Arab Gulf Journal of Scientific Research, 28 (3): 170- 177 (2010)

Research Paper Ref. No 2555

Investigation of Flavonoids Derived from the Butanol Extract of Juniperus procera Leaves

فحص الفلافونويد من أوراق نبات العرعر من خلاصة البيوتانول

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Abstract: Flavonoids are one of the most studied classes of polyphenolic phytochemicals, because of the important qualities associated with their potency, antioxidant qualities and other biological activities. *Juniperus procera* has been used in folk medicine for the treatment of a variety of conditions for many years in the Kingdom of Saudi Arabia. Examination of the butanol extract of the leaves of Juniperus *procera* growing in Saudi Arabia, Enemas region led to the isolation of a new flavonoid using different chromatographic methods (paper, thin layer and column chromatography). The structure of the isolated flavonoid was elucidated using several analytical tools such as NMR, ^{1}H, ^{13}C, IR, UV, as well as m. p. (melting point). The new flavonoid was identified as: 4H-1-Benzopyran-4–one-7-[[6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]-deoxy]-2,3-dihydr-5-hydroxy-2-(3-hydroxy-4–methoxy-phenyl).

Keywords: *Juniperus procera, isolation, extraction, phenolic compound, flavanone.*

المستخلص: تعتبر الفلافونيدات من أهم الفينولات نسبة لأنها اختصت بقوتها كمضادات للأكسدة لنشاطها البيولوجي. نبات العرعر من النباتات الطبية يستخدم في علاج عدد من الأمراض في المملكة العربية السعودية . تمت دراسة خلاصة البيوتانول لأوراق نبات العرعر النامي في منطقة النماص جنوب المملكة العربية السعودية وتم عزل فلافونيد جديد لأول مرة باستخدام الطرق الكروماتوغرافية المختلفة (كروماتوغرافيا الورق والطبقة الرقيقة والعمود الكلاسيكي)، وقد تم التعرف عليه بواسطة التحاليل الطيفية المختلفة (الأشعة فوق البنفسجية، الرنين النووي المغناطيسي للهيدروجين والكربون 13- والكوزي وطيف الكتلة، وقد اتضح أن هذا المركب هو: 4H-1-Benzopyran-4–one-7-[[6-O-(6-deoxy-alpha-L-mannopyranosyl)-(beta-D-glucopyranosyl]-deoxy]-2,3-dihydr-5-hydroxy-2-(3-hydroxy-4 –methoxy-phenyl).

كلمات مدخلية : *نبات العرعر، استخلاص، فصل ، مركبات فينولية، فلافانون.*

INTRODUCTION

Flavonoids are phenolic compounds widely present in plants and foods of plant origin (Harborne, 1973; Markham, 1982; Nuutila, *et al*. 2002; Argaez, *et al*. 2007). They contain fifteen carbon atoms in their basic nucleus- flavan, arranged in a C_6-C_3- C_6 configuration consisting of two aromatic rings (A and B) linked by a three carbon unit which may or may not form a third flavone ring (C) (Markham, 1982). There are six major subgroups: flavones, flavonols, flavanones, anthocyanins, chalcones, and isoflavonoids. (Figure1). Flavonoids encompass a large group of polyphenolic substances that have antibacterial, anti-inflammatory, antiallergic, antimutagenic,

antiviral, antineoplastic and vasodilator effects (Coelho, *et al*. 2006; Zheng, *et al*. 2007; Mediavilla, *et al*. 2007).

Juniperus procera called Arar and commonly known in English as African juniperus, is a coniferous tree native to the mountain of eastern Africa from East Sudan to Zimbabwe and the southwest of the Arabian Peninsula (widely distributed throughout the southern part of Saudi Arabia) (Gaber, *et al*. 1992).The Arar tree has two kinds of leaves, spreading needle-like and imprecated scale–like (Migahid, 1978). It is a medium-sized tree reaching 20 – 50 m (rarely 40 m) (Migahid, 1978) and has been used locally as a traditional remedy for tuberculosis and Jaundice (Gaber, *et al*. 1992).

Previous work (Ilias, *et al*. 1995; Tsutomu, *et al*. 2004), *has numerous references to Juniperus procera*. In this study, we are interested in only the flavonoids from the leaves of *Juniperus procera*. One flavonoid was isolated from ethyl acetate extract this flavonoid was identified as ; 3',4',3,7-tetrahydroxy flavone (Figure 2) isolated for the first time from leaves of this plant (Adil, *et al*. 2010).

This paper reports the isolation and characterization of the flavanone glycosidealso isolated from the leaves of *Juniperus procera* (Figure 3).

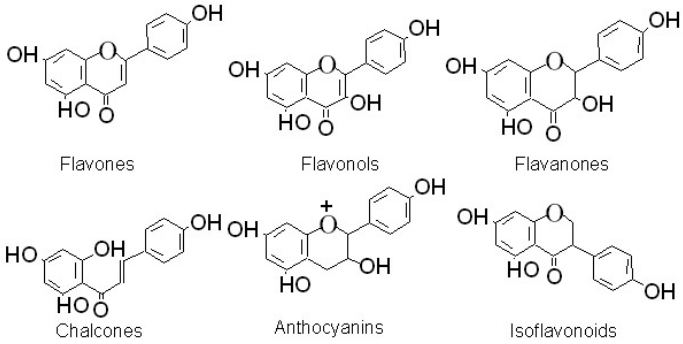

Fig. 1. Chemical structures of flavonoids subgroups.

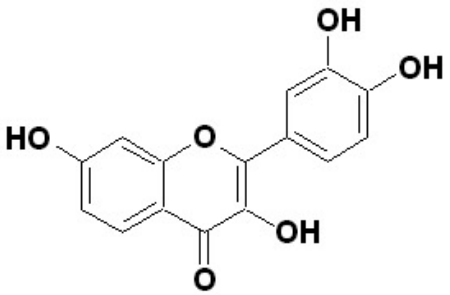

Fig. 2. 3',4',3,7-tetrahydroxy flavone.

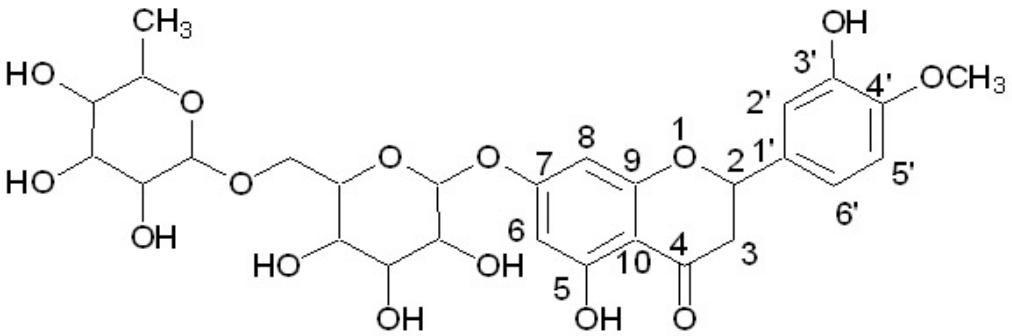

Fig. 3. 4H-1-Benzopyran-4–one-7-[[6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]-deoxy]-2,3-dihydr-5-hydroxy-2-(3-hydroxy-4 –methoxy-phenyl) (I).

MATERIALS AND METHODS

Plant Material

The leaves of *J. procera* were collected from the Enemas region in southern of Saudi Arabia during November 2006 and identified by Dr. Jacob Thomas of the Department of Botany and Microbiology. A voucher specimen was deposited in the herbarium of the Department of Botany at King Saud University, Riyadh (KSU- 20233).

Extraction and Preparation of Methanol Extract

3.75 Kg of air-dried leaves of the plant were percolated with 85% methanol (15 L) at ambient temperature for five days. The solvent was removed in *vacuo* and the residue (150g) dissolved in 50% aqueous methanol (300ml) was subjected to fractionation using petroleum ether (40^0-60^0C) (5.5 g), chloroform (6.2 g), ethyl acetate (8 g) and butanol (13 g). TLC on procoated silica gel 60 F_{254} plates (layer thickness 0.5 mm, 20×20 cm, Merck, Germany), using MeOH: $CHCl_3$ (1:1 *V: V*) as an eluant and preparative PC was conducted on filtrac No 7 paper using n-butanol-acetic acid- water (40:10:22) as the mobile phase. Investigation showed the butanol fraction contained flavonoid fraction and individually tested positive for glycoside .

Isolation and Identification of Flavonoid

The n-butanol extract (2.5 g) was chromatographed on 500 gm silica gel (Kieselgel 60 mesh 70-230, Astem) for column (100×*5cm*) using MeOH: $CHCl_3$ (1:1 *V: V*) as eluant. The column fractions (300 ml) were collected and combined with the bases on their PC and TLC patterns, flavonoids (F_2 - F_8) were unambiguously determined by means of spectroscopic methods (IR, UV,GC-MS ^{1}H NMR, ^{13}C and 2D experiments COSY) and compared to those previously reported (Markham. 1982; Agrwal, 1989; Harborne, 1996).

EXPERIMENTAL

General Experimental Procedures

Analytical grade solvents were used. The UV spectra were recorded on a Perkin Elmer–Lambda 2 spectro-photometer and a UV lamp was used for localizing of fluorescent spots on TLC and PC. The IR spectra were recorded as KBr disks, using Shimadzu IR-8400 spectrophotometer. Nuclear Magnetic Resonance spectra were recorded on a JEOL DELTA ESP400 MHZ NMR spectrophotometer. Melting point (Mps) were determined using Kofler hot-stage apparatus and uncorrected Mass spectra were recorded on a SHIMADZU GC/MS-GP5050 spectro-photometer.

4 H - 1- Benzopyran - 4 – one – 7 -[[6 - O - (6 – deoxy - alpha - L - mannopyranosyl) - beta -D-glucopyranosyl]- deoxy]- 2,3 – dihydr - 5 – hydroxy - 2-(3 - hydroxy - 4 – methoxy -phenyl) : (17 *mg*)**,** dull yellow powder, R_f=0.37 m.p 320˚*C*. UV- visible spectral data for compound (I) (Table. I). IR: KBr disc showed ν 632, 744 (C-H, Ar) , 1068 (C – O , ether), 1517, 1604 (C=C ,Ar),1647 (C=O), 2937 (-OMe) and 3477 cm^{-1} (OH). ^{1}H-NMR (400 MHz, DMSO-d_6): δ(*ppm*), Figure (4) showed δ 6.93 (3H, m, H-2', H-5'-H-6'), δ 6.14 (2H, m, H-6, H-8) , δ 5.47 (1H, m, H-1'''); 5.41(1 H, d, *J* = 5.12 Hz, H-1'''); 5.19 (2H, m , H-2", H-2'''), 4.97 (1H, m, H-3"); 4.69(1H, m, H-3'''); 4.61 (1H. m, H-4"); 4.52 (3H,m H-4''', H-5''', H-5'''), 3.77 (3H, s, OCH_3 - 4'); 3,42 (7H, m, 2"-OH, 2'''-OH, 3"- OH, 4"-OH· H-6") 2.75 (2H. m, H-3); 1.08 (3H, d, *J*= 5.88, CH_3). The a broad singlet at 12.02 was due OH group where form hydrogen bonding (Figure 4).

GC-MS, m/z (%): 302(M^+, 100), 301(46), 179(31.8), 150(50), 153(34), 137(56.8), 135(46), 124(20), 107(18) (Figure5).

Table 1. UV- visible Spectral Data for Compound I (λmax).

Shift reagent	λ_{max}
MeOH	284, 327 (sh)
$AlCl_3$	242, 285
$AlCl_3$/ HCl	305, 381
NaOMe	252,299,331,361,410
NaOAC	304, 381
NaOAc/H_3BO_3	286, 324

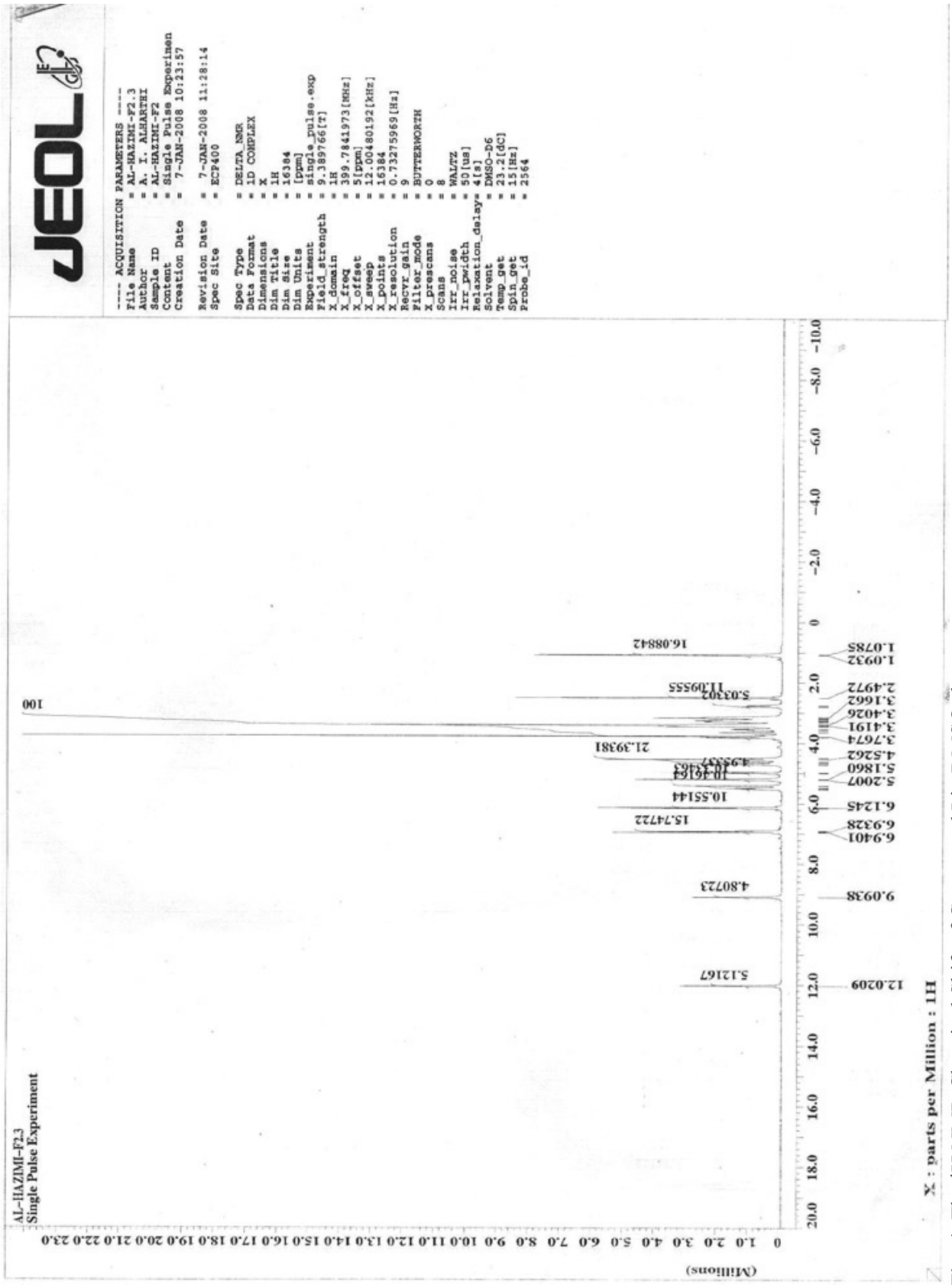

Fig. 4. The ^{1}H NMR Chemical Shift of Compound I in DMSO-d_6.

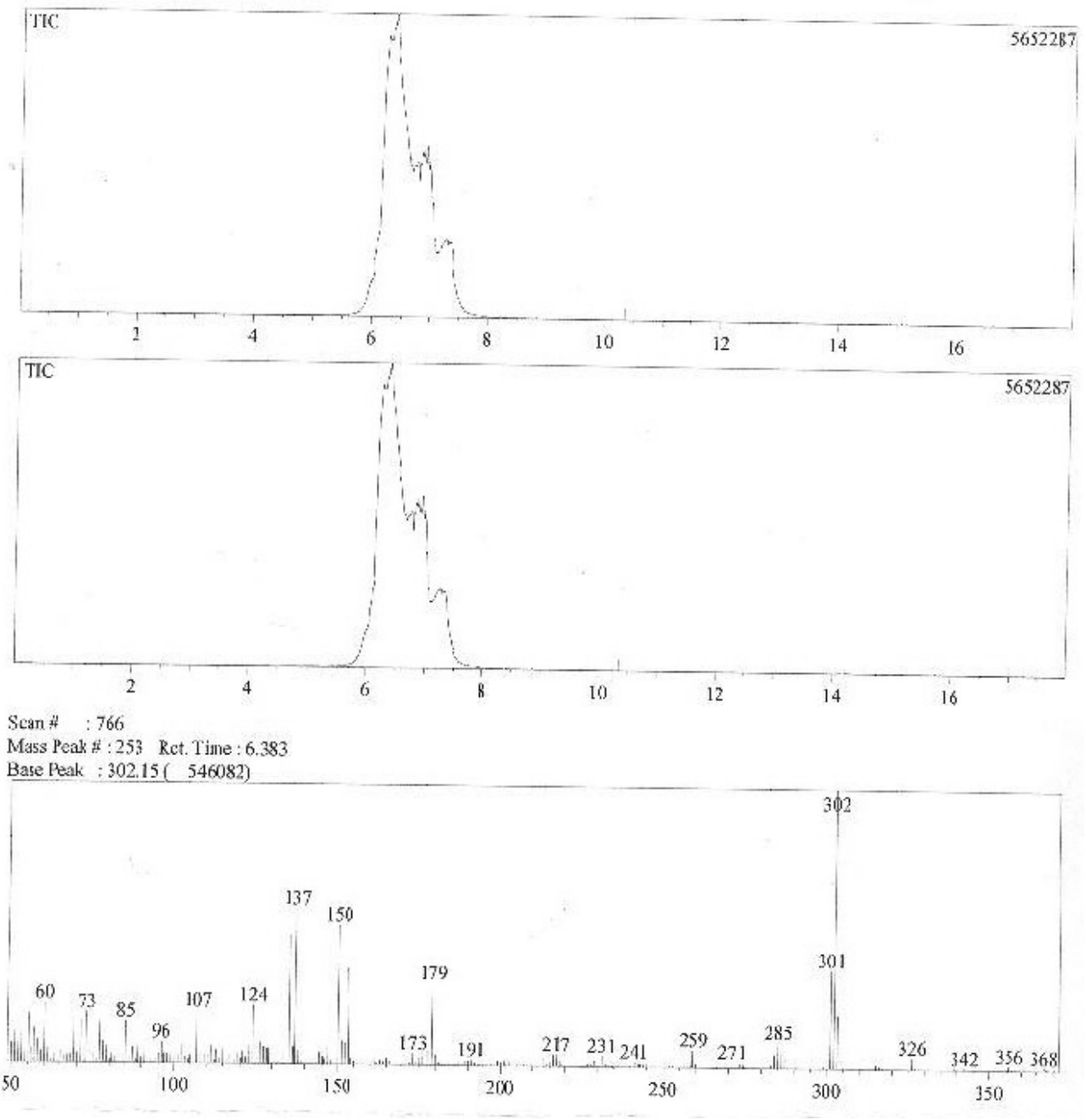

Fig. 5. Mass Spectral Data for Compound 1.

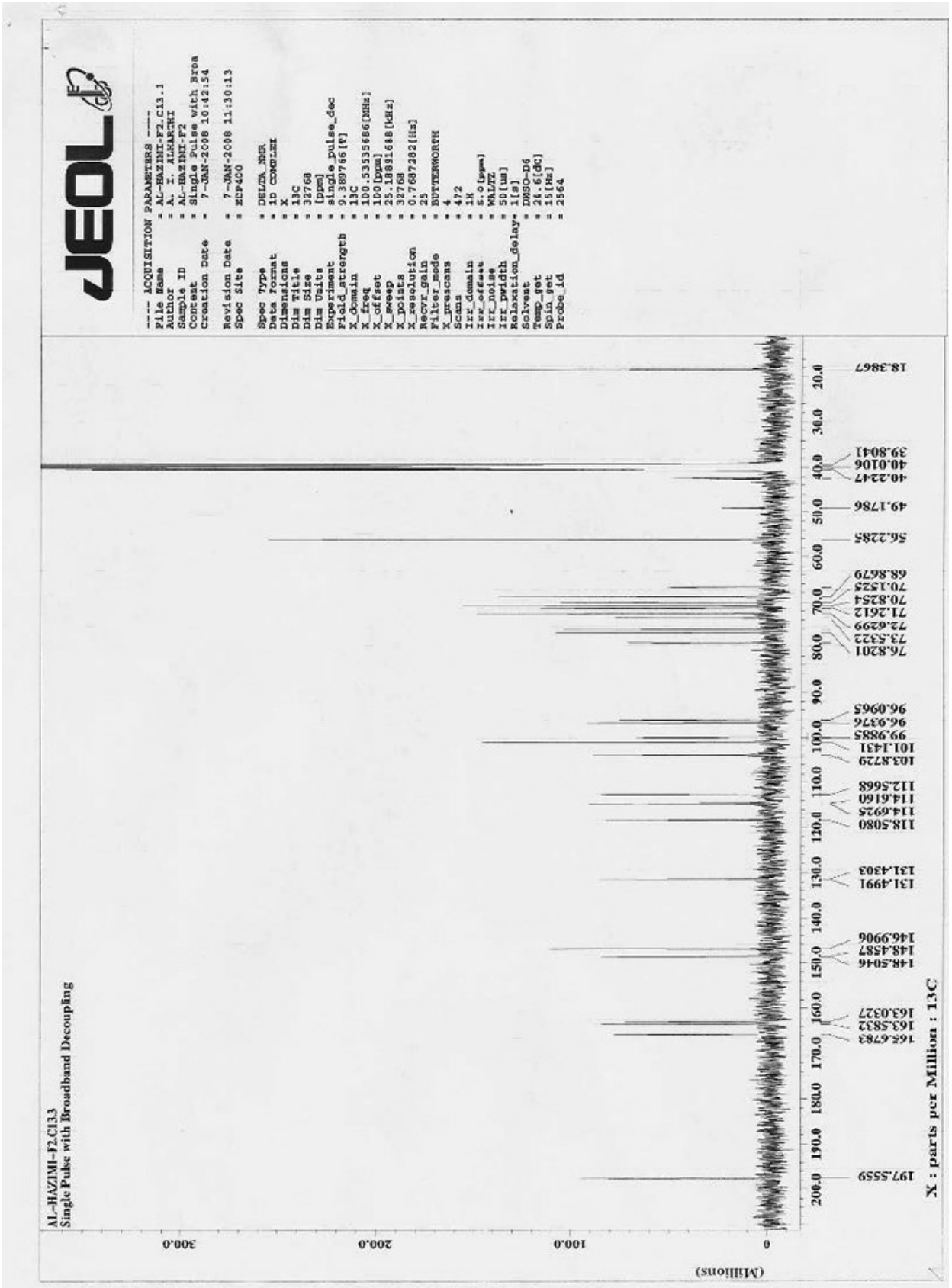

Fig. 6. The ^{13}C NMR Chemical Shift of Compound (I) in DMSO-d_6

^{13}C NMR (DMSO- d_6) (Fig.6): δ 165.6 (C-2); δ 103.8 (C-3) δ 197.5(C-4); δ 165.6(C-5) δ 96.9(C-6); δ 163.0 (C-7); δ 96.0 (C-8); δ 163.0 (C-9); δ 103.8(C-10); δ 118.5(C-1'); δ 131.4(C-2"); δ 114.6 (C-3'); δ 148.5 (C-4'); δ 114.6 (C-5'); δ 131.5 (C-6'); δ 103.8(C-1"); δ 72.6(C-2"); δ 73.5 (C-3"); δ 68.8 (C-4"); δ 76.8 (C-5"); δ 56.2(C-6"); δ 101.1(C-1"'); δ 70.8 (C-2"'); δ 70.1 (C-3"'); δ 72.0(C-4"'); δ 56.2 (C-5"'); δ 18.4 (C-6"') .(Table 2).

Table 2. ^{1}H and ^{13}C Spectral Values δ of Compound (I).

Carbon No	^{1}H NMR	^{13}C NMR		
2	-	165.6		
3	2.49	103.8		
4	-	197.5		
5	-	163.0		
6	6.14	96.9		
7	-	163.5		
8	6.14	96.0		
9	-	163.1		
10	-	103.8		
1'	-	118.5		
2'	6.93	131.4		
3'	-	114.6		
'	-	148.5		
5'	6.93	114.6		
6'	6.93	131.5		
1''	-	103.8	1'''	101.1
2''	-	72.6	2'''	70.8
3''	-	73.5	3'''	70.1
4''	-	68.8	4'''	72.0
5''	-	76.8	5'''	56.2
6''	-	56.2	6'''	18.4
CH_3	1.08(d)	18.3		
OCH_3	3.77	66.6		
7-OH	12.0	-		

RESULTS AND DISCUSSION

One flavonoid (Figure 3) was isolated from butanol extracts of *Juniperus procera*. This compound was identified by comparing its ^{1}H NMR, ^{13}C NMR , ^{1}H-^{1}H COSY NMR and UV spectrum in methanol with different shift reagents with published data (Markham, 1982; Agrwal, *et al*. 1989; Markham, *et al*. 1989) .

The isolated compound was identified as a flavonoid which was clear from its UV absorption in methanol with shift reagents Band **I** at longer wavelength (284 – 327(sh) nm. A positive ferric chloride test suggested that compound (1) was a phenolic flavanone (Mabry, *et al*. 1970a). The ^{1}H NMR spectrum showed three signals at δ 5.41(1H, *dd*, *J*= 12.8, 2.9Hz), δ 3.1(1H, *dd*, *J*= 16.4, 12.8Hz, and δ 2.74(1H, *dd*, *J* = 16.4, 12.8 Hz) characteristic H-2, H-3_{ax} and H-3_{eq}. respectively of flavanone moiety (Mabry, *et al*. 1970a).Two meta-coupled doublets (*J* = 2.3 Hz), at δ 6.12 and 6.14, each integrating for one proton were assigned to H-6 and H-8 , respectively. It also showed signals for one aromatic methoxyl group at δ 3.77 illustrating a long correlation with carbon at 163.0 and was assigned to C-5. A cross- correlation was shown with H-6 (δ 6,12) in its COSY spectrum as a flavonone. The acid hydrolysis of Compound (I) was carried out with 2 mol L^{-1} hydrochloric acid (100^{0} C, 2h),(Ilina et al., 2004) revealing the presence of the sugar glucose- rhamnose, all of which were identified by PC and TLC. The aglycone was found to be identical substituted as position **7** as indicated with their UV spectra upon addition of diagnostic shift reagents. The bathochromic shift (+3) in band **II** upon addition of sodium acetate indicated the absence of a free 7-OH group. When boric acid was added to the methanolic solution in the presence of sodium acetate, band **I** did not shift, indicating the absence of B- ring catechol system.

The presence of 3'-OH was observed from bathochromic shift (48nm) in band **I** with decrease of intensity upon addition of NaOMe. This was confirmed From ^{1}H NMR, with no signals at (doublet 6.8-6.91ppm) (Nimmanapalli, *et al*. 2007)

The shift bathochromically (20) nm in band **I** upon addition of aluminium chloride with hydrochloric acid indicated the presence of free 5-OH group.

^{1}H-NMR showed two sugars glucose-rhamnose. The ^{1}H-NMR spectra of compound (I) revealed the presence of doublet signal at 5.1 ppm (*J* = 5.9 Hz) representing glucose moiety. Another doublet signal at 4.5 ppm (J=3.4 Hz) characteristic to rahamnose was confirmed by the presence of

singlet peak of 3 proton at 1.08 ppm (CH_3 of rahamnose) (Nimmanapalli, *et al*. 2007). They are attached to each other at position 7 (UV data) so there is gluco (6"- 1"') rhamnose sugar moiety in this compound. A singlet peak at 3.77 ppm referring 3 aliphatic protons of $–OCH_3$ attached to position 4' in this compound, identifying this compound with this data as compound (I).

4H-1-Benzopyran – 4 –one -7 - [[6-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyl]-deoxy]-2,3-dihydr - 5-hydroxy - 2 -(3-hydroxy - 4 – methoxy –phenyl).

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Ref. No. (2555)
Rec. 29/3/2010
In-revised form: 27/10/2010