CHAPTER 4

AN ACYLATED PHLOROGLUCINOL WITH ANTIMICROBIAL PROPERTIES FROM *HELICHRYSUM CAESPITITIUM*

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An acylated phloroglucinol with antimicrobial properties from *Helichrysum caespititium*

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Abstract

A new acylated form of a phloroglucinol with significant antimicrobial properties was isolated by bioactivity guided fractionation from *Helichrysum caespititium* (Asteraceae). The structure elucidation, and conformation of the new phloroglucinol, 2-methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl) phenyl]but-2-enyl acetate, was established by high field NMR spectroscopic and MS data. The compound inhibited growth of *Bacillus cereus, B. pumilus, B. subtilis* and *Micrococcus kristinae* at the very low concentration of 0.5 μg/ml and *Staphylococcus aureus* at 5.0 μg/ml. Six fungi tested were similarly inhibited at low MICs, *Aspergillus flavus* and A. *niger* (1.0 μg/ml), *Cladosporium cladosporioides* (5 μg/ml), *C. cucumerinum* and *C. sphaerospermum* (0.5 μg/ml) and *Phytophthora capsici* at 1.0 μg/ml.

Keywords: *Helichrysum caespititium*; Asteraceae; Antimicrobial; Phloroglucinol

1. Introduction

Many *Helichrysum* (Asteraceae) species have been examined for their chemical components. These include 38 species from South Africa (Jakupovic, Kuhnke, Schuster, Metwally & Bohlmann, 1986; Jakupovic, Zdero, Grenz, Tsichritzis, Lechmann, Hashemi-Nejad, & Bohlmann, 1989b; Meyer, Afolayan, Taylor & Erasmus, 1997) eight from Madagascar (Randriaminahy, Proksch, White & Wray, 1992), several from Spain (Tomas-Baberan, Msonthi & Hostettmann, 1988; Tomas-Barberan, Iniesta-Sanmarin, Tomas-Lorente, & Rumbero, 1990) and many species from Australia (Jakupovic, Schuster, Bohlmann, Ganzer, King & Robinson, 1989a) The fact that different *Helichrysum* species produce different secondary metabolites (acetophenones, flavonoids, phloroglucinols) as a biochemical defence mechanism (chemical barrier) against bacteria and fungi is of great interest, since it indicates the use of different metabolic pathways. As part of a programme to investigate the medicinal potential of South African *Helichrysum* species we examined *H. caespititium* (DC.) Harv. for possible biological activity. The Southern Sotho inhale the smoke for relief of head and chest colds and also use it as a dressing for open wounds during circumcision rites. Caespitin (**1**) was previously isolated from this species (Dekker, Fourie, Snyckers & Van der Schyf, 1983) and shown to have antimicrobial properties. The antimicrobial activity guided fractionation of the acetone extract of the aerial parts of *H. caespititium* led to the isolation of the new phloroglucinol derivative 2-methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl)-phenyl]but-2-enyl acetate (**2**). Evaluation of the antimicrobial activity of compound **2** against ten bacteria showed significant biological activity against all the Gram-positive bacteria tested. In addition, the growth of the six fungi tested, was significantly inhibited at very low MIC values.

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2. Results and discussion

2.1. Structure elucidation of 2

The structure of **2** was established through the usual spectroscopic techniques including ^{1}H- and ^{13}C-NMR analysis. With the aid of DEPT, COSY and HETCOR pulse sequences, the multiplicity of the proton peaks, their relationship to one another and the identity of each carbon and hydrogen could be established, respectively. The process of identification was facilitated by the article (Dekker et al., 1983) on the compound caespitin; and the identification of acylphloroglucinols from *Helichrysum* species (Jakupovic et al., 1989b; Bohlmann & Mahanta, 1979). The presence of a free phloroglucinol nucleus was suspected on the grounds of three phenolic carbons being shifted far downfield in the ^{13}C-spectrum (C-2′, C-4′ and C-6′ at 164.0, 161.6 and 160.2 ppm, respectively). The single aromatic proton attached to C-5′, had the anticipated proton (∂5.98) and carbon (95.7 ppm) shifts. The ^{13}C-spectrum left no doubt that a carbonyl carbon and ester carbon were present (211.8 and 173.6 ppm, respectively). It remained to establish what other substituents were on the side chains. The 2-methylpropanoyl moiety at C-3′ showed the anticipated septuplet for the $CHMe_2$ group (∂3.96) and the doublet for the geminal dimethyl substituents (∂1.17). An initial problem was posed by the nature of substituents present on C-1′, but a closer examination of the proton NMR spectrum revealed that the side chain of **2** was not unlike that of the 3,3′-dimethylallyl group (Ar–$CH_2CH{=}CMe_2$) present in the compounds described by Bohlmann and Mahanta (1979). In case of compound **2**, however, the one terminal methyl group had been replaced by $–CH_2OCOCH_3$. The upfield ^{13}C-shift position of the methylene group at C-4 (benzylic to the aromatic ring and allylic to the side chain alkene) at 21.5 ppm was unusual, but in keeping with the findings of Tomas-Barberan et al. (1990), Dekker et al. (1983) and Bohlmann and Mahanta (1979) on similar moieties.

2.2. Significance of structure

The claim that **2** is a new compound is based on the finding that (Jakupovic et al., 1989b) describes an acylated phloroglucinol with molecular formula $C_{17}H_{22}O_6$ but ascribes an incorrect structure to it on p. 1120. The structure shown in that paper has an *n*-butyl group at C-3′ instead of the $–COCHMe_2$ group.

2.3. Antibacterial activity

The activity of compound **2** was examined against 10 bacteria by the agar dilution method (Turnbull & Kramer, 1991). The compound significantly inhibited the growth of all the Gram-positive bacteria tested (Table 1) at a concentration of between 0.5 and 5 μg/ml. This phloroglucinol had no activity against all the Gram-negative bacteria tested. These results are in accordance with previous reports (Tomas-Barberan et al., 1990; Dekker et al., 1983) of similar antimicrobial activity of related compounds against Gram-negative bacteria. Most bacillus species are regarded as having little or no pathogenic potential, however, both *Bacillus cereus* and *B. subtilis* have been known to act as primary invaders or secondary infectious agents in a number of cases and have been implicated in some cases of food poisoning (Turnbull & Kramer, 1991). *Staphylococcus aureus*, is a human pathogen, whose infections are amongst the most difficult to combat with conventional antibiotics (Tomas-Barberan et al., 1988, 1990). This study provides a probable

Table 1
Antibacterial activity of the crude acetone extract of the aerial parts of *Helichrysum caespititium* and compound **2** isolated from the extract

Bacterial species	Gram (+/−)	MIC[a]	
		Crude extract (mg/ml)	**2** (μg/ml)
Bacillus cereus	+	1.0	0.5
B. pumilus	+	1.0	0.5
B. subtilis	+	1.0	0.5
Micrococcus kristinae	+	1.0	0.5
Staphylococcus aureus	+	1.0	0.5
Enterobacter cloacae	−	1.0	na[b]
Escherichia coli	−	1.0	na
Klebsiella pneumoniae	−	na	na
Pseudomonas aeruginosa	−	1.0	na
Serratia marcescens	−	na	na

[a] Minimum inhibitory concentration.
[b] Not active.

Table 2
Antifungal activity of the crude acetone extract of the aerial parts of *Helichrysum caespititium* and compound **2** isolated from the extract

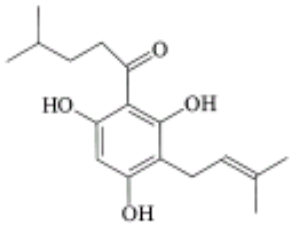
Fungal species	MIC[a]	
	Crude extract (mg/ml)	**2** (μg/ml)
Aspergillus flavus	1.0	1.0
A. niger	0.01	1.0
Cladosporium cladosporioides	0.01	5.0
C. cucumerinum	0.01	0.5
C. sphaerospermum	0.01	0.5
Physophthora capsici	1.0	1.0

[a] Minimum inhibitory concentration.

scientific explanation for the therapeutic potency attributed to *H. caespititium*, claimed by traditional healers in the Free State province of South Africa, for example, during wound treatment in male circumcision rites.

2.4. Antifungal activity

The growth of six fungi, *Aspergillus niger*, *A. flavus*, *Cladosporium cladosporioides*, *C. cucumerinum*, *C. sphaerospermum* and *Phytophthora capsici*, were significantly inhibited at very low MIC's by compound **2** (Table 2). *A. flavus* and *A. niger* are some of the most important fungi responsible for human systemic infections. These organisms were inhibited at 1.0 μg/ml. It is generally agreed that at least one acidic hydroxyl group and a certain degree of lipophilicity are required for biological activity compound (Tomas-Barberan et al., 1990). Lipophilicity is important because many antifungal metabolites exert their toxicity by some membrane associated phenomenon, and it is known that acidic hydroxyl groups may act by uncoupling oxidative phosphorylation. In this case, the antifungal compound isolated from *H. caespititium* bears three acidic hydroxyls (phenolic hydroxyls) and lipophilicity (3′-isobutyrylphenyl and but-2-enyl acetate residues). On the other hand, antibacterial activity, against Gram-positive bacteria seems to be related to the presence of phenolic hydroxyls (phenol itself is a well known antibacterial compound (Tomas-Barberan et al., 1990).



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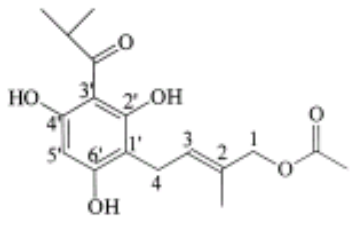

2

3. Experimental

3.1. Plant Material

Shoots of *H. caespititium* were collected from the Drakensberg in the Mount-aux-Sources area in QwaQwa, South Africa during August 1998. A voucher specimen (AM11) of the species was deposited in the herbarium of the National Botanical Institute of South Africa in Pretoria.

3.2. Preparation of extract

Air dried (80 g) plant material was immersed in acetone and shaken on a rotary shaker for 5 min without homogenising it. The extract was filtered and concentrated to dryness under reduced pressure at 40° with a rotary evaporator. After determining the yield (6.4 g (w/w)), the extract was stored at 4° until antibacterial assays commenced.

3.3. Antibacterial activity

An aliquot of the crude extract of *H. caespititium* was serially diluted (ten-fold) to obtain a range of 1.0–0.01 mg/ml in 2% acetone final concentrations. Compound **2** was diluted to final concentrations of 100.0, 10.0, 5.0 and 0.5 μg/ml in 2% acetone. The plant extract and isolated pure compound **2** (sterilised by filtering through a 0.22 μm filter) were added to 5 ml of sterilised nutrient agar in Petri dishes and swirled carefully before congealing. The organisms were streaked in radial patterns on agar plates (Mathekga & Meyer, 1998). Plates were incubated at 37° in the dark and examined after 24 and 48 h. Complete inhibition of growth was required for the extract to be declared bioactive. The controls consisted of Petri dishes containing only nutrient agar and others containing nutrient agar in 2% acetone. Each treatment was analysed in triplicate.

3.4. Antifungal activity

The acetone plant extract as well as compound **2** were subjected to the same treatment as noted above except that instead of streaking bacteria onto the agar, 48 h cultured fungal inoculum disks were carefully deposited at the centre of each Petri dish. Plates were incubated at 25° in the dark and examined after 24 and 48 h. Complete inhibition of growth was similarly required for the extract to be declared bioactive. Controls were likewise prepared containing only nutrient agar or nutrient agar in 2% acetone. Each treatment was analysed in triplicate.

3.5. Isolation and identification of 2

The crude acetone extract of *H. caespititium* was initially subjected to preparative TLC in $CHCl_3$–EtOAc (1:1). The targeted band was recovered and rechromatographed by column chromatography with 100% chloroform on silica gel 60. Direct TLC antibacterial bioassays of the fractions indicated the presence of several antibacterial compounds in the extract. The fraction with the highest antibacterial activity were finally isolated in a pure form by HPLC in H_2O–EtOH (1:1) on a reverse phase Phenomenex column (250 × 4.60 mm; 5 μ). NMR analysis of DEPT, COSY and HETCOR spectra were obtained using standard pulse sequences on a Varian 200 MHz spectrometer. Mass spectra were recorded on a Hewlett-Packard 5988 GC/MS instrument. High resolution mass spectra were obtained from a Kratos MS 80 RF double-focussing magnetic sector instrument.

3.5.1. Compound 2

2-Methyl-4-[2′,4′,6′-trihydroxy-3′-(2-methylpropanoyl)phenyl]but-2-enyl acetate mp 140°; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 1.17 (6H, *d*, J = 6.7 Hz, $CH\underline{Me}_2$), 1.73 (3H, *s*, $CH_3C{=}$), 2.12 (3H, *s*, $\underline{CH_3}CO_2$), 3.40 (2H, *brd*, J = 7.1 Hz, H-4), 3.96 (1H, septuplet, J = 6.7 Hz, $\underline{CH}Me_2$), 4.79 (2H, *s*, H-1), 5.49 (1H, *brt*, J = 7.1 Hz, H-3), 5.98 (1H, *s*, H-5′), 7.90 (2H, *bs*, 2 × ArOH), 12.90 (1H, *bs*, ArOH on C-2′). ^{13}C-NMR (500 MHz, $CDCl_3$): 19.8 (di$\underline{Me}$), 21.5 (C-4), 21.7 ($\underline{Me}C{=}$), 21.7 ($\underline{CH_3}CO_2$), 39.6 ($\underline{CH}Me_2$), 64.8 (C-1), 95.7 (C-5′), 104.5 (C-3′), 106.5 (C-1′), 129.5 (C-3), 130.1 (C-2), 160.2 (C-4′), 161.6 (C-6′), 164.0 (C-2′), 173.6 (CO_2), 211.8 (C=O). GCMS m/z (rel. int.) : 262 (25, M-60), 219 (100, M-60-$CHMe_2$), 177 (8), 115 (6), 109 (7), 69 (10). Preparation of the trimethylsilyl derivative afforded a small peak (1%) at m/z 322 (M-trimethylsilyl ether). HRMS calculated for $C_{17}H_{22}O_6$ requires 322.14164; found 322.14363.

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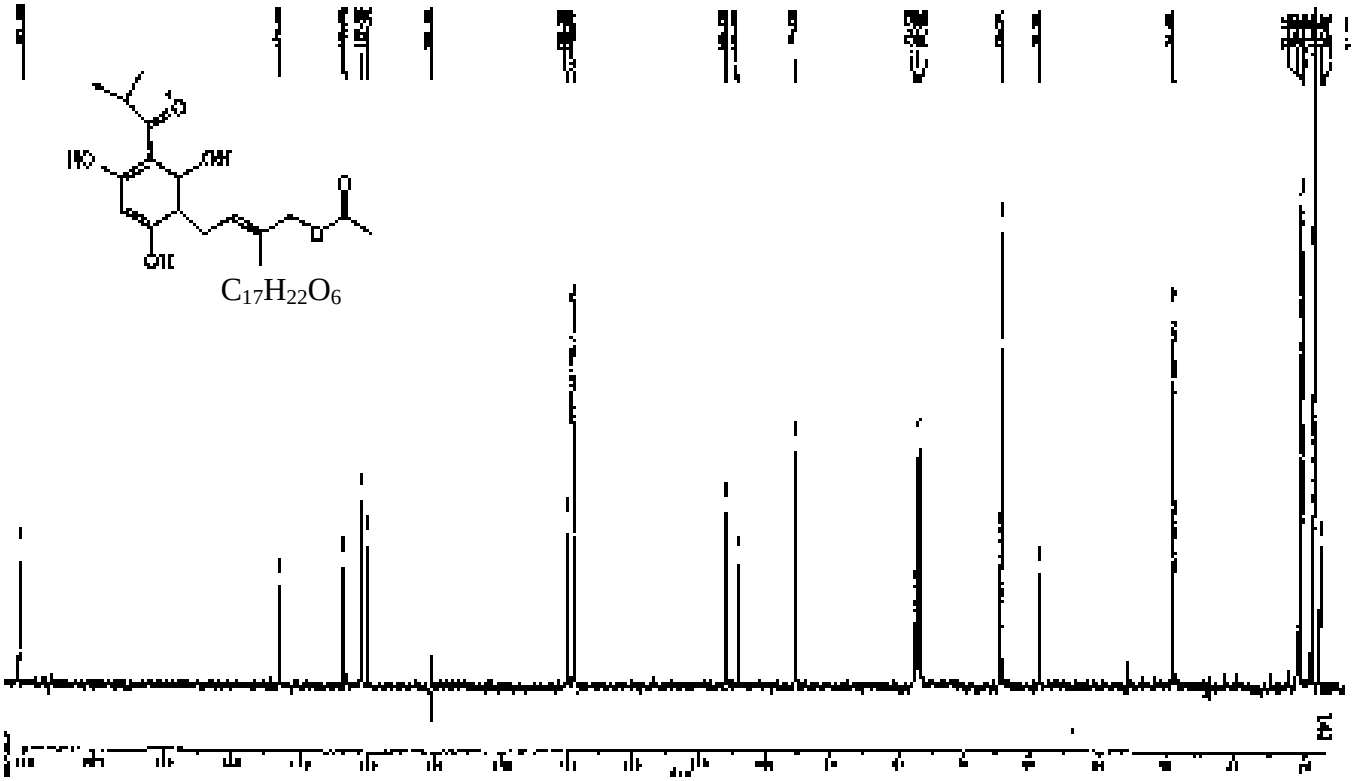

Figure 4.1 ^{13}CNMR of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

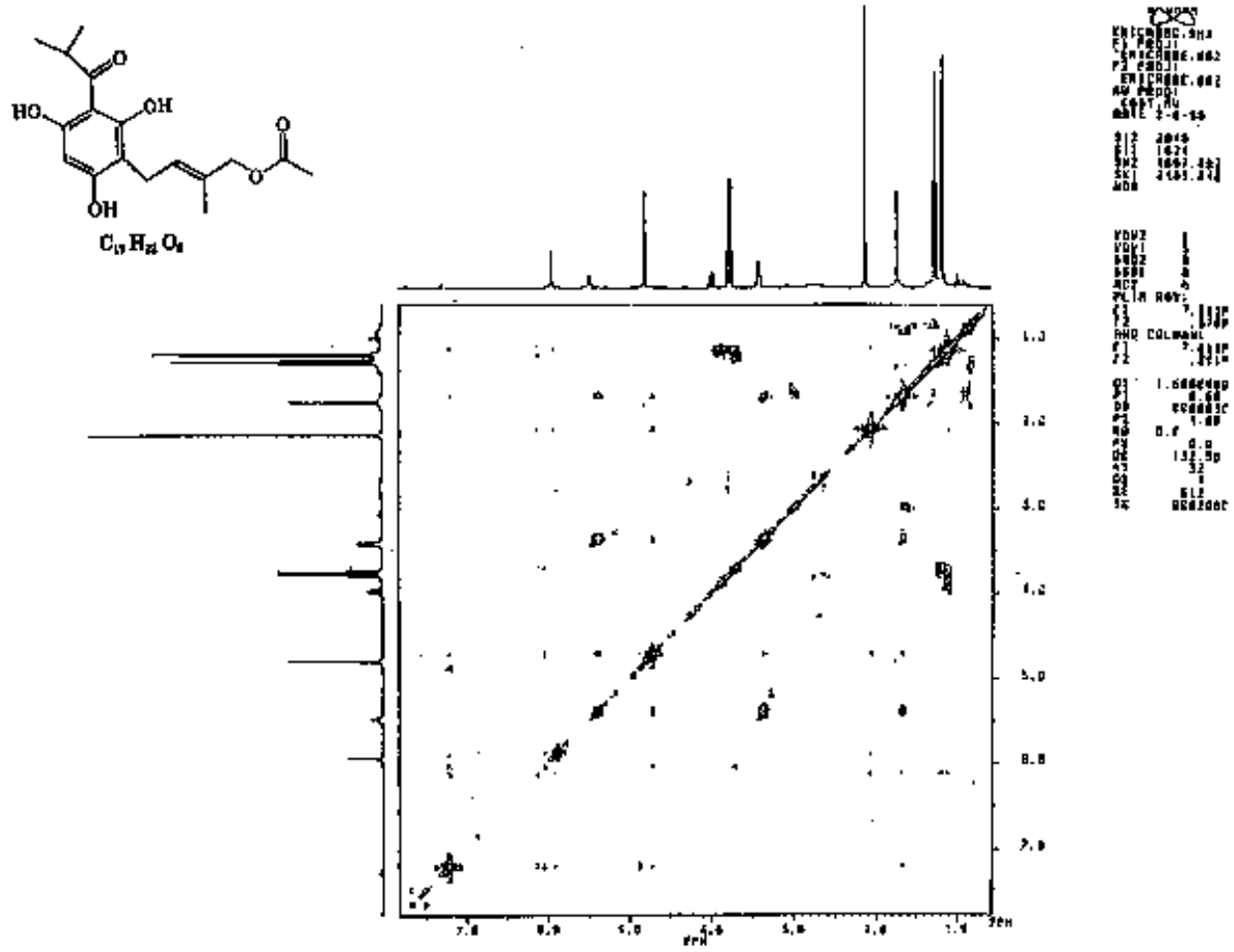

Figure 4.2: COZY of caespitate in $CDCI_3$ MW. 322.14 $C_{17}H_{22}O_6$

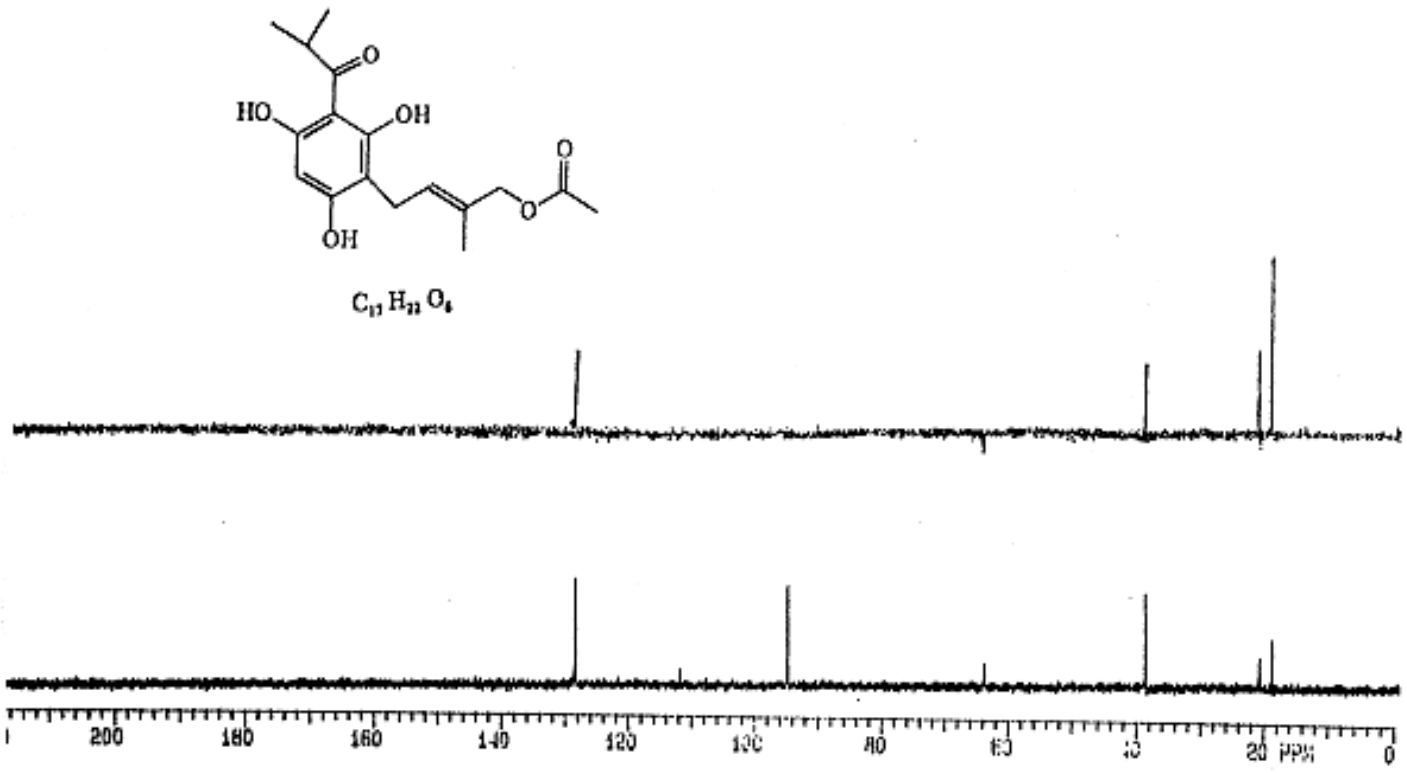

Figure 4.3: DEPT spectrum of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

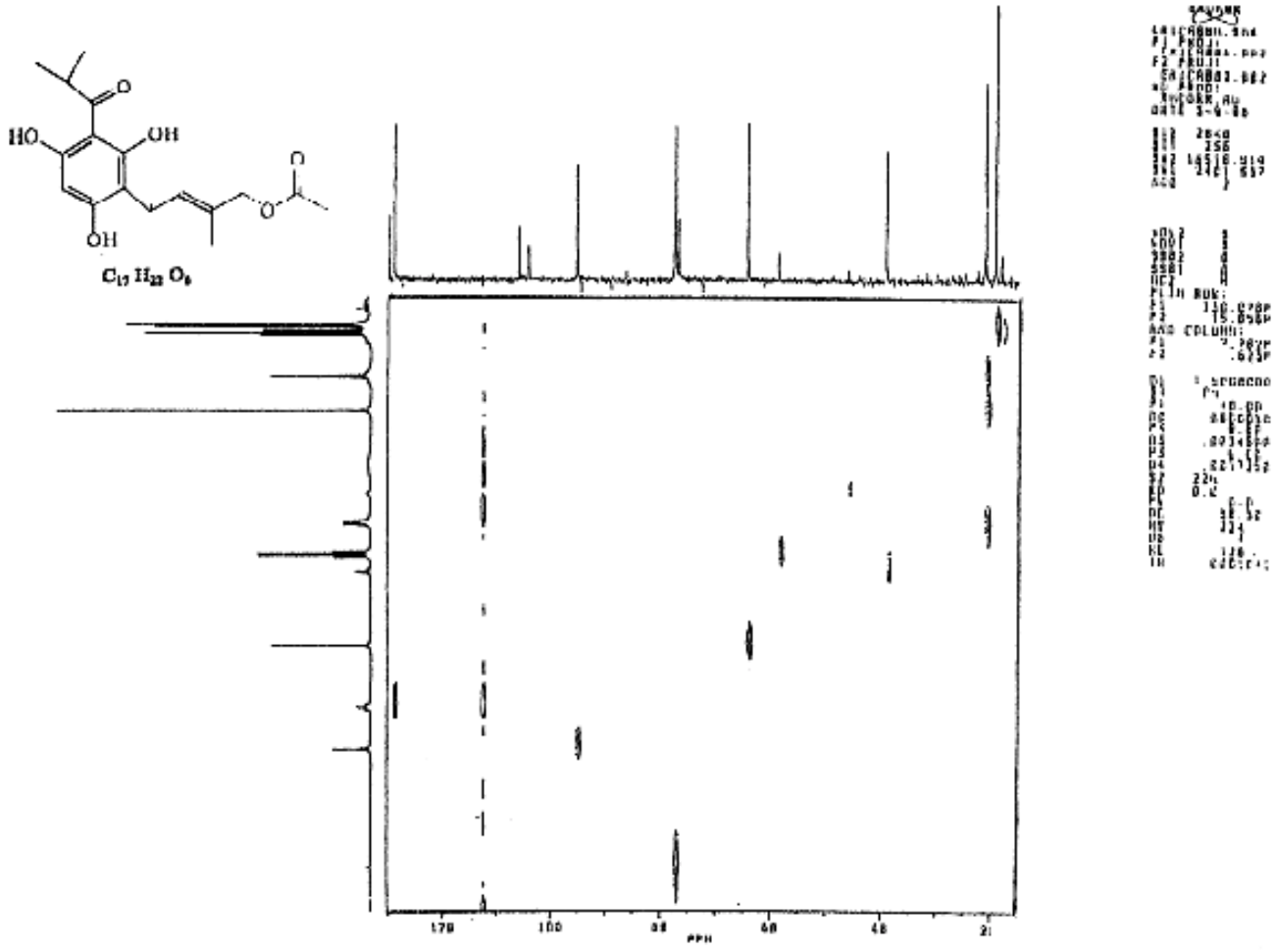

Fig 4.4: HETCOR of caespitate in $CDCI_3$. MW. 322.14 $C_{17}H_{22}O_6$

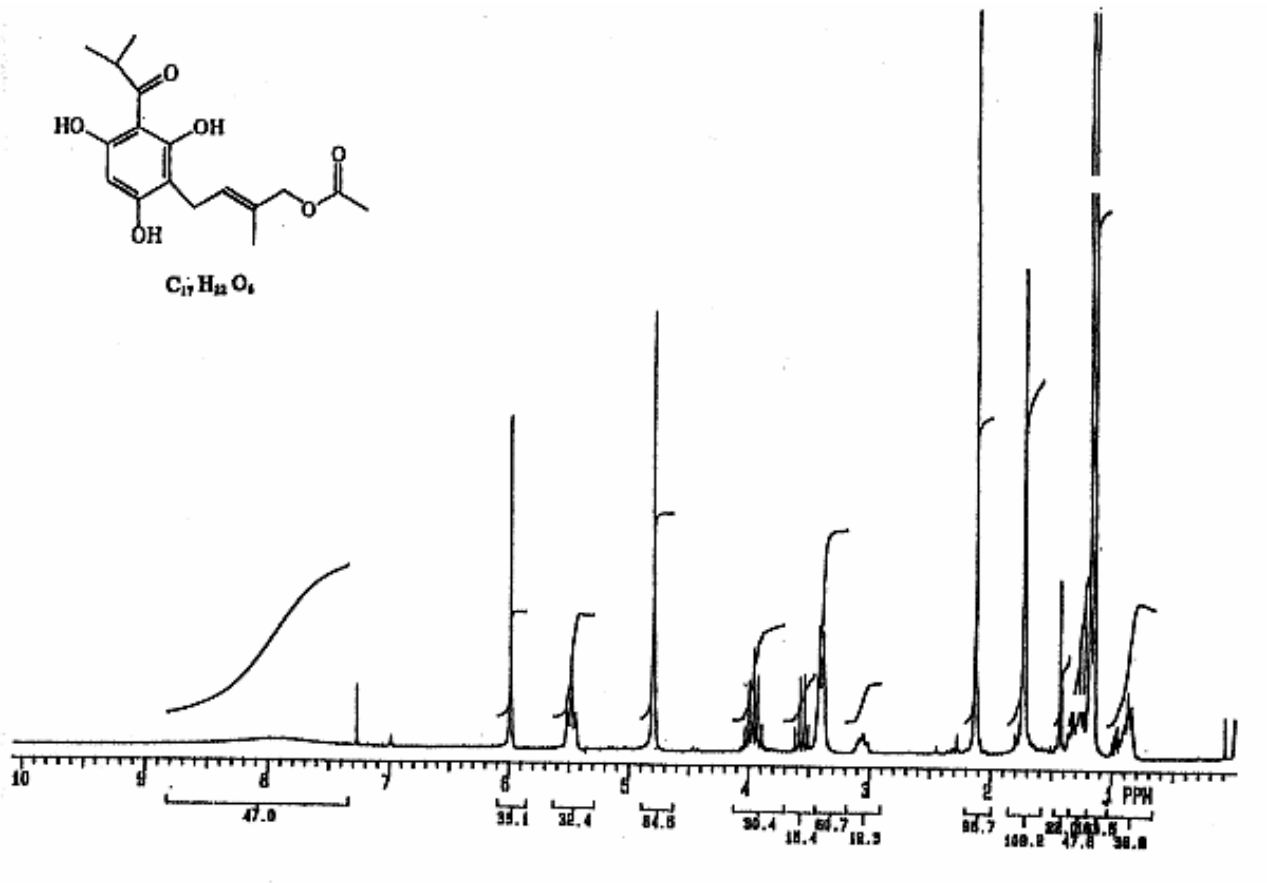

Figure 4.5: H of caespitate in $CDCI_3$ MW. 322.14 $C_{17}H_{22}O_6$

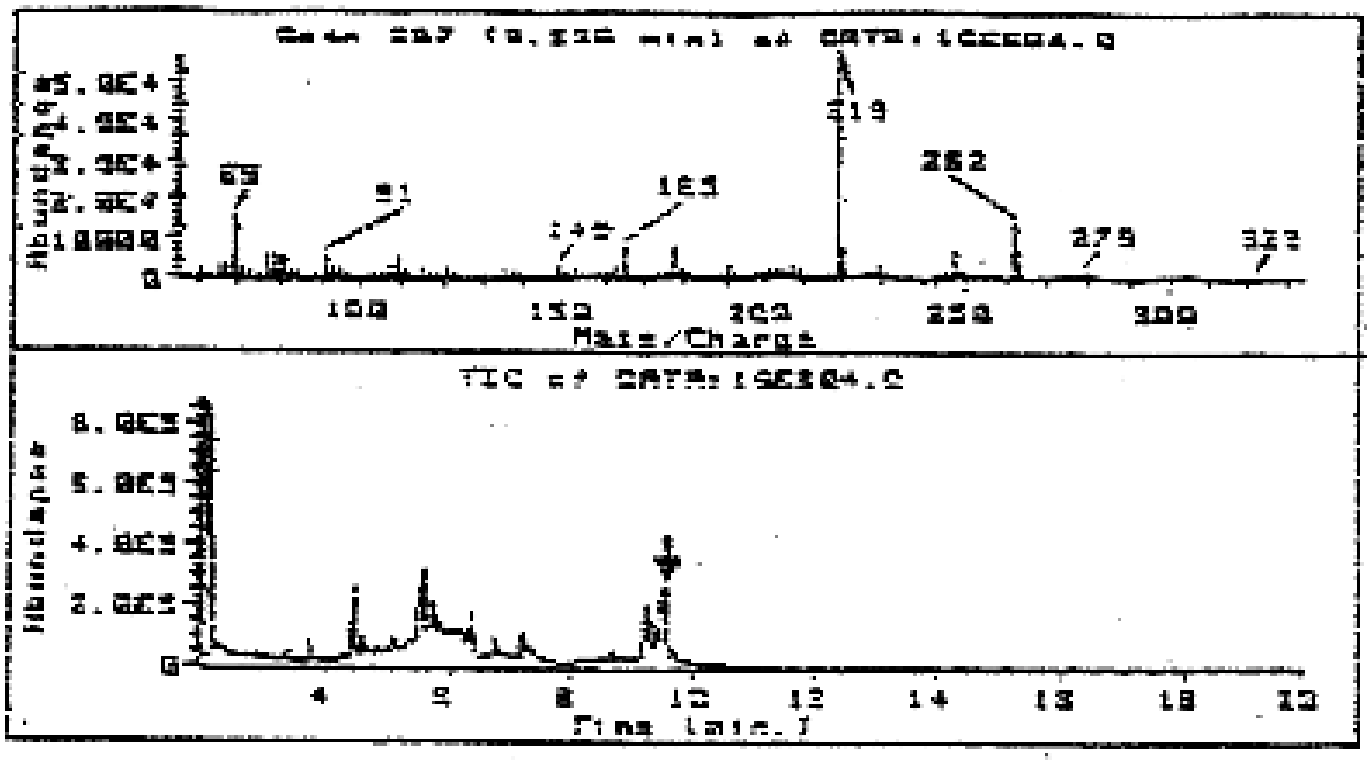

Figure 4.6 GCMS: TMS mass determination of caespitate. MW. 322.14 $C_{17}H_{22}O_6$