Isolation and Identification of Two New Cyclic Amino Acids From The Seeds Of *Zizyphus Spina-Christi* L. (Willd) by Means of ^{1}H-NMR-^{13}C-NMR-HSQC-HMBC AND GC-MS

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Abstract

n this study we isolate two new cyclic amino acids from the seeds of *Zizyphus spina-christi* L. (Willd) 70% methanolic extract. The two compounds were identified by means of ^{1}H-NMR-^{13}C-NMR-HSQC-HMBC and GC-MS as 4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid (less polar and major compound) and 4-hydroxy-4-hydroxymethyl-1-methyl pyrrolidine-2-carboxylic acid (more polar and minor compound) of the ratio 4:3.

Keywords: Amino acids, seeds, *Zizyphus spina-christ,*.
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Introduction

Increased attention has been paid to the genus *Zizyphus* Family Rhamnaceae due to its significant medicinal uses viz hypoglycemic, hypotensive, anti-inflammatory, antimicrobial, antioxidant, antitumor, liver protective and immune function improvement purposes (Nunes et al. 1987; Adzu et al. 2001; Borgi et al. 2007) Nowadays, the development of drugs from natural sources is recommended in order to overcome the side effects of many of the synthetic drugs. Recent research on medicinal plants has generated a great deal of information about the biologically active chemical components that are responsible for the claimed medicinal effects.

Zizyphus species (Rhamnaceae family) are commonly used in folklore medicine for the treatment of various diseases such as digestive disorders, weakness, liver complaints, obesity, urinary troubles, diabetes, skin infections, loss of appetite, fever, pharyngitis, bronchitis, anaemia, diarrhea, and insomnia (Han and Park 1986; Kirtikar and Basu 1984). They are widespread in the Mediterranean region, Africa, Australia, and tropical America. Previous phytochemical studies on the different species of the genus Zizyphus led to the isolation and characterization of cyclopeptide alkaloids, flavonoids, sterols, tannins, and triterpenoid saponins (Ikram et al. 1981; Nawwar et al. 1984). Zizyphus spina-christi (L.) Willd is a wild tree, with spiny branches and small, orange–yellow fruits, commonly found in Jordan, Israel, and Egypt, known in Egypt as Nabq or Sidr (Taeckholm 1974) where it is used to treat blisters, bruises, chest pains, dandruff, fractures, headache, and mouth problems (Ghazanfar 1994). For example, the fresh leaves are applied on a swollen eye at night (Taeckholm 1974). The roots are used to cure and prevent skin diseases (Dalziel 1937).

The root bark infusion is used tradionally in northern Nigeria as a remedy for stomach pain and other gastrointestinal tract ailments. It has been used in folk medicine as a demulcent, a stomachic, for toothaches, as astringent and as mouth wash (Duke 1985). Fruits are used to promote the healing of fresh wounds, for dysentery, bronchitis, coughs and tuberculosis (Hutchens 1973). It is also used to relieve digestive disorders, obesity, urinary troubles and as a potent anti-microbial agent (Shahat et al. 2001 and Nazif 2002). Zizyphus spina-christi (L.) Willd has many contents including peptide and cyclopeptide alkaloids, flavonoids, sterols, tannins, butulinic acid and triterpenoidal saponin glycosides (Ikram et al. 1981; Higuchi et al. 1984; Nawwar et al. 1984; Han et al. 1990; Barboni et al. 1994; Abu-Zarga et al. 1995; Cheng et al. 2000; Shahat et al. 2001; Tripathi et al. 2001).

The present study deals with the investigation and identification of the chemical constituents in 70% methanol extract of the seeds of *Zizyphus spina-christi* L. (Willd).

Materials and methods

Plant material

Ripened seeds of *Z. spina-christi* L. (Willd) plant were collected from Orman garden, Giza, Egypt in April 2002. The plant was identified by Dr. Kamal El Batanony, professor of Taxonomy, Faculty of Science, Cairo University. A voucher specimen (No. 4108) was deposited at the Herbarium unit of the Institute for future reference.

GC-MS system was used

GC-MS-Detector HP 6890 (MS: Ion source: ionization energy: 70eV; Column HP 35, Length: 30 cm, inner diameter: 250 um, layer thickness: 0.25 um;carrier gas: helium, 0.6 ml/min; injector temperature: 250 °C, temperature program: 50 °C: 1 min, 50-300 °C with 15 °C /min)

NMR: Varian Unity Inova 400 MHz Austria.

For spectral analysis (^{1}H-NMR,^{13}C-NMR) and 2D (^{1}H,^{1}H-COSY, HSQC and HMBC) spectroscopy.

Isolation and Identification of two new cyclic amino acids

The air dried powder of *Zizyphus spina-christi* ripened seeds (1 Kg.) was percolated with methanol 70% till exhaustion and evaporated under reduced pressure to yield 89 g dried extract. The methanolic extract was suspended in water (300ml) and partitioned successively with petroleum ether (60-80°C) (6×400ml), chloroform (6×300ml), ethyl acetate (6× 400ml) and butanol (6× 400ml).

Each fraction was dried over anhydrous sodium sulphate and concentrated under reduced pressure to give 15g, 10g, 12g, 10g and 30g (petroleum ether, chloroform, ethyl acetate, butanol and aqueous dried extracts, respectively). The aqueous fraction was screened by two-Dimensional paper chromatography using n-butanol-acetic acid-water (4:1:5,v/v/v) and acetic acid :water (15:85,v/v) s and visualized under UV light before and after spraying with $AlCl_3$ and exposure to ammonia vapour. The results revealed the presence of two spots.

The aqueous fraction was subjected to colum chromatography using microcrystalline cellulose (E. Merck) as adsorbent and elution was carried out with absolute ethanol. Fractions (50 ml each) were collected and concentrated into small volumes. All fractions were screened by PC (Whatmann No. 1) using *n*-butanol-acetic acid- water (4 : 1 : 5, v/v/v) and acetic acid : water (15 : 85, v/v), while similar fractions were pooled and solvents evaporated under reduced pressure.

Paper chromatography showed the presence of two spots starting from fraction 3 to fraction 13: These fractions were combined, eluted and purified on Sephadex LH-20 column using absolute ethanol as eluent, yielding an isolate which appeared as very near two spots with strong violet color under UV light which are isolated by GC-MS.

Results and Discussion

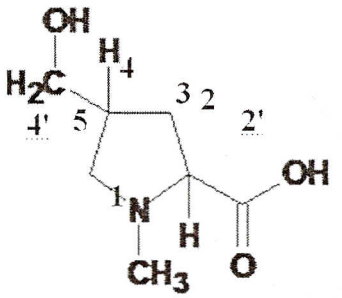
Isolate 1 (30 mg) appeared as two spots ; hR_f values = 75&74 in solvent system *n*-butanol : acetic acid : water (4 : 1 : 5) and 82 & 83 in solvent system 15% acetic acid, respectively. It gave a strong violet colour under UV .

Isolate is a mixture of two compounds (1A&1B) in ratio 1.2:1. Each of them is a saturated compound of 7 carbon atoms with one carboxylic group.The ^{1}H-NMR spectrum in Methanol- d_4+ 25% pyridine -d_5 (Fig. 23) shows signals in a range from 1.9 to 4.3 δ indicating that both are saturated aliphatic compounds. The Dept-HSQC spectrum indicated that each of the two compounds contains three CH_2 groups. Two of every component bare a big difference in the chemical shift (Δδ= 0.15 to 1.36 ppm) of the geminal protons indicating saturated ring system. The shift difference of the third is smaller (Δδ=0.06) for compound 1A and absent for compound 1B. Each compound holds one N-CH_3 group (at δ = 3.07and 2.96). There are two CH groups in compound 1A and one in compound 1B. HMBC spectrum (Fig.28) shows that the ^{13}C-NMR signal at δ =172.3 is excited by two carbonyls belonging to two different compounds. The quartery carbon of tertiary alcohol at δ = 81.0 is attributed to compound 5B.

The structure of compounds 1A &1B was confirmed by GC-MS data of trimethylsilylated isolate 1, which showed two GC peaks indicating two compounds. The molecular ion peak of Compound 1A was not clear, but the signals caused by the decomposition of the molecular ion with loss of hydrogen (m/z=302) so M^+= 303

(odd number which proves that the compound contains one nitrogen atom) methyl(m/z=288) or $COOSi(CH_3)_3$ (base peak at m/z=186, α-cleavage next to the nitrogen, further loss of trimethylsilanol gives the ion at m/z=96.

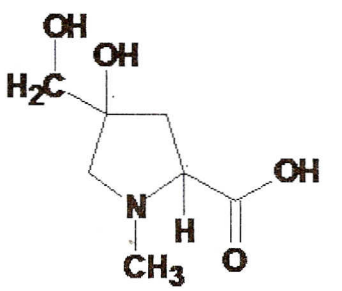
Compound 1B gave a very small molecular ion peak (m/z=391 odd number) and signals caused by loss of methyl (m/z=376), $CH_2OSi(CH_3)_3$ (M/Z=288, α-cleavage next to the oxygen at carbon-4) or $COOSi(CH_3)_3$ (m/z=274). The combination of ^{1}H, ^{1}H-COSY, HSQC and HMBC revealed 2-(4-hydroxymethyl-pyrrolidine) carboxylic acid compound 1A and 2-(4-hydroxy-4-hydroxymethyl -pyrrolidine) carboxylic acid compound 1B, both are new natural products.



2-(4-hydroxymethyl-pyrrolidine) carboxylic acid (Compound 1 A)

Table 1: $^{1}H,^{13}C$-NMR spectral data of Compound 1A

Number of carbon		C(ppm)	H(ppm)
1	CH_3	41.45	2.96(s)
2	CH	72.31	3.90(t,J=8.8Hz)
2'	C_q	172.33	-
3	CH_2	33.02	1.88(m) 2.60(m)
4	CH	39.88	2.66(m)
4'	CH_2	63.53	3.62(dd,J=11.7,2Hz) 3.56(dd,J=10.6,11.2Hz)
5	CH_2	58.79	2.32(dd,J=12.1,11.3Hz) 3.68(dd,J=11.7,7.2Hz)



2-(4-hydroxy-4-hydroxymethyl -pyrrolidine) carboxylic acid (Compound 1B

Table 2: $^{1}H,^{13}C$-NMR spectral data of Compound 1B

Number of carbon		C(ppm)	H(ppm)
1	CH_3	43.76	3.07(s)
2	CH	72.31	4.24(dd,J=11.4,7.3Hz)
2'	C_q	172.33	-
3	CH_2	40.88	2.32(dd,J=13.9,11.3Hz) 2.47(dd,J=13.9,7.2Hz)
4	C_q	81.01	-
4'	CH_2	65.78	3.62(s)
5	CH_2	64.44	3.14(d,J=12.4Hz) 3.96(d,J=12.4Hz)

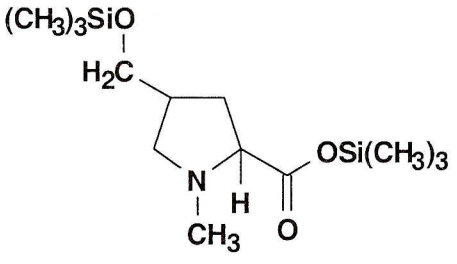
GC-MS spectral data are ascribable to compounds 1A&B

Table 3: GC-MS spectral data of Compound 1A

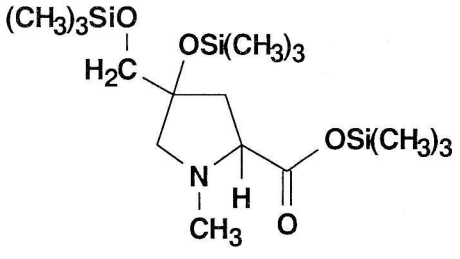
m/z	Fragments
302	$M^+ - H$
288	$M^+ - CH_3$
260	$M^+ - CH_2{=}N{-}CH_3$
186	$M^+{-}COOSi(CH_3)_3$

Table 4: GC-MS spectral data of Compound 5B

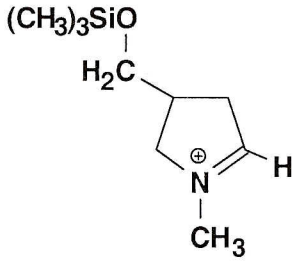
m/z	Fragments
391	M^+
376	$M^+ - CH_3$
348	$M^+ - CH_2{=}N{-}CH_3$
288	$M^+ - CH_2{-}OSi(CH_3)_3$
274	$M^+{-}COOSi(CH_3)_3$



M^+: m/z = 303

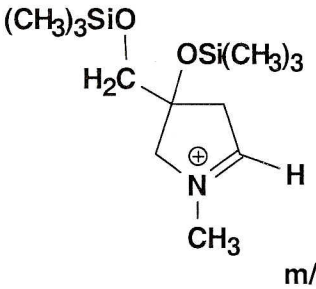


M^+: m/z = 391



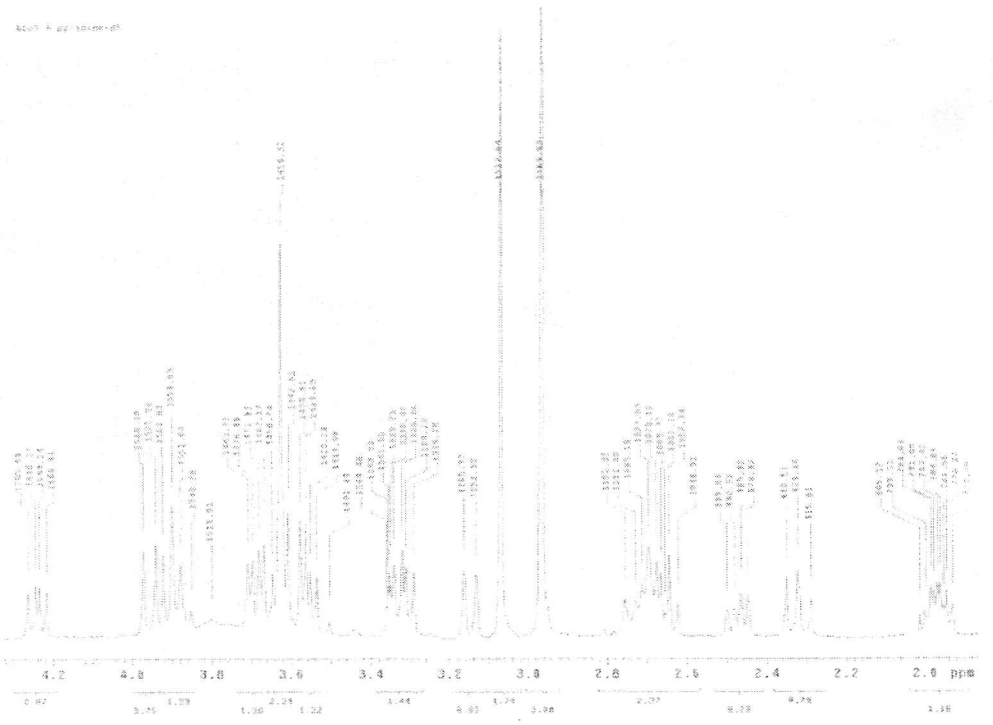
m/z = 186

Compound 1A

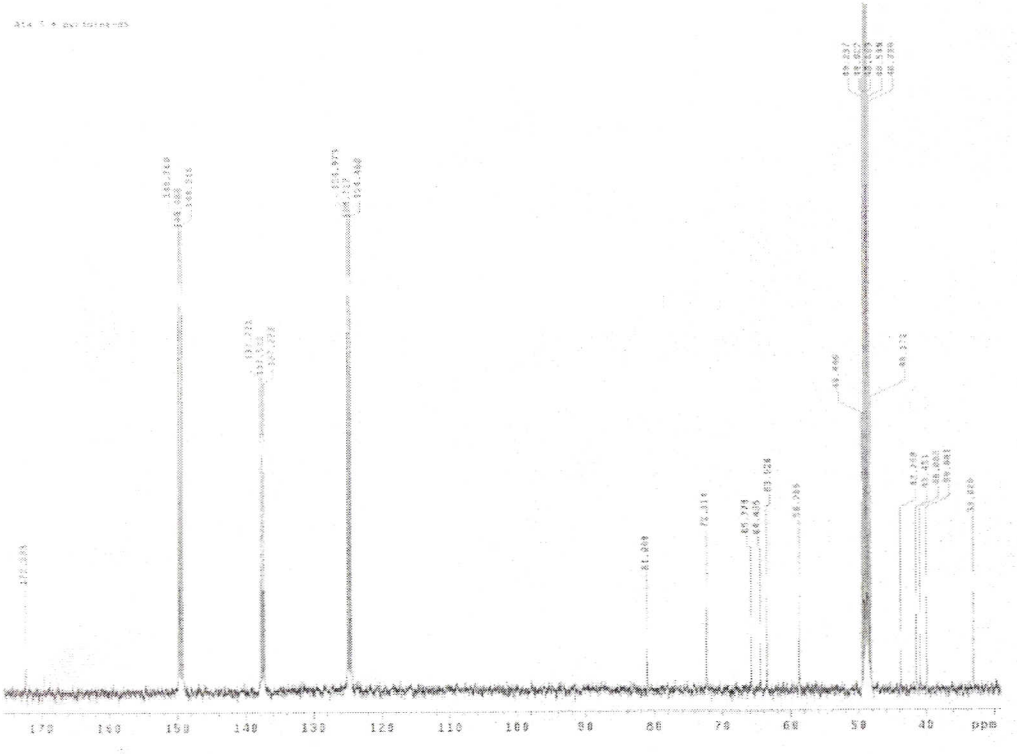


m/z = 274

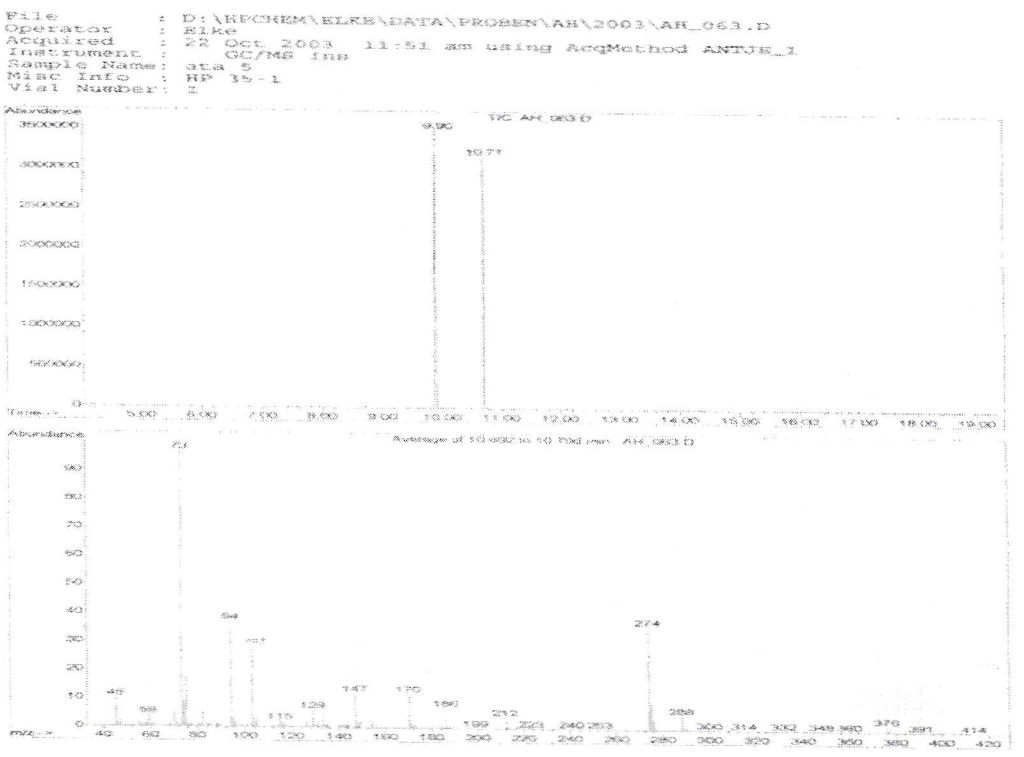
Compound 1B



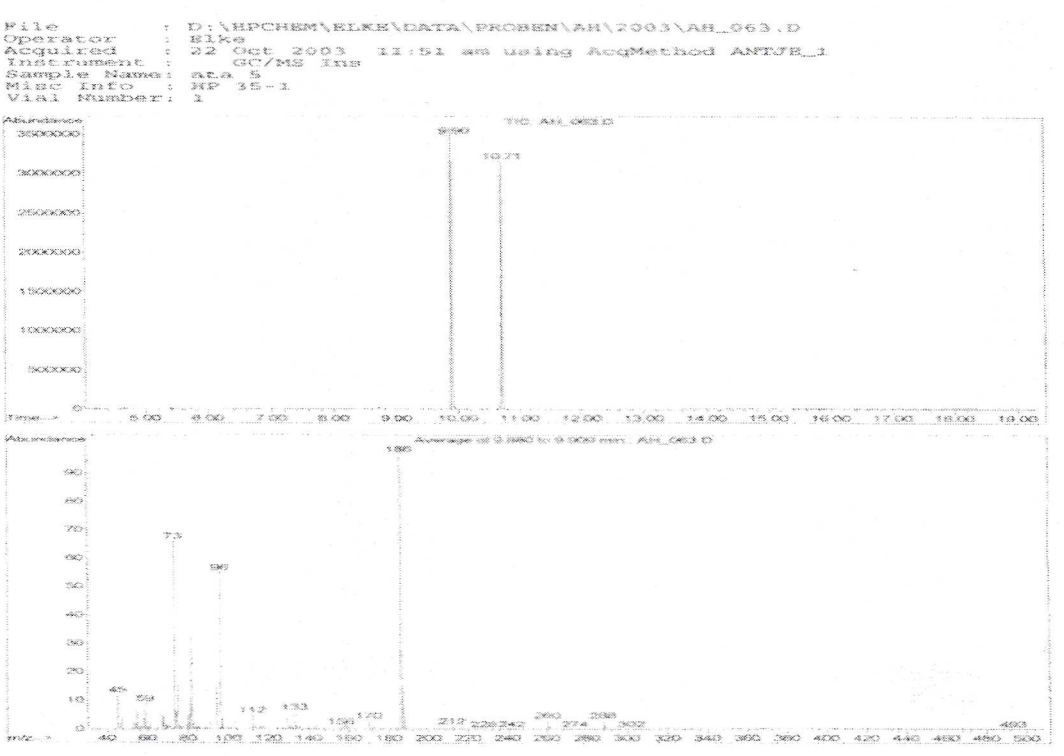
^{1}H-NMR Spectrum



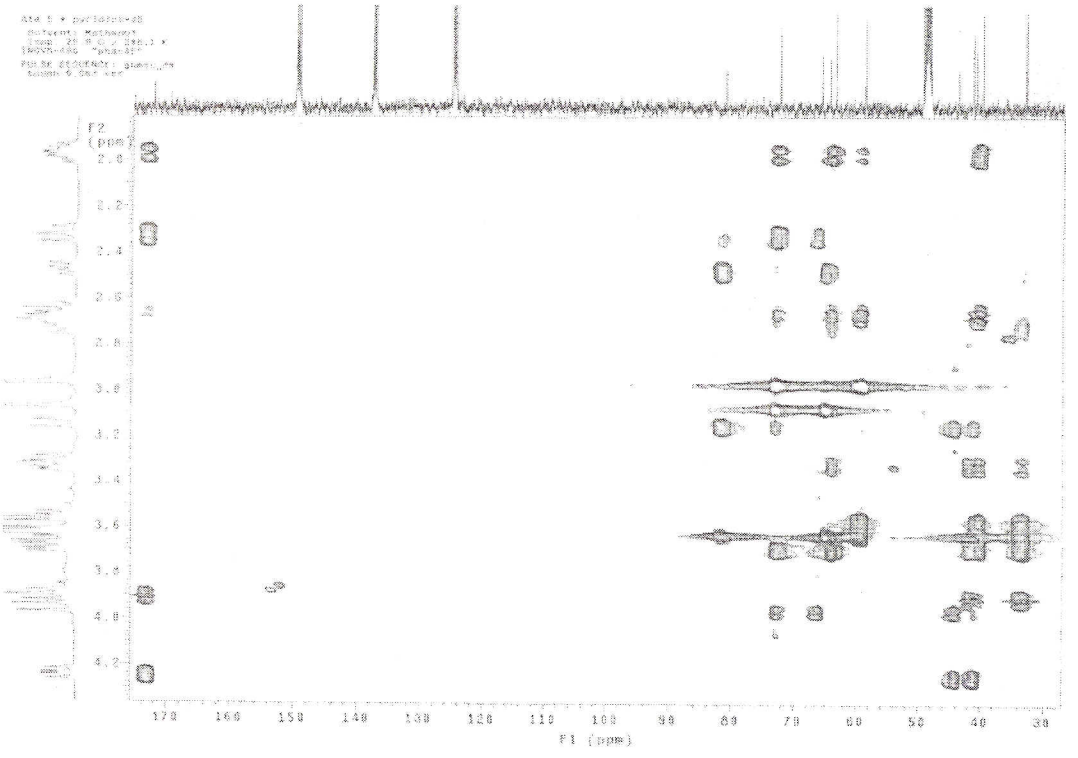
^{13}C-NMR Spectrum



GC-MS of compound 1A



GC-MS of compound 1B



HSQC Spectrum

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