

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

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### ABSTRACT

New flavonoid (1) was isolated from ethanol extract of the barks of *Juniperus phoenicea* by preparative thin-layer chromatography technique. The barks 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, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopic data, including 2D NMR experiments to be 1-[2,4-dihydroxy-3-(2-hydroxy-3-methyl-3-butenyl)-6-methoxyphenyl]-3-(4-hydroxyphenyl)-2-propen-1-one.

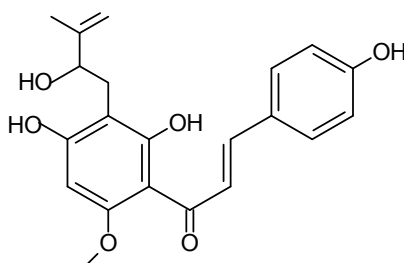
**KEYWORDS:** Isolation, Structural Elucidation, Phytochemical, Flavonoids, *Juniperus Phoenicea*.

### INTRODUCTION

Flavonoids are best known as the red, blue and purple pigments of flowering plants (due to the anthocyanidin sub-group) although the red pigment of some fruits can be due to carotenoids such as lycopene. <sup>[1]</sup> These pigments and the yellow pigments of flavones and flavanols are also responsible for the fall leaves coloration. <sup>[2]</sup> Because of the importance of color in pollinator attraction, flavonoids have an important role in plant reproduction. Flavonoids in plants also serve to protect from ultraviolet (UV) light owing to their high UV absorbance coefficients, and it has been suggested that this property of flavonoids was critical in the evolution of aquatic plants to a terrestrial existence. <sup>[3]</sup> Flavonoids also possess other

critical functions in defense against microorganisms and germination of pollen. <sup>[4,5]</sup> Each group of flavonoids possesses unique chemical properties and has a particular distribution in plants. Anthocyanins (glycosylated anthocyanidins) and proanthocyanidins (polymers that produce anthocyanidins when hydrolyzed) primarily provide color to flowering plants and fruit, and are therefore found in high concentrations in the skin of red grapes, red wine and berries.

Flavan-3-ols, such as catechin, epicatechin gallate are colorless and are found in high concentrations in green tea. Isoflavones are only found in legumes (e.g. soy) and are therefore consumed in high quantities in regions of the world with high soy consumption. Flavanones are found in high levels in citrus fruits, while flavones are present in green leafy spices such as parsley, and flavonols are ubiquitous and found in most fruits and vegetables consumed in the human diet. <sup>[6]</sup> *Juniperus phoenicea* is small tree 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. <sup>[7]</sup> Reported therapeutic uses of juniper include juniper baths for the treatment of neurasthenic neurosis <sup>[8]</sup> and management of scalp psoriasis <sup>[9]</sup> In our search for structurally and biological interesting compounds from plants found in Rania, Kurdistan-Iraq, new flavonoids (1), was isolated from the barks 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, <sup>1</sup>H and <sup>13</sup>C 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. <sup>1</sup>H NMR (Bruker AM-500, 500 MHz) and <sup>13</sup>C NMR (Bruker AC-200, 75 MHz) spectra were acquired in MeOH-d<sub>4</sub> 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, FeCl<sub>3</sub> (3% in dry ethanol). All other chemicals and reagents were analytical grade.

## MATERIALS AND METHODS

**Plant Material:** Barks of *Juniperus phoenicea* 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 Sofowara [10], Trease and Evans [11] and Harborne. [12]

**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 (<35°C) 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% FeCl<sub>3</sub> 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 H<sub>2</sub>SO<sub>4</sub>. 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% NH<sub>3</sub> solution were added to a portion of the aqueous filtrate of the plant extract followed by addition of concentrated H<sub>2</sub>SO<sub>4</sub>. 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% NH<sub>3</sub> solution. A yellow coloration was observed indicating a positive test for flavonoids.

**Test for Alkaloids.** 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 *Juniperus phoenicea*.

**Test for Terpenoids.** Five ml of the extract was mixed with 2 ml of chloroform, and concentrated H<sub>2</sub>SO<sub>4</sub> (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, 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°C. Overnight cultures of each of the bacterial isolates was diluted with sterile normal saline to give inoculums size of 10<sup>6</sup> 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°C for 18–24 h. Amoxicillin (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 Barks of *J. Phoenicea*. Air-dried barks 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 (<35°C) to leave a dark green solid (84 g).

### Isolation of Flavonoids from the Barks 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 n-BuOH–HOAc–H<sub>2</sub>O, (3:1:1). Only one spot was detected by spraying with 3% solution of ferric chloride FeCl<sub>3</sub> 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 n-BuOH–HOAc–H<sub>2</sub>O, (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).

**1-[2,4-dihydroxy-3-(2-hydroxy-3-methyl-3-butenyl)-6-methoxyphenyl]-3-(4-hydroxyphenyl)-2-propen-1-one:** yellow crystalline needles; UV (LC-PDA)  $\lambda_{\text{max}}$  369 nm; IR (film) 3375, 2952, 1622, 1525, 14769 and 1433, 1270 cm<sup>-1</sup>; NMR (360 MHz, MeOH-d<sub>4</sub>); HRESIMS [M - H]<sup>-</sup> 369.1421 m/z calcd for C<sub>21</sub>H<sub>22</sub>O<sub>6</sub> (1.8 ppm); ESIMSMS product ions m/z (% base peak) A fragment 249, B fragment 119, other product ions 295, 219, 181.

## 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 of alcoholic extract of *Juniperus phoenicea***

Tests	Tannins	Alkaloids	Flavonoids	Steroids	Terpenoids	Saponins
Result	+	+	+	-	+	-

Zone of Inhibition (mm)			
Extraction Conc.(mg/mL)	<i>Staphylococcus aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Escherichia coli</i>
200	14	15	15
100	12	10	12
Amoxicillin (25)	17	18	19

Compound 1 was isolated as a yellow crystalline needles from ethanolic extract of the barks 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 n-BuOH–HOAc–H<sub>2</sub>O, (3:1:1) to obtain compound 1. The structure of compound 1 was elucidated on the basis of MS, IR, UV, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopic data, including 2D NMR experiments. The IR spectrum of compound 1 (Fig. 1) showed characteristic absorption bands at  $\nu$  (KBr) 3375 (OH), 2952 (CH-stretching), 1622 (C=O), 1525, 1476 and 1433 (C=C, Ar), 1270 (CO) cm<sup>-1</sup>. The presence of conjugated carbonyl at 1622 cm<sup>-1</sup> indicated that compound 1 belongs to: flavones, flavonols, chalcones or aurones.

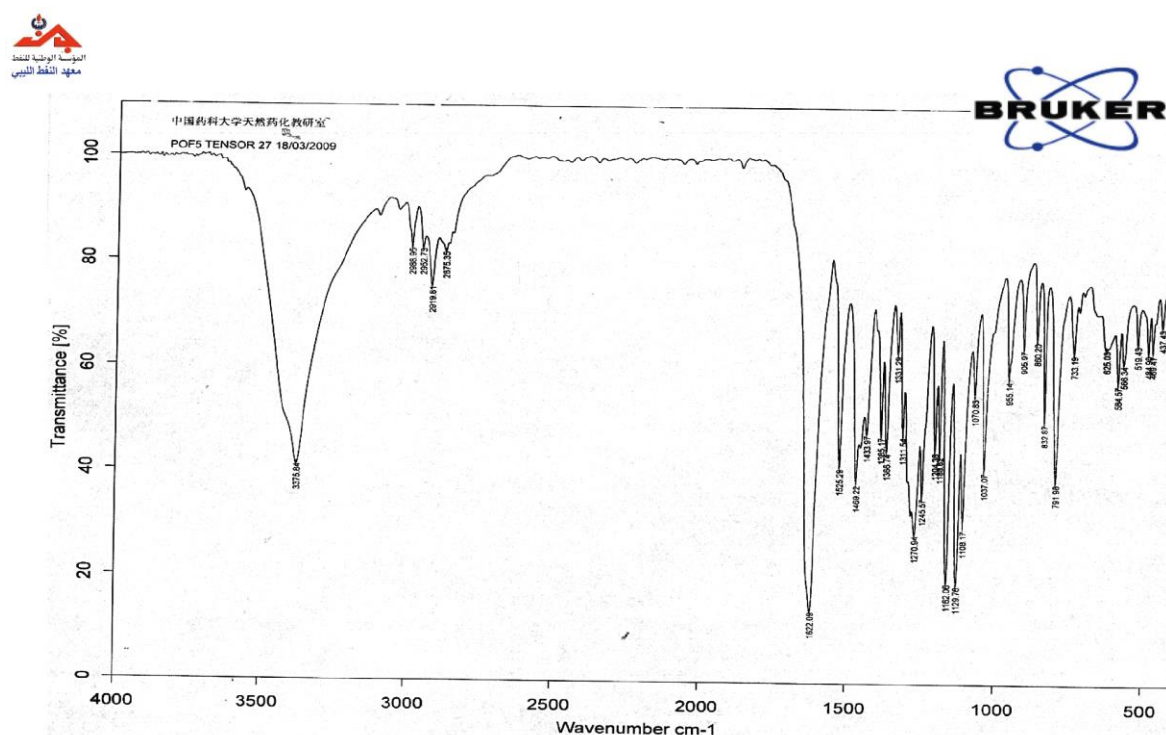
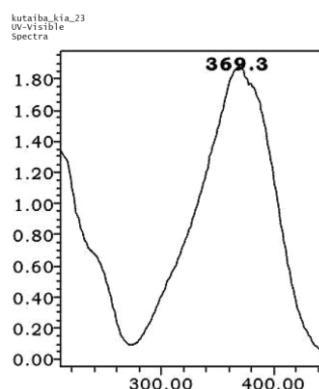


Figure 1. IR (KBr, disc) spectrum of 4.

The UV spectrum of compound 1 in MeOH (Fig. 2) showed characteristic intense absorption band at 369 (Band I) and a diminished absorption band near 240 (Band II) nm, indicating the presence of a chalcone skeleton<sup>13</sup>.





**Figure 2.** UV spectrum of compound 1.

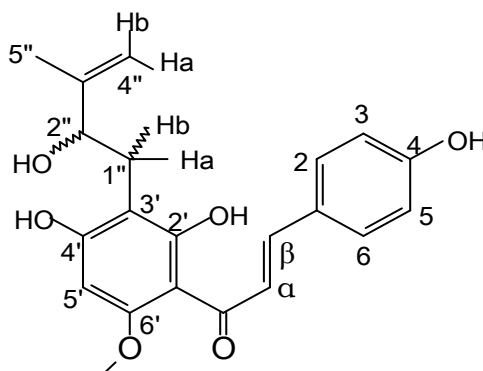
The chalcone skeltone of compound 1 can easily be distinguished from the remaining classes of flavonoids by the two set of doublets at  $\delta$  7.79 and 7.68 (each 1H, d,  $J=15.5$  Hz) in the  $^1\text{H}$ -NMR spectrum and its corresponding carbons resonating at  $\delta$  128.4 and 143.5 in the  $^{13}\text{C}$  NMR spectrum, assigned to  $\alpha$ - and  $\beta$ -carbon atoms respectively. <sup>[14-16]</sup>

The  $^1\text{H}$  NMR spectrum (Fig. 3) of compound 1 showed a number of signals characteristic of chalcone and 2-hydroxy-3-methyl-3-butenyl moieties. <sup>[17-19]</sup> The chalcone skelton of 1 was identified by a characteristic two  $^1\text{H}$  doublets with same coupling constants ( $J = 15.5$  Hz) resonating at  $\delta$  7.79 and 7.68 assigned to the olefinic  $\alpha$ - and  $\beta$ -protons respectively. Two 2H doublets resonating at  $\delta$  7.5 ( $J_{2,3/6,5} = 8.5$  Hz) and 6.83 ( $J_{3,2/5,6} = 8.5$  Hz) are due to the aromatic H-2,6 and H-3,5, respectively, of the B-ring and their coupling constants showed that they are ortho-coupled. A singlet at  $\delta$  3.90 (s, 3H) was assigned to a methoxy group. Another singlet resonating at  $\delta$  6.03 (s, 1H) was assigned to H-5'. The presence of a 2-hydroxy-3-methyl-3-butenyl moiety was identified by the geminal protons of the C-4'' terminal double bond resonated at  $\delta$  4.79 and 4.70 as two singlets, while the protons of the C-1'' methylene group resonated separately as a double doublet at  $\delta$  2.96 (H-1''a,  $J = 13.6$  and 5.5 Hz) and 2.83 (H-1''b,  $J = 13.6$  and 7.5 Hz). The oxymethine proton of the moiety was observed as a doublet ( $J = 8.9$  Hz) at  $\delta$  4.33, and the methyl group protons (H-5'') resonated at  $\delta$  1.80.

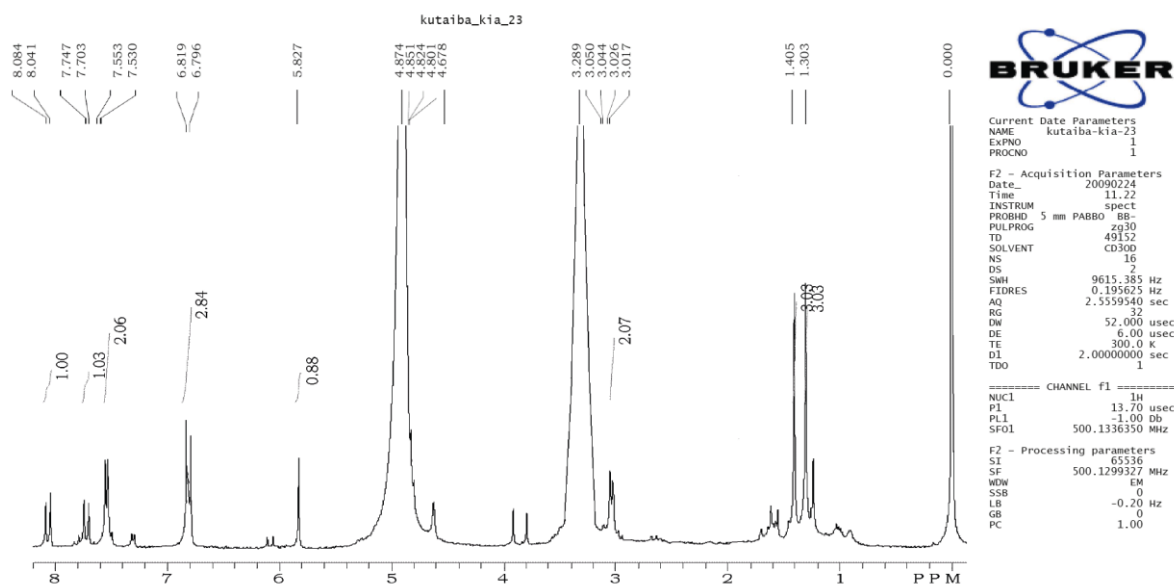
$^1\text{H}$	ppm	Mult (J)	Integ
H- $\alpha$	7.79	d (15.5)	1H
H- $\beta$	7.68	d (15.5)	1H
H-2,6	7.50	d (8.5)	2H
H-3,5	6.83	d (8.5)	2H
H-5'	6.03	s	1H
H-4''a	4.79	s	1H



H-4''b	4.70	s	1H
H-2''	4.33	dd (7.8, 5.5)	1H
6'-OMe	3.90	s	3H
H-1''a	2.96	dd (13.6, 5.5)	1H
H-1''b	2.51	dd (13.6, 7.5)	1H
CH <sub>3</sub> -5''	1.80	s	3H



NUCLEAR MAGNETIC RESONANCE (NMR) Lab.



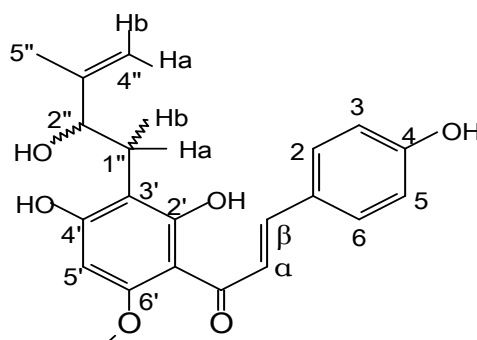
**Figure 3.1H NMR spectrum of compound 1 in MeOH-d<sub>4</sub>.**

The <sup>13</sup>C-NMR spectrum (Fig. 4) of compound 1 showed resonances for twenty one carbon atoms with two methyl, two 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  $\delta$  C 128.4 ( $\alpha$ -C) and 143.5 ( $\beta$ -C). The downfield signal at  $\delta$ C194.1 was assigned to the ketonic carbonyl carbon. The downfield chemical shift of the C=O ketonic carbon indicated the presence of an O-methy group at the adjacent C-6'

position in A-ring. <sup>[16]</sup> The other signals at  $\delta$ C 106.4 (C-1'), 166.7 (C-2'), 106.7 (C-3'), 164.9 (C-4'), 92.1 (C-5'), 162.9 (C-6'), 29.8 (C-1''), 76.7 (C-2''), 148.8 (C-3''), 110.9 (C-4'') and 17.9 (C-5'') further supported the presence of a chalcone skeleton and 2-hydroxy-3-methyl-3-butenyl substituted A-ring. The carbons of the aromatic B-ring resonated at  $\delta$ C 125.8 (C-1), 131.3 (C-2,6), 116.9 (C-3,5) and 161.1 (C-4), indicated a C-4 substituted B-ring. The methyl carbon signal at  $\delta$ C 56.2 (C-6') indicated the presence of O-methyl group in the molecule.

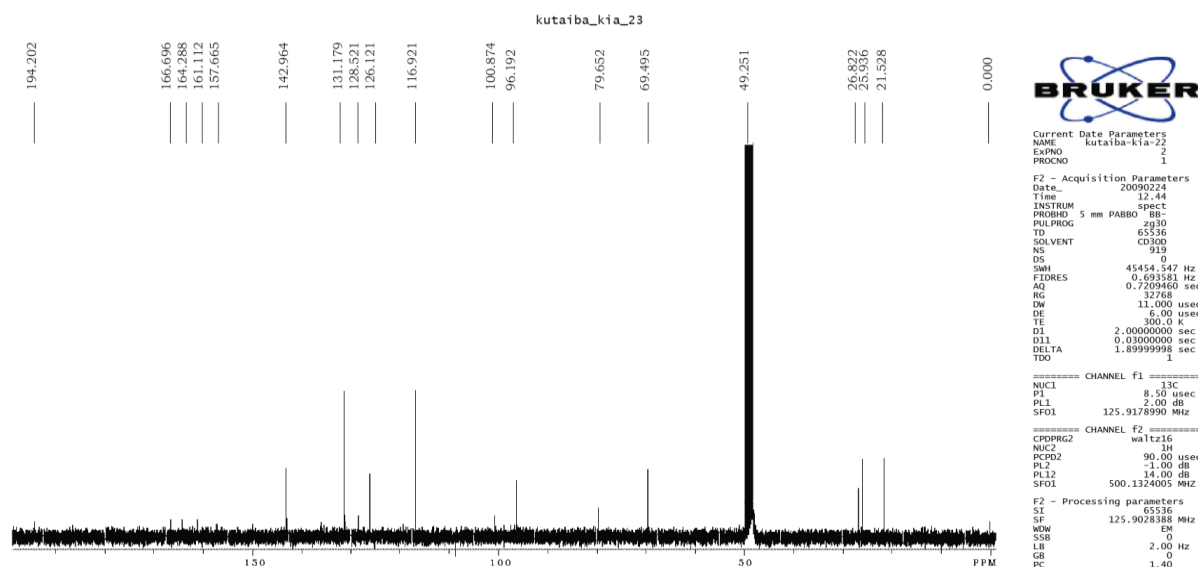
The complete <sup>13</sup>C-NMR chemical shift data for 1, when compared with the reported data, indicated that the aglycone is a chalcone having 2-hydroxy-3-methyl-3-butenyl as a side chain<sup>20</sup>. The complete <sup>13</sup>C-NMR and multiplicity data of compound 1 are presented in Figure 4.

<b><sup>13</sup>C</b>	<b>ppm</b>	<b>HMQC</b>	<b>HMBC</b>
C- $\beta$	143.5	X	H- $\alpha$ ;H-2.6
C- $\alpha$	128.4	X	H- $\alpha$ ;H- $\beta$ ;H-3,5
C=O	194.1		H- $\alpha$ ;H- $\beta$ , H-5'
C-1'	106.4		H-5'
C-6'	162.9		H-5';6'-OMe
C-5'	92.1		
C-4'	164.9		H-5';H1''a,b
C-3'	106.7		H-1''a,b;H-2''; H-5'
C-2'	166.7		H-1''a,b
C-1	125.8		H- $\beta$
C-2,6	131.3	X	H-2',6'; H- $\beta$
C-3,5	116.9	X	H-3,5; H-2',6'
C-4	161.1		H-2,6; H-3,5
C-1''	29.8	X	H-2''
C-2''	76.7	X	H-4''a,b; H1''a,b; Me-5''
C-3''	148.8		Me-5'';H-2''; H-1''a,b
C-4''	110.9	X	Me-5''; H-2''
C-5''	17.9	X	H-4''a,b;H-2''
6'-OMe	56.2	X	
MeOH	49.03		
TMS	0.0		

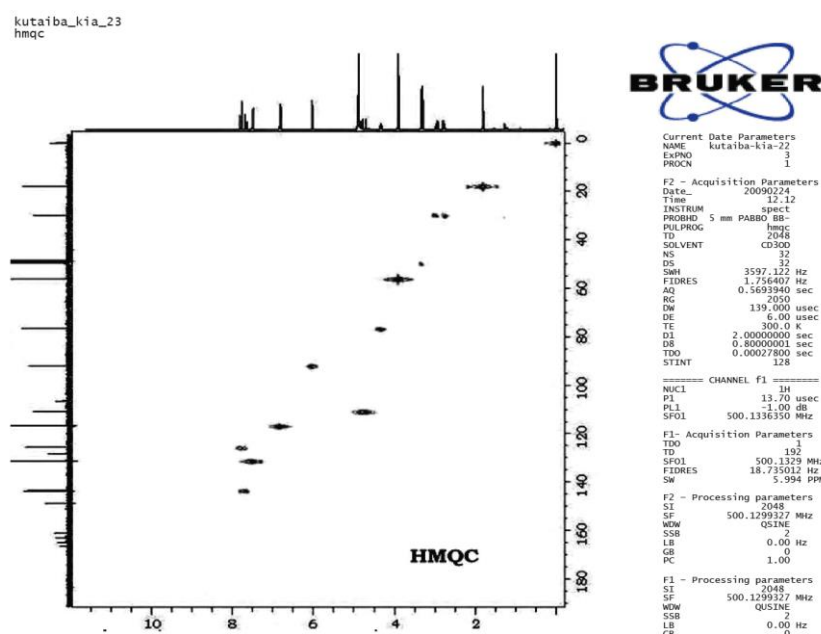




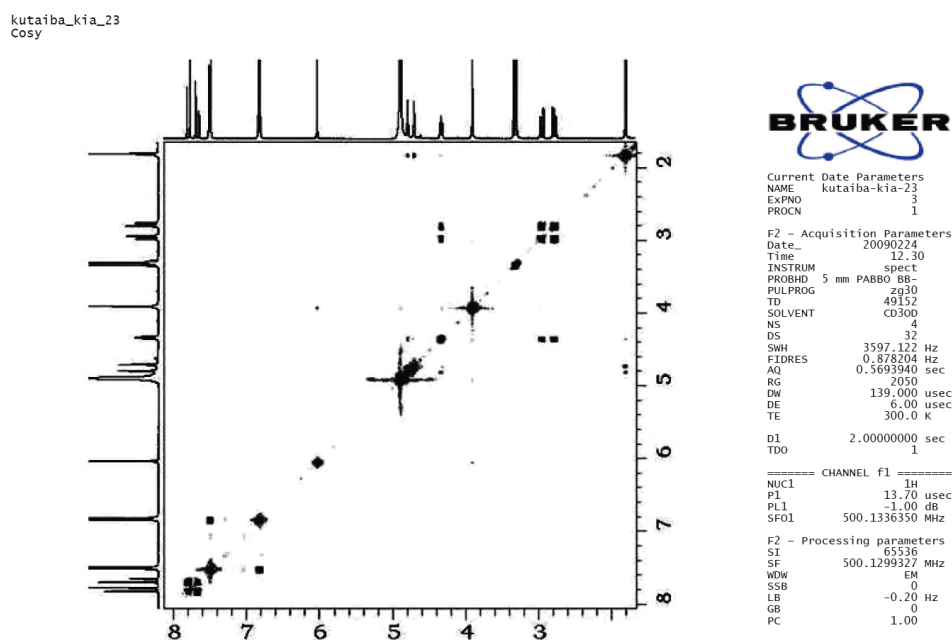
## NUCLEAR MAGNETIC RESONANCE (NMR) Lab.

Figure 4.  $^{13}\text{C}$  NMR spectrum of compound 1 in  $\text{MeOH-d}_4$ .

The HMQC technique was used to establish the direct one-bond  $^1\text{H}$ - $^{13}\text{C}$  connectivities. The protons of rings A, B and C i.e. H-5' ( $\delta\text{H} 6.03$ ),  $\alpha$ -H ( $\delta\text{H} 7.79$ ),  $\beta$ -H ( $\delta\text{H} 7.68$ ), H-2,6 ( $\delta\text{H} 7.50$ ) and H-3,5 ( $\delta\text{H} 6.83$ ) showed one-bond correlations with C-5' ( $\delta\text{C} 92.1$ ),  $\alpha$ -C ( $\delta\text{C} 128.4$ ),  $\beta$ -C ( $\delta\text{C} 143.5$ ), C-2,6 ( $\delta\text{C} 131.3$ ) and C-3,5 ( $\delta\text{C} 116.9$ ), respectively (Fig. 5). Protons signals of the prenyl moiety, i.e. H-1"a ( $\delta\text{H} 2.96$ ), H-1"b ( $\delta\text{H} 2.78$ ), H-2" ( $\delta\text{H} 4.33$ ), H-4"a ( $\delta\text{H} 4.79$ ), H-4"b ( $\delta\text{H} 4.70$ ) and H-5" ( $\delta\text{H} 1.80$ ) showed direct connectivities with C-1" ( $\delta\text{C} 29.8$ ), C-2" ( $\delta\text{C} 76.7$ ), C-4" ( $\delta\text{C} 110.9$ ) and C-5" ( $\delta\text{C} 17.9$ ), respectively.

Figure 5.  $^1\text{H}$ - $^{13}\text{C}$  HMQC NMR spectrum of 1 in  $\text{MeOH-d}_4$ .

<sup>1</sup>H-<sup>1</sup>H COSY NMR spectrum of compounds 1 showed coupling between the doublets of H-2,6 ( $\delta$ H 7.50), H-3,5 ( $\delta$ H 6.83) of aromatic B-ring (Fig. 6). The  $\alpha$ -H ( $\delta$ H 7.79) showed correlation with the  $\beta$ -H ( $\delta$ H 7.68). Interactions of the C-1" methylene protons ( $\delta$ H 2.96 and 2.78) with the C-2" methine proton ( $\delta$ H 4.33) was observed. The latter (H-2") showed coupling with the C-5" methyl protons ( $\delta$ H 1.80). Interactions of the C-4" methylene protons ( $\delta$ H 4.79 and 4.70) with the C-5" methyl protons ( $\delta$ H 1.80) was observed.



**Figure 6.** <sup>1</sup>H-<sup>1</sup>H COSY NMR spectrum of compounds 1 in MeOH-d<sub>4</sub>.

Structure 1 was finally assembled with the help on Heteronuclear Multiple Bond Connectivity (HMBC) experiment (Fig. 7). The H-5' ( $\delta$ H 5.93) showed two-bond couplings with C-4' ( $\delta$ C 164.9) and C-6' ( $\delta$ C 162.9), three-bond couplings with C-3' ( $\delta$ C 106.7) and C-1' ( $\delta$ C 106.4). The H-2,6 ( $\delta$ H 7.50) of aromatic B-ring showed coupling with C-2,6 ( $\delta$ C 131.3), C-3,5 ( $\delta$ C 116.9), C-4 ( $\delta$ C 161.1) and  $\beta$ -C ( $\delta$ C 143.5), while H-3,5 signal ( $\delta$ H 6.83) was found to be coupled with C-3,5 ( $\delta$ C 116.9), C-4 ( $\delta$ C 161.1) and  $\alpha$ -C ( $\delta$ C 128.4). The  $\alpha$ -H ( $\delta$ H 7.79) showed coupling with  $\alpha$ -C ( $\delta$ C 128.4),  $\beta$ -C ( $\delta$ C 143.5) and C=O ( $\delta$ C 194.1), while H- $\beta$  ( $\delta$ H 7.68) signal was found to be coupled with C-2,6 ( $\delta$ C 131.3), C- $\alpha$  ( $\delta$ C 128.4), C-1 ( $\delta$ C 125.8) and C=O ( $\delta$ C 194.1). The O-methyl protons ( $\delta$ H 3.90) were found to be coupled with C-6' ( $\delta$ C 162.9), indicating that the O-methyl group was present at C-6' of ring A. The attachment of the 2-hydroxy-3-methyl-3-butenyl moiety at C-3' of ring A was inferred from the HMBC interaction of C-1" methylene protons H-1"a,b ( $\delta$ H 2.96, 2.78) with the C-3' signal ( $\delta$ C 106.7) of the aglycone. The H-1"a,b ( $\delta$ H 2.96, 2.78) showed heteronuclear interactions with

C-3' ( $\delta$ C106.7), C-2' ( $\delta$ C166.7), C-4' ( $\delta$ C 164.9), C-2'' ( $\delta$ C 69.495) and C-3'' ( $\delta$ C 148.8). The H-2'' ( $\delta$ H4.33) showed coupling with C-3' ( $\delta$ C 106.7), C-1'' ( $\delta$ C 29.8), C-3'' ( $\delta$ C 148.8), C-4'' ( $\delta$ C110.9) and C-5'' ( $\delta$ C17.9), while Me-5'' ( $\delta$ H1.80) signal was found to be coupled with C-2'' ( $\delta$ C 76.7), C-3'' ( $\delta$ C 148.8) and C-4'' ( $\delta$ C 110.9). The H-4''<sub>a,b</sub> ( $\delta$ H4.79, 4.70) showed heteronuclear interactions with C-2'' ( $\delta$ C76.7) and C-5'' ( $\delta$ C17.9).

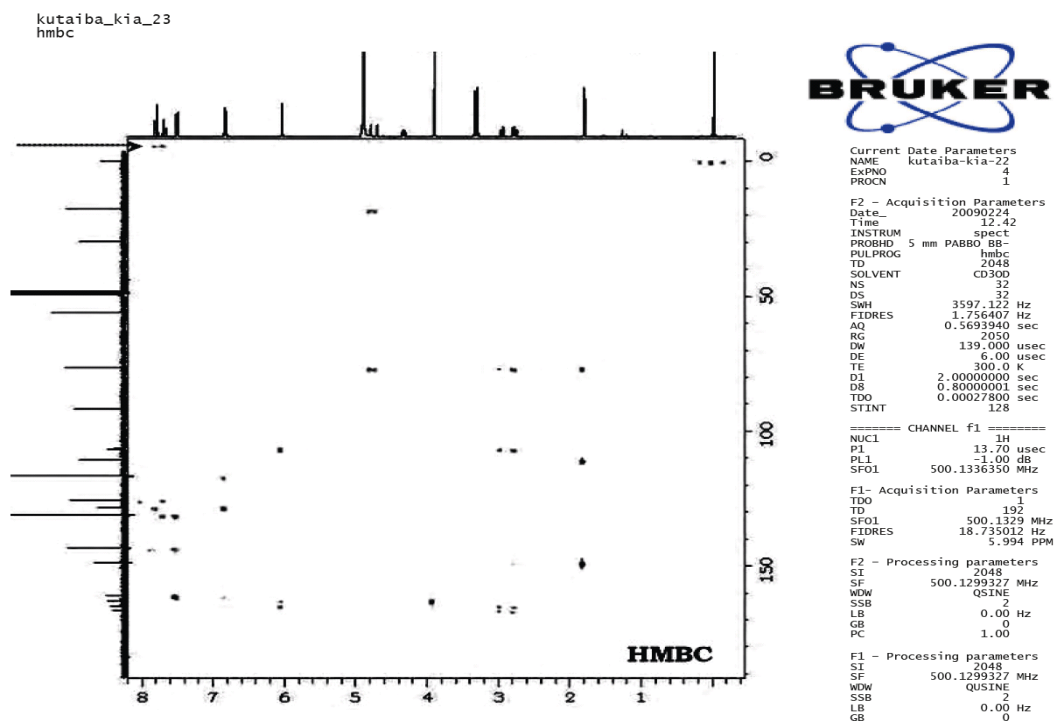


Figure 7.  $^1\text{H}$ - $^{13}\text{C}$  HMBC NMR spectrum of 1 in  $\text{MeOH-d}_4$ .

The high-resolution mass spectrum of 1 (Fig. 8) showed a  $[\text{M}-1]$ -ion at 369.1421 corresponding to a molecular formula of  $\text{C}_{21}\text{H}_{22}\text{O}_6$ . The electrospray ionization mass spectrum (ESI-MS) of 1 showed several fragments characteristic of a prenylated chalcone skeleton. The major peaks at  $m/z$  249 (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-hydroxy-3-methyl-3-butenyl moiety and methoxy group on ring A and a hydroxyl group on ring B of the aglycone. <sup>[17,18]</sup>

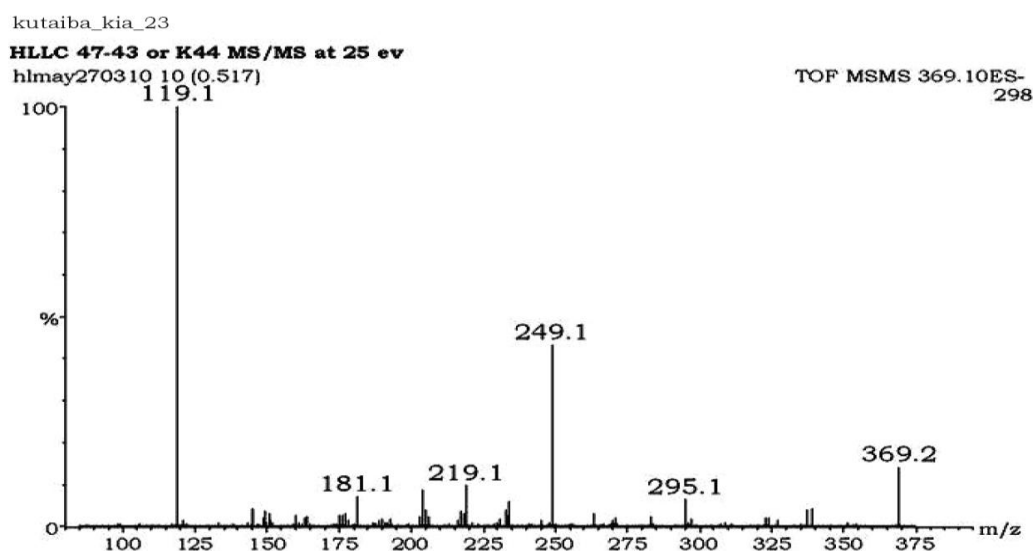


Figure 8. ESI-MS<sup>2</sup> spectrum of compounds 1.

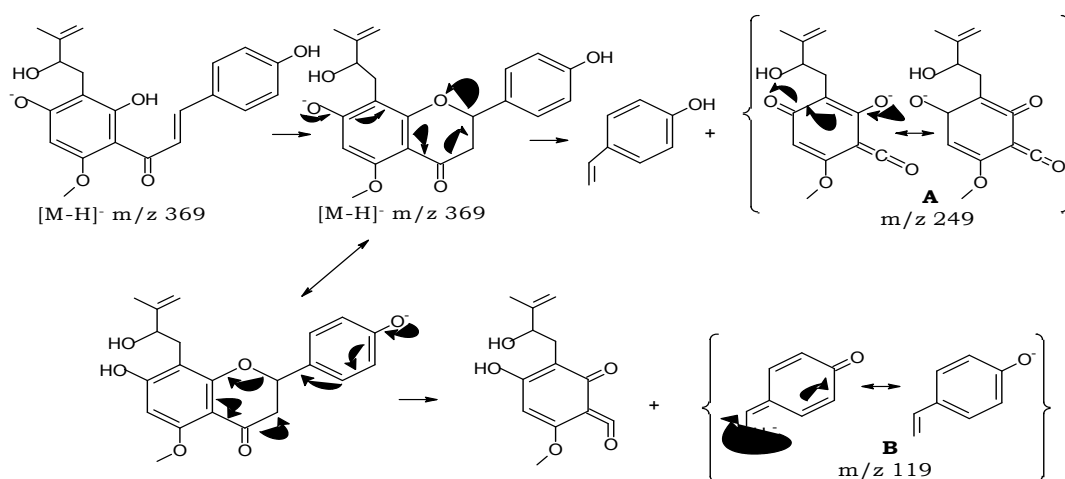


Figure 8. Fragmentation pathways to A and B fragments of compound 1.

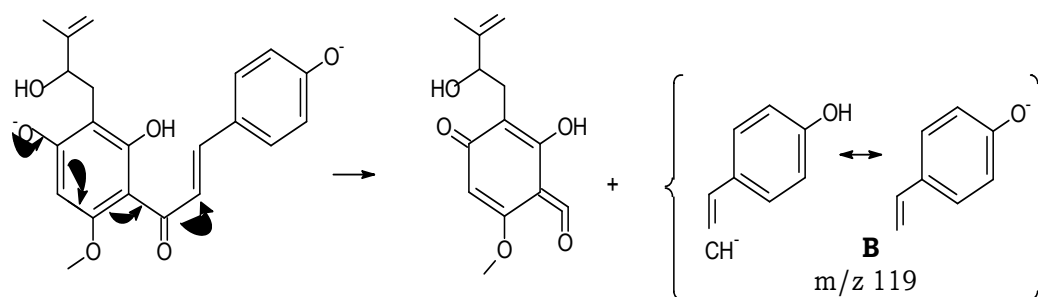


Figure 10. Alternative pathway to B fragments observed as prominent peak at m/z 119 in the negative ion ESMS spectrum of compound 1.

On the basis of the above spectral evidences, the structure of compound 1 was deduced to be 1-[2,4-dihydroxy-3-(2-hydroxy-3-methyl-3-butenyl)-6-methoxyphenyl]-3-(4-hydroxyphenyl)-2-propen-1-one.

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