FREE RADICAL SCAVENGERS FROM THE LEAVES OF *Acacia saligna* Labill

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ABSTRACT

The Ethylacetate extract of *Acacia saligna* Labill leaves (Mimosaceae) showed radical scavenging activity in the 1,1-diphenyl -2-picrylhydrazyl (DPPH) decoloration assay. Bioassay-guided isolation led to five known flavonoid compounds including Apigenin (A), Luteolin (B), (+) Catechin (C), Quercetrin (D) and Myricetrin (E). The characterization of these compounds was achieved by various chromatographic and spectroscopic methods.

Key Words: **Free radical scavenging activity, Natural antioxidant, *Acacia saligna*, Mimosaceae, Flavonoids.**

INTRODUCTION

Free radicals are atoms that have at least one unpaired electron, thus making them unstable and highly reactive. Free radicals then roam throughout the body causing damage to cells by stealing stable electron partners from other cells, causing more free radicals, more instability, and more damage. This cell damage can impair the body's ability to fight illness. Furthermore, oxygen-derived free radicals i.e., reactive oxygen species (ROS) are well recognized to be the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and hepatotoxicity (Guyton *et al.*1997, Halliwell and Gutteridge 1999 and Aniya *et al.* 2005). Thus a scavenger ROS is expected to prevent this free radical mediated diseases.

The use of antioxidants, both natural and synthetic, in the prevention and cure of various diseases is gaining wide importance in the medicinal filed. Currently there is considerable interest in the natural antioxidants activities than the synthetic ones such as butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) because they are seen as being safer and causing fewer adverse reaction (Han *et al*.2004). So far, there is a great world interest in finding new, safe and economic natural antioxidants with high activity from natural sources to replace synthetic ones (Subhadhirasakul *et al*.2003 and Han *et al*. 2004)

Acacia saligna (Mimosaceae) is an extremely rugged tree, adaptable to derelic land and exceptionally arid conditions in Australia and North Africa (El-Lakany 1987). It grows rapidly and is used for fixing sand dunes, stabilizing drift sands (El-Bagoury *et al.* 1995 and Abou-Deya 1999) and beutification projects. Its leaves are palatable and can be used either freshly or dried as supplementary feed for sheep and goats (Abou El-Nasr *et al.* 1996 and Howard *et al.* 2002). Water and ethanolic extracts of the aerial part of this plant exhibited potent molluscicidal activity against *Biomphalaria alexandrina* snails (Ahmed *et al.* 1999). Previous phytochemical investigation of the aerial parts of this plant has led to the isolation of coumarin and steroidal components from the ethanol extract (Ahmed *et al.* 1999) and hydrocarbons, fatty alcohols, aldehydes,

triterpenes and sterols form the hexane extract (El-Sawi *et al.* 2003). In this paper we report for the first time from this plant the isolation and structural elucidation of five flavonoids responsible for the radical scavenging activity of the ethylacetate extract of *Acacia saligna* leaves.

MATERIALS AND METHODS

Plant material

Acacia Saligna leaves were collected in April 2003 from the experimental farm of the Faculty of Agriculture, Fayoum, Egypt and identified by the Botany Department, Faculty of Science, Cairo University. A Voucher specimen (A.S.4) was deposited in the Herbarium of the Biochemistry Department, Faculty of Agriculture, El-Fayoum Branch, Cairo University.

Extraction

The ground air-dried leaves (1.2kg) were extracted successively with Pet. Ether 40-60 °C (5 L), $CHCl_3$ (5 L), EtOAc (6 L) and MeOH (6 L) at room temperature (27°C + 2). Each extract was concentrated under reduced pressure to give 8.5, 18.3, 12.1 and 60.0g residues, respectively.

Testing for free radical scavenging activity

The free radical scavenging effect of the crude extracts and compounds was assessed by the decoloration of a methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical according to Brand-Williams *et al.* 1995. The degree of decoloration indicates the free radical scavenging efficiency of the substances. A methanol solution of DPPH serves as a control. The percentage of DPPH decoloration was calculated as follows:

Decoloration (%) = 1- (Absorbance with compound at 517 nm / Absorbance of the blank) X 100.

A freshly prepared DPPH solution (20mg/L) was used for the assay. The percentage DPPH decoloration of the extracts and the compounds were triplicatly assessed at 100, 50 and 10 μg/ml.

Analytical Thin Layer Chromatography (TLC)

TLC analysis was performed on precoated silica gel plates (Kieselgel G-60 F-254, 0.25 mm Merck) using the following solvent systems:

1- n-Butanol-Acetic acid-Water (4:1:5) upper layer.
2- Chloroform-Methanol-Water (70:30:5).
3- Ethyl acetate-Acetic acid-Formic acid-Water (100:11:11:27).
4- Dichloromethane-Methanol-Water (50:25:5).
5- Chloroform-Acetone (50:6).
6- Chloroform-Methanol (80:20).

Zones were detected under UV light (254nm and 365nm) and by spraying with: concentrated H_2SO_4 followed by heating at 105°C for 5 min. or with 1% methanolic diphenylboryloxyethyl amine followed by 5% methanolic polyethyleneglycol 400. Sugars were detected by spraying with naphthoresorcinol phosphoric acid followed by heating at 105°C for 10 min.

Isolation of the bioactive components

The bioactive ethyl acetate extract was subjected to the isolation of the bioactive components as follows:

Nine grams of the EtOAc residue were subjected to column chromatography over silica gel (200g, 230-400 mesh, Merck) and eluted with the following solvent mixtures of $CHCl_3$: MeOH:H_2O (95:5:0;90:10:0;80:20:0 and 70:30:5 1L for each eluent). Ten fractions of each eluent were collected. The eluates were combined on the basis of similarty of TLC profiles to afford 11 fractions and were then tested for free radical scavenging activity. The bioactive fraction No. 4,6,8,10 and 11 were further purified several times over Sephadex LH-20 and Silica gel columns as shown in Fig. (1) yielded five compounds A–E.

Structure Identification of the isolated compounds

The isolated compounds were characterized by chemical investigation (detection tests and acid hydrolysis) and spectroscopic methods.

Chemical Investigations

Detection Tests

The phytoconstituent classes of the extracts and the isolated compounds were detected according to the methods described by Farnsworth 1966.

Acid hydrolysis of the isolated compounds

Acid hydrolysis was performed in a sealed tube at 100°C for 2h with 2mg of the compound in 2ml of 2N HCl. The aglycone was extracted with Et_2O and analyzed by TLC with system 5.

The aqueous layer was neutralized with N,N-dioctylamine (10% in $CHCl_3$). After evaporation to dryness, the sugars were identified by TLC with system 4 by comparison with authentic samples.

Spectroscopic Methods

Nuclear Magnetic Resonance (NMR) Spectroscopy.

^1H and ^{13}C NMR spectra were recorded in CD_3OD on a varion Mercury VXR 300 (300 MHz for ^1H and 75 MHz for ^{13}C) the chemical shifts are given in ppm.

Mass Spectroscopy (MS)

Mass spectrum was recorded on a GC MS.QP1000 EX Shimadzu Mass spectrometer at 70e.V.

The NMR and MS spectra were carried out in Faculty of Science Cairo University.

Ultraviolet Spectroscopy (UV)

The spectra were registered with a spectrophotometer CeCil 3000 series according to Mabry *et al.* 1970**.**

RESULTS AND DISCUSSION

The air-dried and powdered leaves of *Acacia saligna* were exhaustively extracted with Pet. ether, $CHCl_3$, EtOAc and MeOH. The free radical scavenging effect of each extract was tested. Only the EtOAc extract showed strong activity than the other extracts (Table 1). This extract was subjected to silica gel column chromatography to afford 11 fractions (see Materials and Methods). The bioassy-guided of these fractions indicated that fractions No. 4,6,8,10 and 11 were the active fractions among the 11 tested fractions of the EtOAc extract. TLC examination of each bioactive fraction in systems 1 and 3 showed it to be a mixture of several compounds containing one major compound. These major compounds gave positive colour test for flavonoids but their R_f values were different from each others.

The bioactive fractions were repeatedly chromatographyed over Sephadex LH-20 and Silica gel as shown in Fig. (1) to yield five compounds (A,B,C,D and E).

Table 1: Free radical scavenging effect of the extracts and isolated compounds from *Acacia saligna* leaves.

Extract/ compound	%DPPH decoloration µg/ml extract or compound		
	100	50	10
Extracts			
Pet. Ether	23.4	20.3	6.5
$CHCl_3$	37.6	18.2	11.2
EtOAc	96.6	72.9	33.4
MeOH	34.2	21.5	8.7
Isolated compounds			
A	34.3	31.7	29.2
B	100.0	100.0	83.3
C	100.0	81.2	36.5
D	95.9	86.3	41.1
E	82.3	57.5	34.9

Compound A:

It was obtained as a yellow amorphous powder, which gave positive reaction with diphenylboryloxyethylamine test on TLC suggesting that it is a flavonoid compound. The mass spectrum (Fig. 2) showed molecular ion peak at m/z 270, which demonstrated that its molecular formula is $C_{15}H_{10}O_5$.

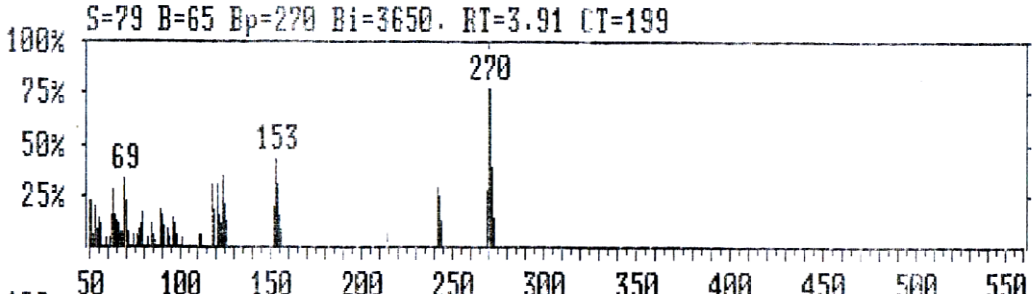

Fig. (2) The mass spectrum of compound A

This compound showed a UV absorption curve similar to that of Apigenin (Two bands at λ 267 and 336nm). This structure (Fig. 3) was clearly deduced by detection of the two meta-coupled protons, H-6 and H-8 of ring A (δ 6.19 d, J=

2.1Hz, δ 6.43 d, J= 2.1 Hz, respectively), a singlet resonance for H-3 (δ 6.56, s) and the typical AA` - XX` pattern for a para-substituted aromatic ring B (δ 6.93, d, J= 9 Hz H-3`, 5` and δ 7.84, d, J=8.7 Hz H-2`, 6`) in the ^{1}H-NMR spectrum (Fig. 4) as well as by comparison, the ^{13}C-NMR spectrum of this compound (Fig. 5 and Table 2) with literature data (Markham *et al.* 1978).

Compound	**R1**	**R2**	**R3**
A	H	H	H
B	H	OH	H
D	O-rhamnoside	OH	H
E	O-rhamnoside	OH	OH

Fig. (3) Structural formulae of the isolated compounds(A,B,D and E).

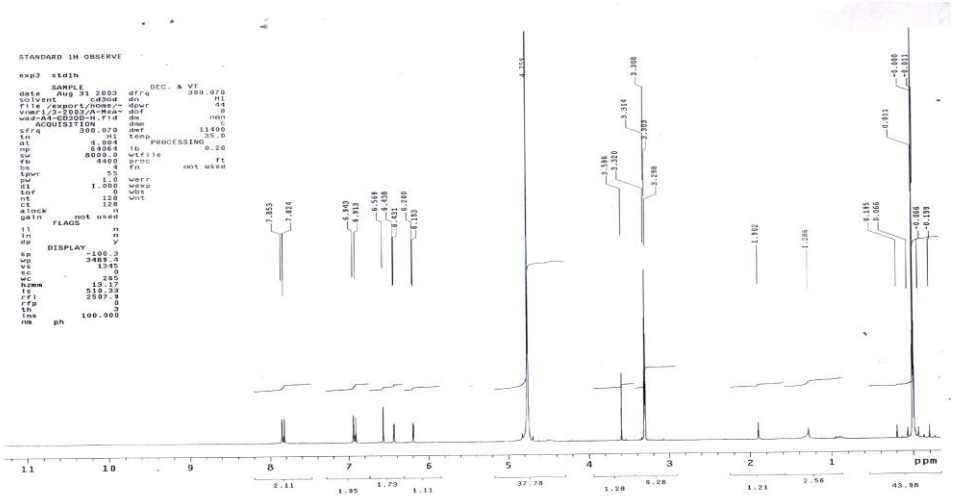

Fig. (4) ^{1}H-NMR spectrum of compound A in CD_3OD.

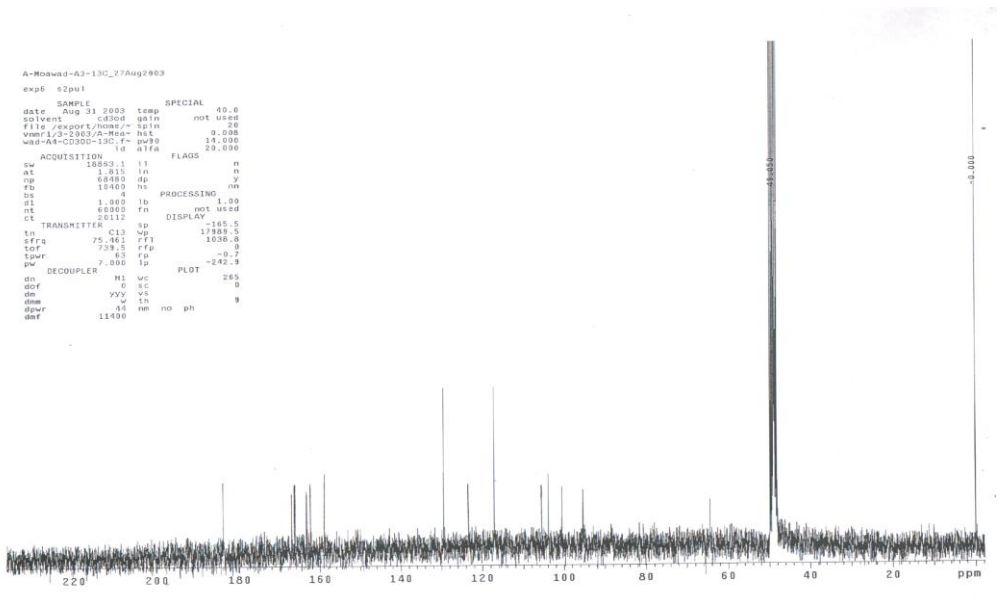

Fig. (5) ^{13}C-NMR spectrum of compound A in CD_3OD

Table 2: ^{13}C-NMR spectral data (ppm) of the five isolated compounds A-E in CD_3OD

C-atom No	A	B	C	D	E
2	166.45	166.97	82.86	158.59	158.57
3	103.83	103.83	68.83	136.59	136.31
4	183.84	183.78	28.51	179.64	179.67
5	163.20	163.18	157.53	163.21	163.19
6	100.47	100.47	96.45	100.04	99.99
7	166.30	166.30	157.79	166.31	166.24
8	95.28	95.28	95.63	94.89	94.84
9	159.52	159.52	156.90	159.33	159.45
10	105.11	105.11	100.84	105.86	105.85
1`	123.71	123.71	132.26	123.05	122.02
2`	114.17	114.17	115.35	116.45	109.64
3`	147.13	147.13	146.24	146.45	146.89
4`	151.19	151.19	146.24	149.85	137.96
5`	116.85	116.85	116.19	117.03	146.69
6`	120.32	120.32	120.09	122.90	109.69
sugar					
1	--	--	--	103.58	103.64
2	--	--	--	73.35	73.44
3	--	--	--	72.23	72.22
4	--	--	--	72.07	72.07
5	--	--	--	71.95	71.93
6	--	--	--	17.68	17.70

Compound B

It was obtained as a yellow fine crystal, which gave positive colour test for flavonoids on TLC and showed two bands at λ 253 and 349 nm in the UV spectrum. In the mass spectrum of this compound (Fig. 6), the base peak $[M]^+$ was observed at m/z 286 (100%), along with other diagnostic fragments $[M-H]^+$ 285 (14.5%), $[M+H]^+$ 287 (43.9%), $[M-CO]^+$ 258 (20.1%), $[A_1(C_7H_4O_4)+H]^+$ 153 (31.2%), $[B_2(C_8H_6O_2)]^+$ 134 (17.8%) and $[A_1-CO]^+$ 124 (13.9%), which suggested compound B to be luteolin Fig (3).

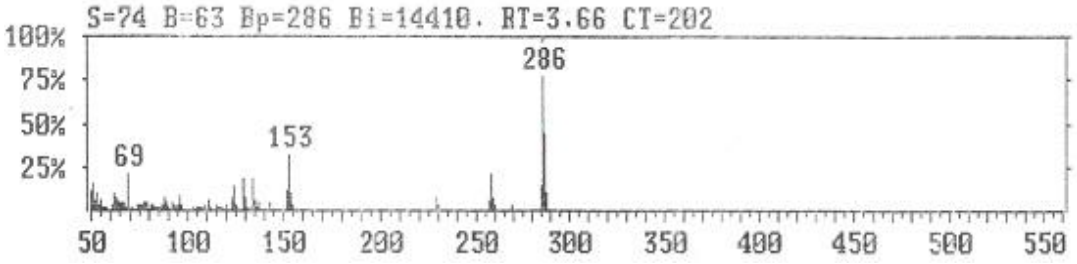

Fig. (6) The mass spectrum of compound B

The structure was further confirmed by the presence of a pair of meta coupled doublets of one proton each at δ 6.19 (J= 2.1 Hz) and δ 6.41 (J= 1.8 Hz) attributed to H-6 and H-8 protons of ring A, one proton singlet at δ 6.51 for H-3 and another three aromatic protons at δ 6.90 (1H, d, J= 8.7 Hz, H-5`) and δ 7.36 (2H, d, J= 5.7 Hz H-2`, 6`) attributed to the protons of the B ring substituted at C-3 and C-4 in the ^{1}H-NMR spectrum of compound B (Fig. 7) in addition to comparing the ^{13}C-NMR spectrum (Fig. 8 and Table 2) with literature data (Markham *et al.* 1978).

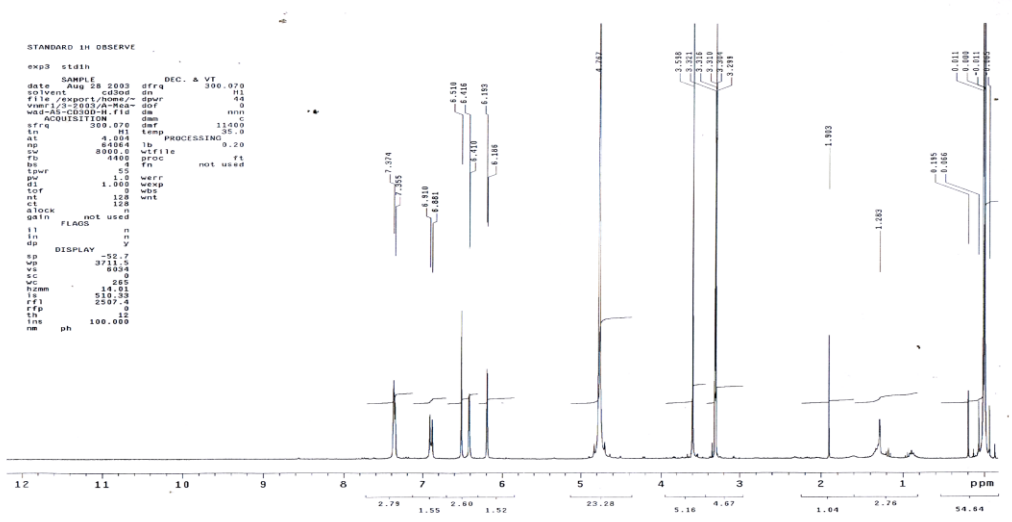

Fig. (7) ^{1}H-NMR spectrum of compound B in CD_3OD

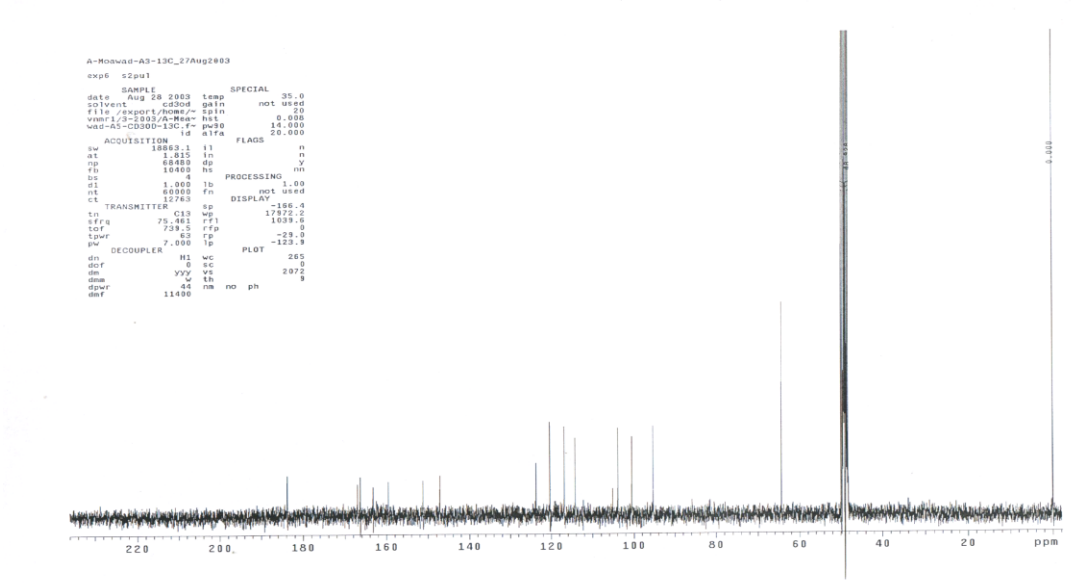

Fig. (8) ^{13}C-NMR spectrum of compound B in CD_3OD

Compound C

It was obtained as white needles, which gave positive colour test for flavonoids on TLC. Its structure was characterized as Flavan-3-ol accordance with the following considerations:-

The mass spectrum of the compound (Fig. 9) showed a molecular ion peak $[M]^+$ at m/z 290 corresponding to molecular formula $C_{15}H_{14}O_6$ and its UV spectrum exhibited a distinct maximum at 284 nm.

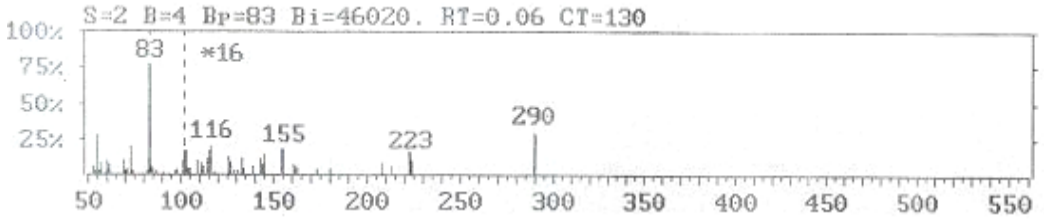

Fig. (9) The mass spectrum of compound C

The ^{1}H-NMR spectrum (Fig. 10) showed signals for five aromatic protons, three of which formed an ABX system at δ 6.73 (1H, d, J=1.8 Hz, H-6`), δ 6.77 (1H, d, J=8.1 Hz, H-5`) and δ 6.84 (1H, d, J= 1.8 Hz, H-2`) attributed to the protons of the B ring substituted at C-3 and C-4. The other two formed an AX system at δ 5.87 (1H, d, J= 2.1 Hz) and δ 5.94 (1H, d, J= 2.4 Hz) assigned to H-6 and H-8 of ring A. The spectrum also showed two protons signals at δ 2.54 (1H, d, J= 8.1, 16.2 Hz, H-4), δ 2.84 (1H, dd, J= 5.4, 16.2 Hz, H-4`), one proton signal at δ 3.98 (1H, m, H-3) and doublet signal at δ 4.57 (1H, d, J= 7.8 Hz, H-2).

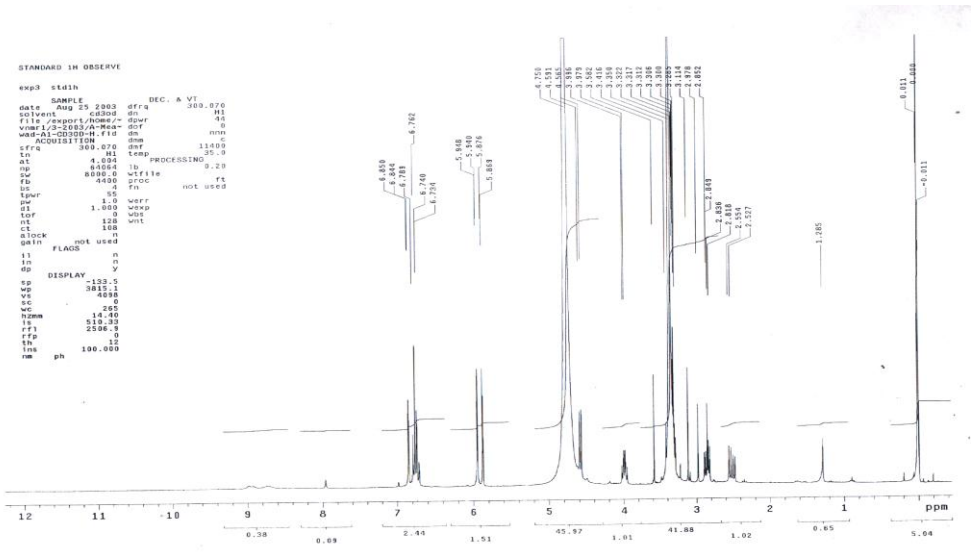

Fig. (10) ^{1}H-NMR spectrum of compound C in CD_3OD

The ^{13}C-NMR chemical assignments of this compound (Fig. 11 and Table 2) confirmed the above assumption and are in full agreement with literature values reported for the flavanol (+) Catechin (Fig. 12) previously isolated from *Acacia nilotica* (Khalid *et al.* 1989) and from *Loranthus kaoi* (Lin and Tzelin 1999).

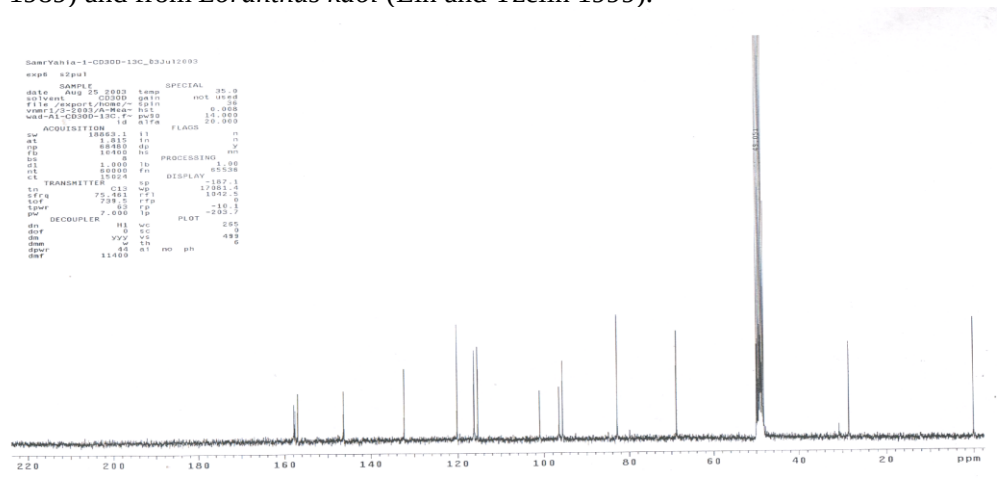

Fig. (11) ^{13}C-NMR spectrum of compound C in CD_3OD

Fig. (12) Structural formula of compound C ($C_{15}H_{14}O_6$).

Compound D

It was obtained as a yellow amorphous powder and gave positive colour test for flavonoids. The mass spectrum (Fig. 13) indicated a molecular ion peak at m/z 448 corresponding to the molecular formula $C_{21}H_{20}O_{11}$.

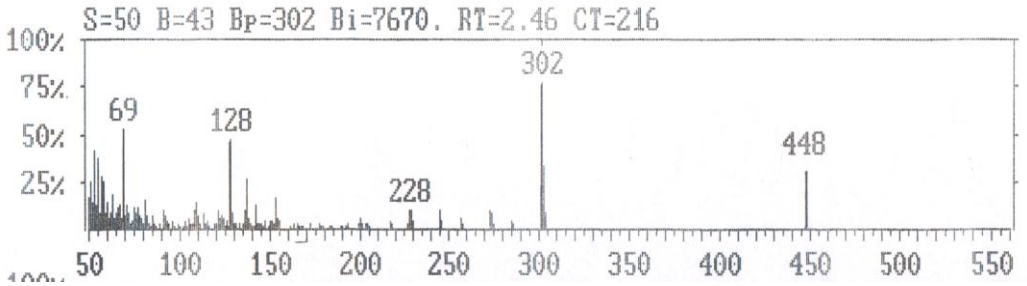

Fig. (13) The mass spectrum of compound D

On acidic hydrolysis L-rhamnose was identified by TLC as the sole sugar moiety in this compound. The presence of α-rhamnose sugar was confirmed by the ^{1}H-NMR spectrum Fig. (14) due to the appearance of only one anomeric proton signals at δ 5.35 (1H, d, J= 1.5 Hz) and methyl group at δ 0.94 (3H, d, J= 5.7 Hz-C6`) in addition to the fragment ion peak at m/z 302 (M-146) in the mass spectrum.

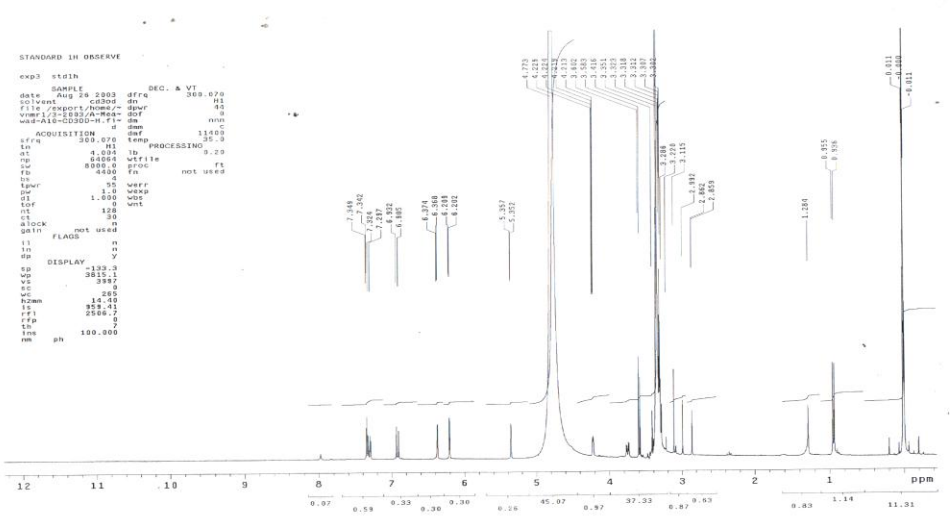

Fig. (14) ^{1}H-NMR spectrum of compound D in CD_3OD

The ^{13}C-NMR spectrum of this compound (Fig. 15 and Table 2) showed 21 carbon signals out of which 6 carbons accounted for the sugar moiety (Rha). The remaining 15 carbons were, due to the aglycone moiety. The aglycone was clearly deduced as quercetin by the ^{1}H-NMR spectrum since a pair of meta coupled doublets of one proton each at δ 6.20 (J= 2.1 Hz) and δ 6.37 (J= 2.1 Hz), ascribed to H-6 and H-8 protons of ring A. Another pair of ABX type signals at δ 6.91 (1H, d, J= 8.1 Hz, H-5`), δ 7.34 (1H, d, J= 2.1 Hz, H-2`) and δ 7.32 (1H, dd, J= 8.1, 2.1 Hz, H-6`) corresponding to the protons of B ring.

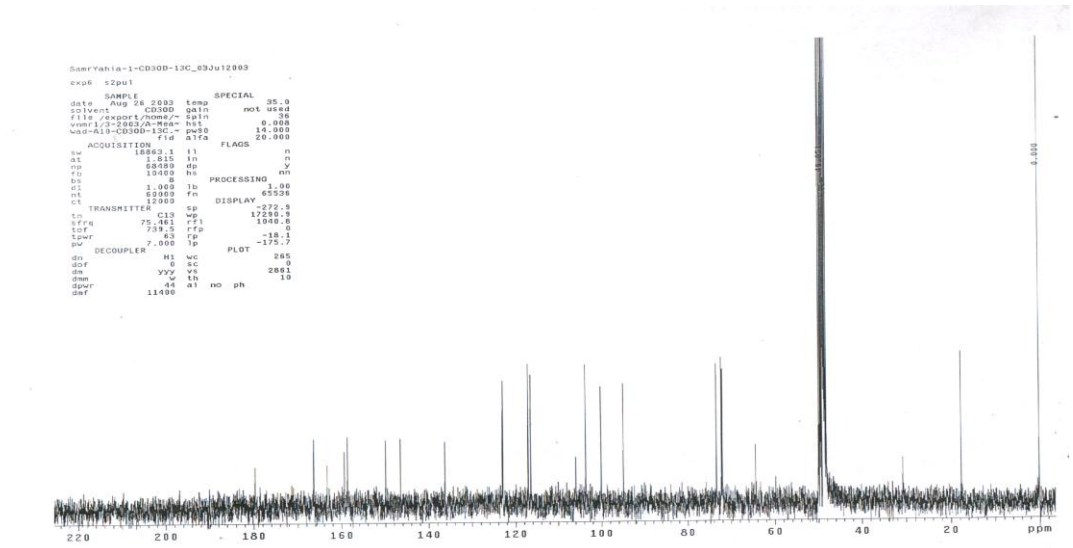

Fig. (15) ^{13}C-NMR spectrum of compound D in CD_3OD

The glycosylation site at C-3 hydroxy was confirmed by the ^{13}C-NMR spectrum through the down field resonance of C-2 at δ 158.59 ppm and the up field signals of C-3 at δ 136.24 ppm in comparing with Quercetin data (Markham, 1989 and Agrawal, 1992). Thus the structure of D (Fig. 3) was characterized as Quercetin 3-O-α-L rhamnoside (Quercitrin). This result was confirmed by comparison the present spectroscopic data with those previously reported for quercitrin (Le-Dévéhat *et al.* 2002).

Compound E

It was obtained as a yellow amorphous powder and gave positive reaction with diphenylboryloxyethylamine test on TLC suggesting that it is a flavonoid compound. Its molecular formula $C_{21}H_{20}O_{12}$ was determined from the mass spectrum (Fig. 16) in which the [M]+ ion was observed at m/z 464.

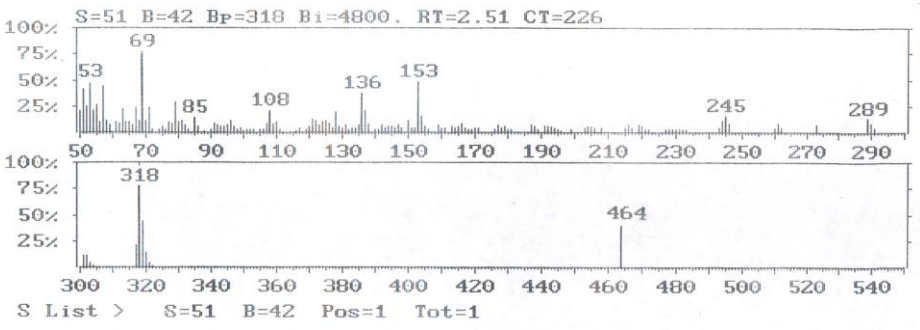

Fig. (16) The mass spectrum of compound E

Acid hydrolysis of compound E yielded L-rhamnose as a sole sugar moiety (TLC). Only one anomeric proton at δ 5.32 (1H, d, J= 1.5 Hz) and methyl group at δ 0.96 (3H, d, J= 6.0 Hz) in the ^{1}H-NMR spectrum (Fig. 17) in addition

to the fragment ion peak at m/z 318 $(M-146)^+$ were further confirmed the presence of α-L Rhamnose only as a sugar moiety.

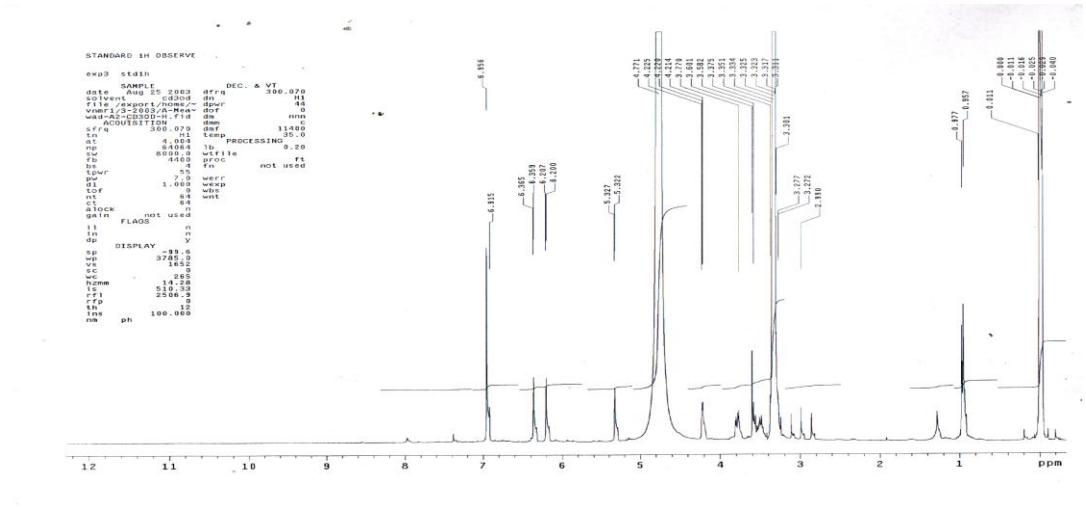

Fig. (17) ^{1}H-NMR spectrum of compound E in CD_3OD

The aglycone was established as Myricetin by the appearance of a pair of meta coupled doublets of one proton each at δ 6.2 (J= 1.5 Hz) and δ 6.36 (J= 1.8 Hz) assigned to H-6 and H-8 protons of ring A and a doublet signal of two protons at δ 6.93 (2H, d, J= 12.9 Hz) ascribed to H-2` and H-6` of B ring. The ^{13}C-NMR chemicals sifts (Fig. 18 and Table 2) of the C-2 and C-3 positions (δ 158.57 and δ 136.31 ppm) indicated that the rhamnose must occupy the hydroxyl at C-3 (Markham, 1989).

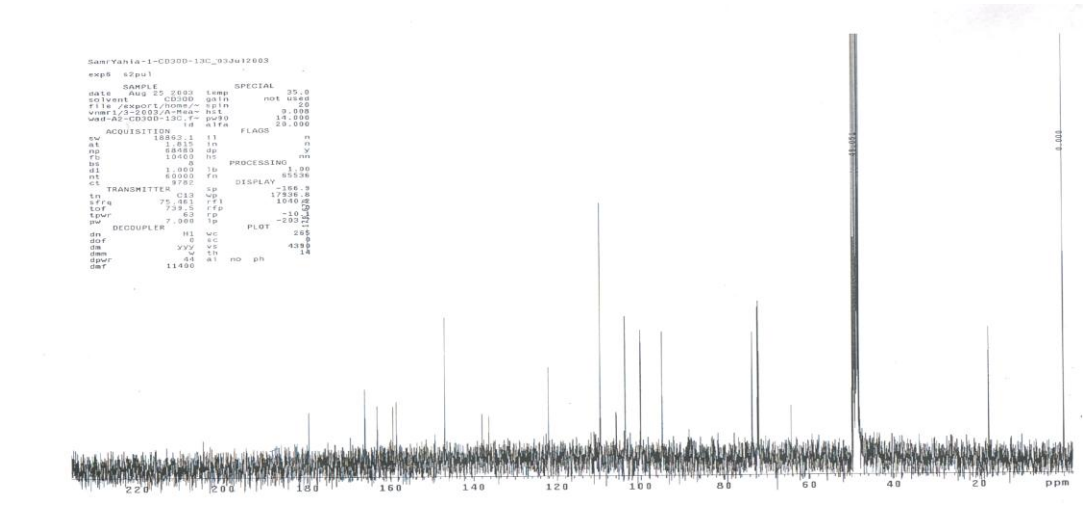

Fig. (18) ^{13}C-NMR spectrum of compound E in CD_3OD

On the basis of the above findings compound E (Fig. 3) was determined to be Myricetin-3-O-rhamnoside (Myricetrin). This result was confirmed by comparison the present spectroscopic data with those previously reported for Myricetrin (Markham *et al.* 1978).

Free radical scavenging activity:

The ethylacetate extract of *A.saligna* showed a strong free radical scavenging effect in the DPPH decolouration assay (Table-1). The decolouration percentages were 96.6, 72.9 and 33.4% at the three concentrations examined of ethylacetate extract i,e. 100, 50 and 10 μg/ml, respectively.

A moderate activity as antioxidant was observed in both $CHCl_3$ and MeOH extracts, with decolouration percentages of 37.6 and 34.2 respectively at a concentration 100μg/ml. However Pet. Ether extract of *A. saligna* was less active as free radical scavenging activity investigated using a DPPH colour reduction assay.

Assay-guided isolation of DPPH-active fractions led to the identification of flavonoids isolated from the ethylacetate by Column Chromatography on silica gel and Sephadex LH-20.

Five bioactive compounds A, B, C, D and E were identified by ^{1}H and ^{13}C-NMR, MS and UV spectroscopy.

The most potent compound as free radical scavenger was compound (B), surprisingly reduced the colour of DPPH to 100% decolouration at both concentrations used 100 and 50 μg/ml, and 83.3% with concentration of 10 μg/ml. The spectral data confirmed that compound (B) has Luteolin structure.

The second effective compound as free radical scavenger is compound C. It also showed 100% decolouration in DPPH-assay at concentration 100 μg/ml. The spectral analysis indicated that compound C is (+) Catechin.

Compound D and E showed free radical scavenging activities (95.9% and 82.3% decolouration of DPPH) at concentration of 100 μg/ml respectively. Quercetin glycoside (compound D) and Myricetin glycoside (compound E) were identified by chemical and spectral investigations.

It is interesting to note that the scavenging activity of the butanol extract of *Dyera costulata* leaves was due to the flavonoid Quercetin3-o-α-L-rhamnoside (Subhadhirasakul *et al.* 2003). Its antioxidant activity (50% at 9.37μM) was about 8 times higher than that of the well known synthetic antioxidant BHT (50% at 80.78μM). This flavonoid was the third potent isolated compound in this study, compound D (Table 1).

The less effective compound was (A) (34.3% colour reduction in DPPH assay) with Apigenin structure.

In vitro, flavonoids are often powerful inhibitors of lipid peroxidation with several mechanisms i.e. ROS/RNS (reactive oxygen species/ reactive nitrogen species) scavengers, inhibitors of damage haem protein/peroxide mixture, metal ion binding agents and inhibitors of lipoxygenase and cyclooxygenase enzymes (Halliwell and Gutteridge 1999).

The degree of hydroxylation and relative positions of –OH groups are of primary importance as free radical scavengers, as complexers of pro-oxidant metals and/or quenchers of the formation of singlet oxygen.

The action of flavonol antioxidation is multi-functional. Flavonols form complexes with metals. Chelation occurs at the 3-hydroxy, 4-keto grouping

and/or at the 5-hydroxy, 4 keto group, when the A-ring is hydroxylated in the 5-position, also an O-quinol grouping on the B-ring can demonstrate metal complex activity (Pratt 1992).

The position and the degree of hydroxylation is primary importance in determining antioxidant activity, there is general agreement that ortho-dihydroxylation of the B-ring contributes markedly to the antioxidant activity of flavonoids (Daniel 1989 and Rice *et.al.*1996).

The para quinol structure of B-ring has been shown to impart even greater activity than the ortho quinol structure; while the meta configuration has no effect on antioxidant activity. However, para and meta hydroxylation of B-ring apparently does not occur commonly in nature.

Flavonoids are widely distributed in plants and other plant products and average total daily consumption in the Netherland was estimated as at least 23 mg of which 16 mg/day in quercetin, more than the average daily intake of vitamin E (7-10 mg) (Awad 1994 and Halliwell and Gutteridge 1999).

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مزيلات الشقوق الحرة من أوراق نبات الاكاسيا

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الشقوق الحرة المنفردة من نواتج التمثيل الغذائى تسبب الكثير من الأضرار للخلايا فتضعف من مقاومة الجسم للأمراض، فضلاً عن أنها تُعتبر أحد المسببات للعديد من الأمراض الخطيرة مثل السرطان، السكر، تصلب الشرايين والتهاب المفاصل.

لذا اكتسب استخدام مضادات الأكسدة أهمية كبيرة لحماية الجسم من هذه الأمراض لما لها دوراً هاماً كمزيلات لهذه الشقوق الحرة.

ولما كانت مضادات الأكسدة الطبيعية اكثر أماناً واقل في تأثيراتها الجانبية عن مضادات الأكسدة المخلقة لذلك اصبح هناك اهتمام كبير فى إيجاد أنواع جديدة،آمنة، اقتصادية وعالية الفاعلية من المصادر الطبيعية لتحل محل مضادات الأكسدة المخلقة.

وفى هذه الدراسة أظهر مستخلص خلات الأيثيل لأوراق نبات الأكاسيا فعالية كمزيل للشقوق الحرة من خلال تقدير تأثيره على إزالة اللون لمركب ثنائى فينيل بكريل هيدرازيل (DPPH). وقد تم إخضاع هذا المستخلص لفصل المكونات المسئولة عن هذه الفعالية باستخدام طرق التحليل الكروماتوجرافى ثم التعرف على تركيبها الكيميائى بإستخدام طرق التحليل الطيفى (الأشعة فوق البنفسجية، الرنين المغناطيسى، تقدير الكتلة) والطرق الكيميائية (التحليل بالحامض، التحليل الوصفى).

ولقد تم فصل خمسة مركبات تنتمى الى الفلافونويدات وهى المسئوله عن فعالية هذا المستخلص وهى: ليتولين، كاتشين، كيرسيترين، ميرسيترين وابيجنين.