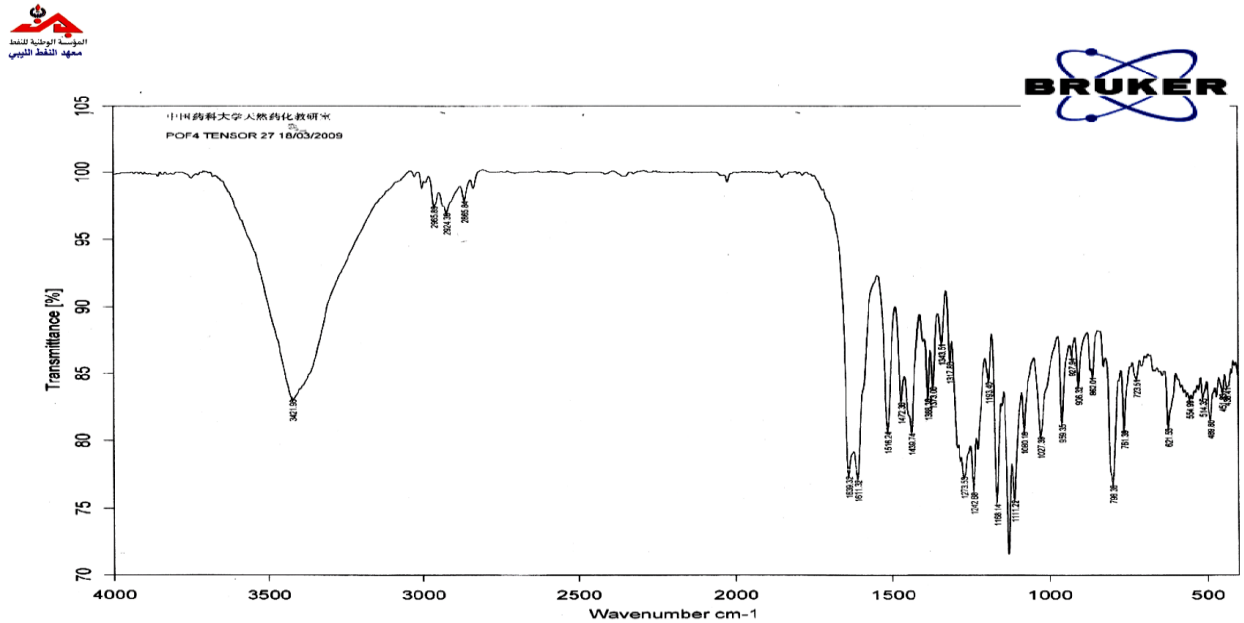


Figure 1. IR (KBr, disc) spectrum of 1.

The UV spectrum of compound 1 in MeOH (Fig. 2) showed characteristic intense absorption band at 371 (Band I) and a diminished absorption band at 240 (Band II) nm, indicating the presence of a chalcone skeleton. [16]

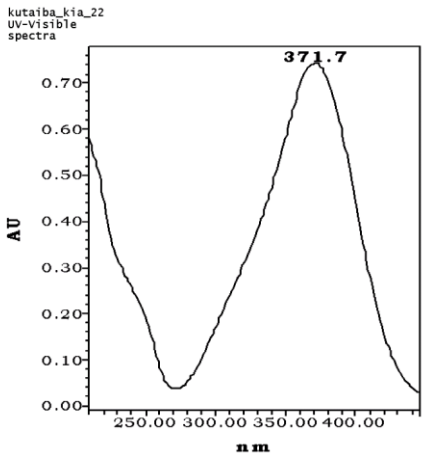


Figure 2. UV spectrum of compound 1.

The chalcone skeltone of compound 1 can easily be distinguished from the remaining classes of flavonoids by the two set of doublets at δH 7.94 and 7.63 (each 1H, d, J=15.5 Hz) in the 1H-NMR spectrum corresponding to carbons resonating at δC 126.12 and 142.96 in the 13C-NMR spectrum, assigned to α- and β-carbon atoms respectively. [17-19] The 1H NMR spectrum (Fig. 3) of compound 1 showed a number of signals characteristic of chalcone and cyclyzed (pyrano) prenyl moieties. [19-21] The chalcone skelton of 1 was identified by a characteristic two 1H doublets with same coupling constants (J = 15.5 Hz) resonating at δ 7.94 and 7.63 assigned to the olefinic α- and β-protons respectively. Two 2H doublets resonating at δ 7.5 (J2,3/6,5 = 8.5 Hz) and 6.83 (J3,2/5,6 = 8.5 Hz) were due to the aromatic H-2,6 and H-3,5, respectively, of the B-ring and their coupling constant showed that they were ortho-coupled. A singlet resonating at δ 5.93 (s, 1H) was assigned to H-5'. The presence of a 2,2-dimethyl-3-hydroxydihydropyran moiety was identified by the protons of the C-1″ methylene group resonating separately as two double doublets at δ 2.85 (1H, dd, J = 5.5 and 16.9 Hz, H-1″a,) and 2.51 (1H, dd, J = 6.9 and 16.9 Hz, H-1″b). The oxymethine proton of the moiety was observed as a double doublet at δ 3.79 (1H, dd, J = 6.8 and 5.5 Hz). While the two methyl signals typically appeared as singlets at δ 1.45 and 1.39 (each 3H, s).

1H	ppm	Mult (J)	Integ
H-α	7.94	d (15.5)	1H
H-β	7.63	d (15.5)	1H
H-2,6	7.50	d (8.5)	2H
H-3,5	6.83	d (8.5)	2H
H-5'	5.93	s	1H
H-2"	3.79	dd (6.8, 5.5)	1H
H-1″a	2.85	dd (16.9, 5.5)	1H
H-1"b	2.51	dd (16.9, 6.9)	1H
CH_3-5"	1.45	s	3H
CH_3-4"	1.39	s	3H

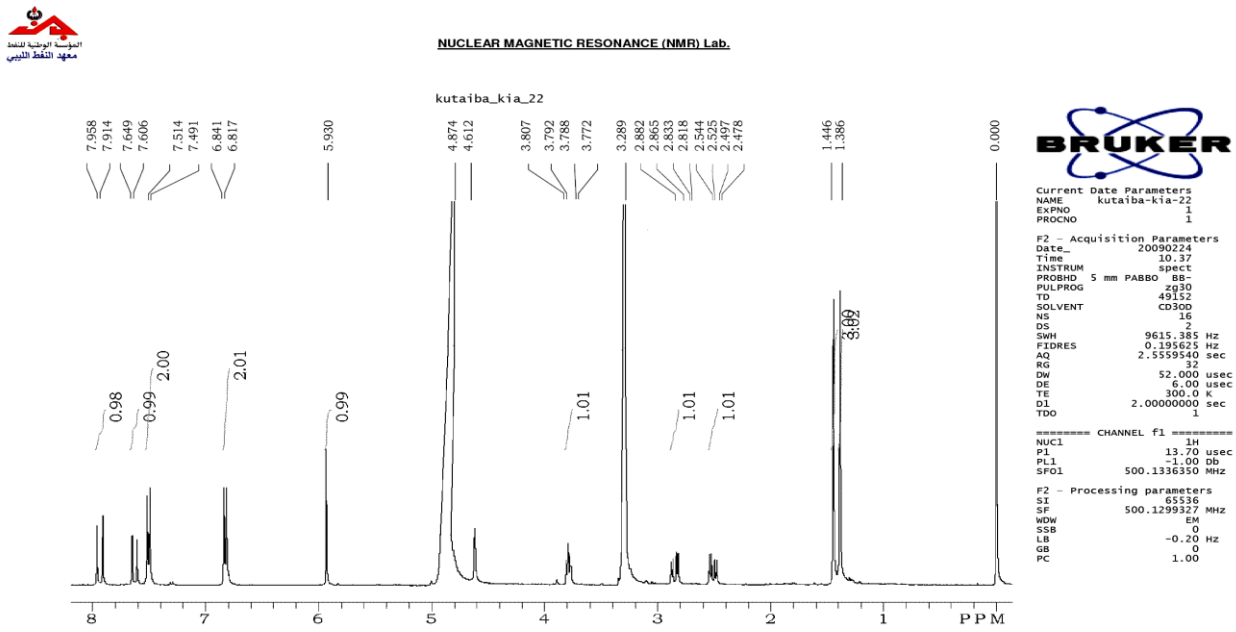
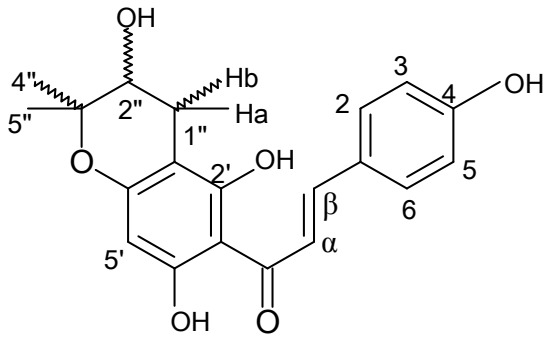


Figure 3. 1H NMR spectrum of compound 1 in MeOH-d4.

The 13 C-NMR spectrum (Fig. 4) of compound 1 showed resonances for twenty carbon atoms with two methyl, one methylene, eight methine and nine quaternary carbons in the molecule. The chalcone skeleton of 1 was further supported by the presence of two characteristics olefinic carbons resonating at δC 126.121 (α-C) and 142.964 (β-C). The downfield signal at δC 194.20 was assigned to the ketonic carbonyl carbon. The downfield chemical shift of the C=O ketonic carbon indicated the presence of a hydroxyl group at the adjacent C-6' position in ring A 19 . The other signals at δC 106.2 (C-1'), 157.166 (C-2'), 100.874 (C-3'), 164.288 (C-4'), 96.192 (C-5'), 166.696 (C-6'), 26.822 (C-1"), 69.495 (C-2"), 79.652 (C-3"), 21.528 (C-4") and 25.936 (C-5") further supported the presence of a chalcone skeleton and 2,2-dimethyl-3-hydroxydihydropyran substituted A-ring. Similarly the carbons of the aromatic B-ring resonated at δC 128.521 (C-1), 131.179 (C-2,6), 116.921 (C-3,5), supporting the presence of a C-4 substituted B-ring in the chalcone skeleton. The hydroxyl bearing C-4 resonated at δC 161.11. A careful study of the 13 CNMR data and its comparison with the reported data again indicated that the aglycone was a chalcone. [22,23] The complete 13 C-NMR and multiplicity data of compound 1 are presented in Figure.

13C	ppm	HMQC	HMBC
C-β	142.964	X	H-2.6
C-α	126.121	X	
C=O	194.20		H-α, H-β
C-1'	106.2		H-5'

C-6'	166.2		(H-5')
C-5'	96.191	X	H-2,6; H-3,5
C-4'	164.288		H-5'
C-3'	100.874	X	H-1"b; H-5'
C-2'	157.166		
C-1	128.521		H-3,5; H-α
C-2,6	131.179	X	H-2',6'; H-β
C-3,5	116.921	X	H-3,5
C-4	161.11		H-2,6; H-3,5
C-1"	26.822	X	
C-2"	69.495	X	Me-4",5"; H1"b
C-3"	79.652		Me-4",5"
C-4"	21.528	X	Me-5"
C-5"	25.936	X	Me-4"
MeOH	49.O3	X	
TMS	0.0		



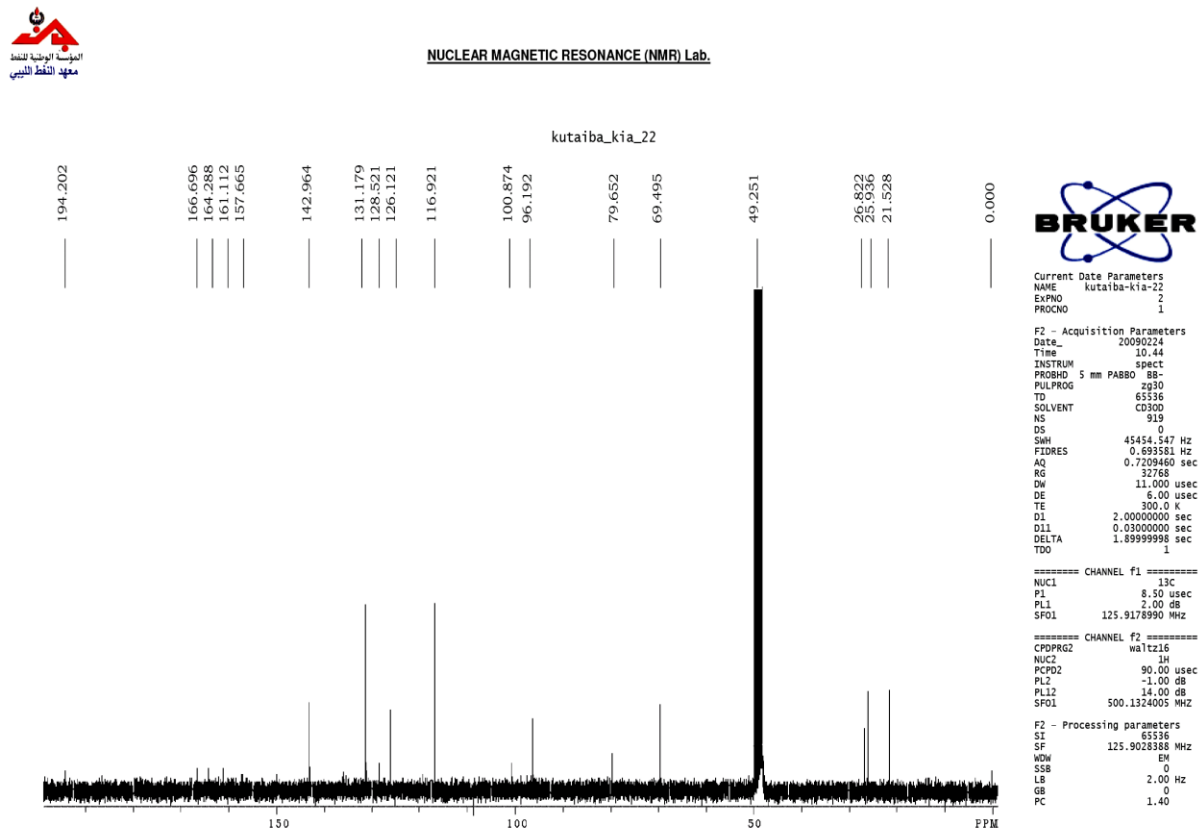


Figure 4. 13C NMR spectrum of compound 1 in MeOH-d4.

The HMQC technique was used to establish the direct one-bond 1 H-13C connectivities. The protons of rings A, B and C i.e. H-5' (δH 5.93), α-H (δH 7.94), β-H (δH 7.63), H-2,6 (δH 7.50) and H-3,5 (δH 6.83) showed one-bond correlations with C-5' (δC 96.192), α-C (δC 126.121) and β-C (δC142.964), C-2, 6 (δC 131.179) and C-3, 5 (δC 116.921), respectively (Fig. 5). Similarly protons signals of the 2,2-dimethyl-3-hydroxydihydropyran moiety, i.e. H-1"a (δH 2.85), H-1"b (δH 2.51), H-2" (δH 3.79), H-4" (δH 1.39) and H-5" (δH 1.45) also showed direct connectivities with C-1" (δC 26.822), C-2" (δC 69.495), C-4" (δC 21.528) and C-5" (δC 21.528), respectively.

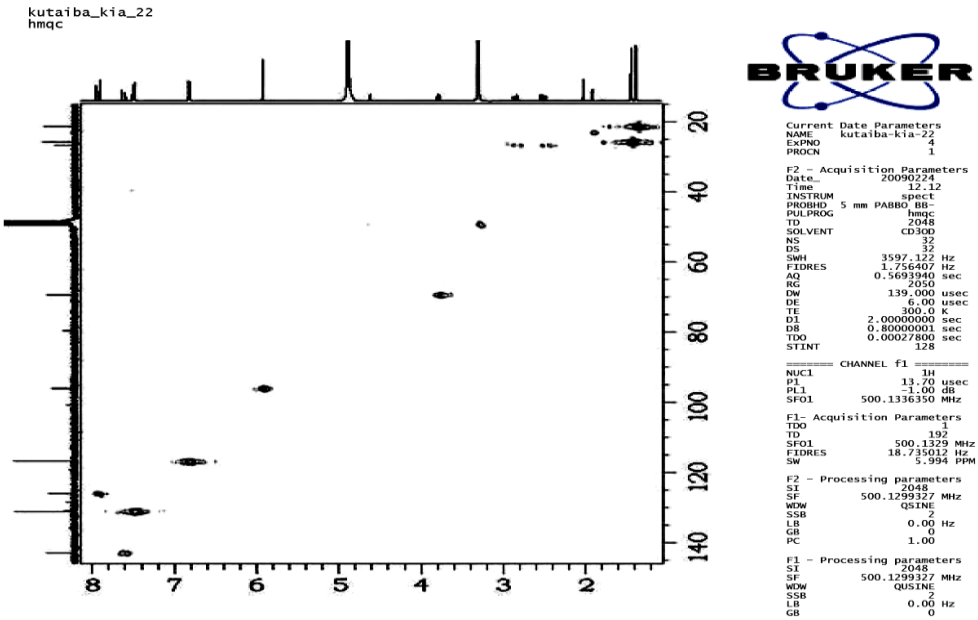


Figure 5. ^{1}H-^{13}C HMQC NMR spectrum of 1 in MeOH-d_4.

1H-1H COSY NMR spectrum of compounds 1 showed couplings between the doublets of H-2,6 (δH 7.50), H-3,5 (δH 6.83) of aromatic B-ring (Fig. 6). The α-H (δH 7.94) showed correlation with the β-H (δH 7.63). Interactions of the C-1" methylene protons (δH 2.85 and 2.51) with the C-2" methine proton (δH 3.79) was observed.

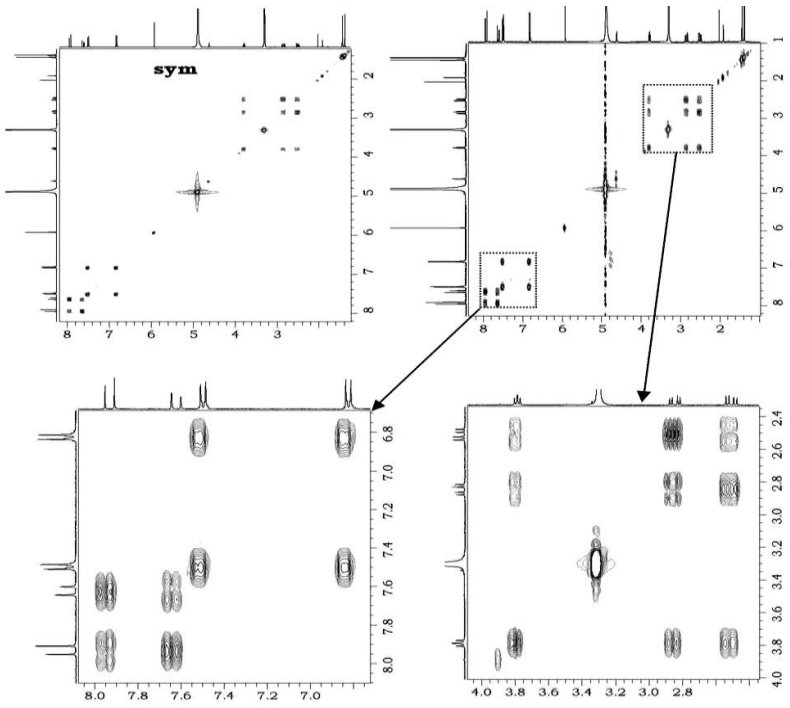


Figure 6.H-1H COSY NMR spectrum of compounds 1 in MeOH-d4.

Structure 1 was finally assembled with the help on Heteronuclear Multiple Bond Connectivity (HMBC) experiment (Fig. 7). The H-5' (δH 5.93) showed two-bond coupling with C-4' (δC164.288) and C-6' (δC 166.696), three-bond coupling with C-3' (δC 100.874) and C-1' (δC 106.2). The H-2,6 (δH 7.50) of aromatic B-ring showed coupling with C-2,6 (δC 131.179), C-4 (δC 161.11) and β-C (δC 142.964), while H-3,5 signal (δH 6.83) was found to be coupled with C-3,5 (δC116.921), C-4 (δC 161.11) and C-1 (δC 128.521). The α-H (δH 7.94) showed coupling with C-1 (δC128.521) and C=O (δC 194.20), while β-H (δH 7.63) signal was found to be coupled with C-2,6 (δC131.179) and C=O (δC 194.8). The attachment of the 2,2-dimethyl-3-hydroxydihydropyran moiety at C-3' of ring A was inferred from the HMBC interaction of C-1" methylene proton H-1"b (δH2.51) with the C-3' signal (δC 100.874) of the aglycone. The H-1"b (δH 2.51) showed heteronuclear ineractions with C-3' (δC 100.4) and C-2" (δC 69.495). The H-4" (δH 1.39) showed coupling with C-2" (δC 69.495), C-3" (δC 79.652) and C-5" (δC 25.936), while H-5" (δH 1.45) signal was found to be coupled with C-2" (δC 69.495), C-3" (δC 79.652) and C-4" (δC 21.528).

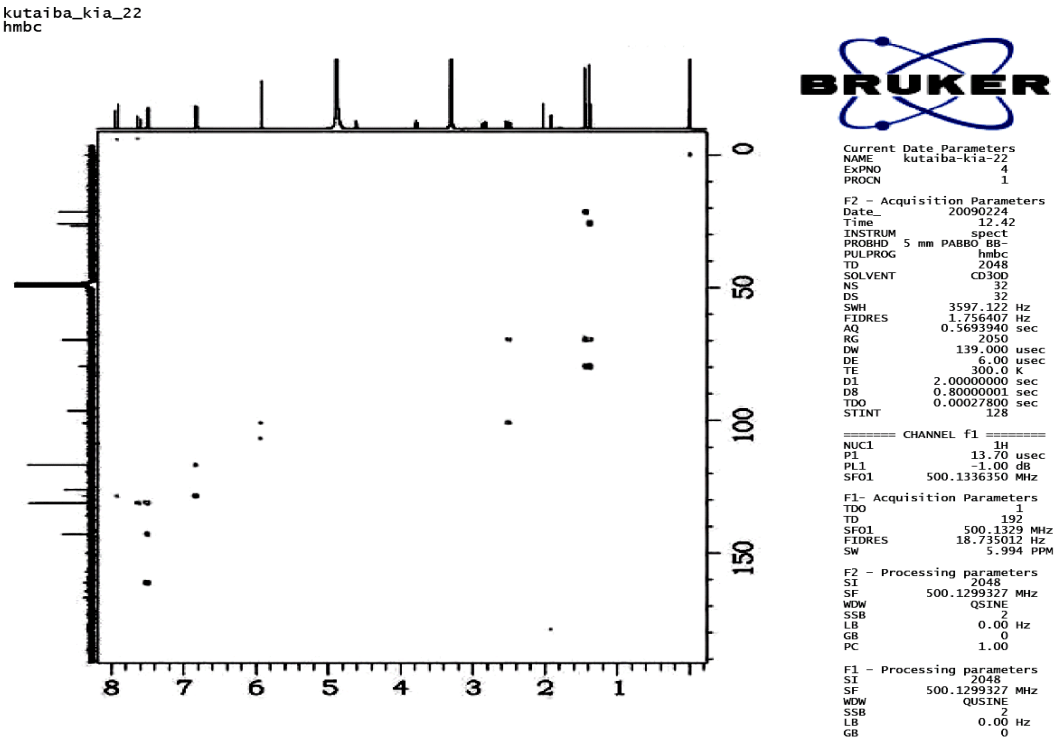


Figure 7. 1H-13C HMBC NMR spectrum of 1 in MeOH-d4.

The high-resolution mass spectrum of 1 (Fig. 8) showed a [M-1]-ion at 355.1182 corresponding to a molecular formula of C20H20O6. The electrospray ionization mass spectrum (ESI-MS) of 1showed several fragments characteristic of a prenylated chalcone

skeleton. The major peaks at m/z 235 (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 a 2,2-dimethyldihydropyran moiety and hydroxy group on ring A and a hydroxyl group on ring B of the aglycone20,21.

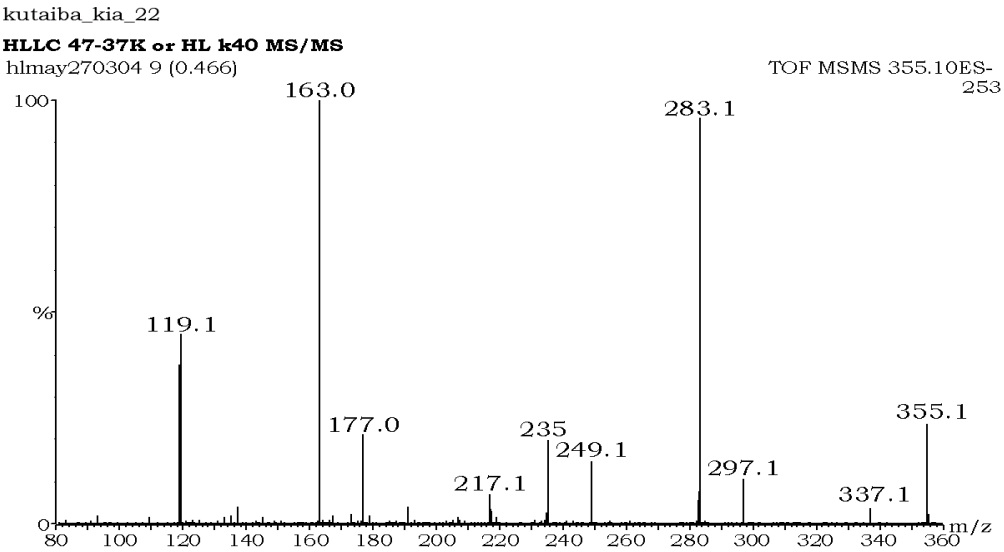


Figure 8. ESI-MS2 spectrum of compounds 1.

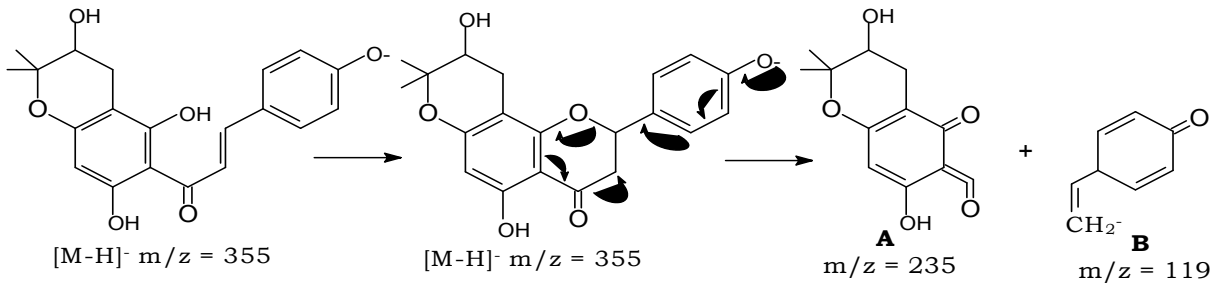


Figure 9. Important mass fragmentation of compound 1.

On the basis of the above spectral evidences, the structure of compound 1 was deduced to be 1-(3,4-dihydro-3,5,7-trihydroxy-2,2-dimethyl-2H-1-benzopyran-6-yl)-3-(4-hydroxyphenyl)-2-propen-1-one.

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