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# Effect of C-3 Modification of Oleanolic Acid on *Candida* spp., *Trichophyton tonsurans* and *Microsporum canis* Inhibition

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**Abstract: Background:** Natural product compounds are the source of numerous chemotherapeutic agents. Investigations were carried out to evaluate the therapeutic properties of a C-3 modified oleanolic acid, 3β-acetoxyoleanolic acid (JH23), in the continue search for lead compounds. **Results:** The well-in-plate agar diffusion method used for the inhibition zone determination, showed that compound JH23 had a more enhanced activity as compared to JH16 (oleanolic acid) the parent nucleus, on most of the test organism; *Candida guielemondi* (JH23, 23±0.04; JH16, 22±0.03), *Candida albicans* (JH23, 29±0.05; JH16, 21±0.12), *Candida krusei* (JH23, 26±0.04; JH16, 23±0.001), *Candida tropicalis* (JH23, 30±0.02; JH16, 23±0.01), *Trichophyton tonsurans* (JH23, 29±0.03; JH16, 19±0.02) and *Microsporum canis* (30±0.01; JH16, 16±0.04). **Conclusion:** The results showed that functionality modification of organic compounds could lead to compounds with enhanced bioactivity.

**Key words:** 3β-acetoxyoleanolic acid, oleanolic acid, *Candidasis* inhibition, antifungal activity

## INTRODUCTION

There is a general consensus among the scientific community that natural products have been playing a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases. Indeed, the vast majority of chemotherapeutic agents are based on natural products and this fact anticipates that new leads may certainly emerge from the tropical plant sources, since biological chemo diversity continues to be an important source of molecular templates in the search for lead drugs. A great deal of experimental data published in the last three decades have confirmed the potential of extracts of medicinal plants and several isolated compounds to cause growth inhibition of various bacterial genera, including multidrug resistant strains. The compound Oleanolic Acid (OA) occurs in numerous varieties of plants, as constituents of medicinal herbs and also forms an integral part of the human diet (Liu, 2005). Many authors have reported the biological activities of OA which includes anti-inflammatory, trypanocidal, anti-HIV and cytotoxic activities (Sultana and Ata, 2008). Preliminary studies confirmed that synthetic derivatives have the potential to become an important new treatment for multiple forms of cancer, some are currently in Phase I clinical trials for the treatment of metastatic or unresectable solid tumors of lymphoma. (Liby *et al*., 2007). Several approaches have been successfully used to improve the biological activity, diminish toxicity and enhance the water solubility of OA (Farina *et al*., 1998; Chen *et al*. 2005). The modification of OA to produce p-coumarate ester analogue resulted in a substantial, eight-fold increase in its antibacterial potential suggesting a strong structure-activity relationship (Tanachatchairatana *et al*. 2008). In turn Rojas and coworkers demonstrated that the OA derivative, aegicerin, extracted from the Peruvian plant *Clavija procera* (Theophrastaceae) possessed very strong activity against a large number of *M. tuberculosis* strains, including clinical isolates resistant to isoniazid and the Multidrug-Resistant (MDR) isolates. MIC values ranged between 1.6 and 3.12 mg $mL^{-1}$ (Rojas *et al*. 2006). From the clinical perspective, very important results were obtained by Horiuchi and coworkers who reported the strong activity of OA and UA isolated from *Salvia officinalis* (Lamiaceae) extract against VRE (vancomycin-resistant enterococci) with MIC value of 8 mg $mL^{-1}$ (Horiuchi *et al*. 2007). This result was confirmed for the reference strain, *Enterococcus faecalis* ATCC 28212 (Fontanay *et al*., 2008). Olean-27-carboxylic acid-type

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triterpenes extracted from the root portion of *Aceriphyllum rossi* (Saxifragaceae) which is a staple food in Korea, exhibited potent antibacterial activity against several strains of MRSA (methicillin-resistant *S. aureus*) and quinolone-resistant *Staphylococcus aureus* (Zheng *et al*., 2008). This result indicated that both the carboxylic group at C-27 and the hydroxyl group at C-24 in aceriphyllic acid are critical for the strong bactericidal activity. OA isolated from the Argentinian legume *Caesalpinia paraguarensis* (Fabiaceae) exhibited good activity against *Bacillus subtilis*, with MICs of 8 mg $mL^{-1}$ (Woldemichael *et al*., 2003). Oleanolic acid was also shown to be more active than its 3-O-monoglucoside and other glucosides and glucuronides, indicating that the aglycone structure appears necessary and sufficient for antibacterial activity (Szakiel *et al*., 2008). In this study, we report our findings on the effect of C-3 modification of oleanolic acid on some fungi and molds.

## MATERIALS AND METHODS

**Source of oleanolic acid:** The *Syzygium aromaticum* (Cloves) was purchased from an Indian spice shop in Durban, South Africa. It was properly authenticated by a plant Botanist, Mr. Pravin Poorun, a senior plant taxonomist of the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville Campus.

**Isolation of oleanolic acid (JH16):** The plant *Syzygium aromaticum* (3.0 kg) was macerated in ethylacetate (2x), it was filtered and concentrated *in vacuo*. The ethylacetate extract was then defatted using n-hexane (2x) the solid residue (10 g) was then packed into a silica gel column and eluted with ethylacetate: hexane (2:8) ration to afford a white amorphous solid which was identified as oleanolic acid (OA, JH16).

**Preparation of 3β-acetoxyoleanolic acid (JH23):** This was done according to a modified method of Li *et al*. (2009) (Scheme 1), as described; A mixture of oleanolic acid (1.0 g), acetic anhydride (10 mL) and N,N-dimethylaminopyridine (DMAP, 0.1 g) was stirred for 24 h at room temperature. It was then dissolved in water and filtered; the residue was washed with 10% hydrochloric acid to remove any excess DMAP. Afterward it was re-dissolved in dichloromethane (DCM) and water added and separated, the DCM portion was dried using anhydrous Sodium sulphate and concentrated *in vacuo*. It was kept aside to dry at room temperature, the solid product was recrystallized in methanol (MeOH) twice to give a white amorphous powder (540 mg, 54%).

**Spectroscopic analysis**

**Nuclear magnetic resonance (NMR):** The NMR spectroscopic analysis was carried out using Bruker Advance FT-NMR 400 MHZ spectrometer.

**Liquid chromatography-mass spectroscopy (LC-MS):** Liquid Chromatography Mass-spectroscopy (LC-MS) technique was used in the determination of the molecular mass of the compounds, using Agilent Technologies 1200 Series Binary SL (LC-MS) machine.

**Infra-red (IR):** The Infra-Red (IR) spectroscopy determination was carried out using Perkin Elmer Spectrum 100 FTIR Spectrometer.

**Biological analysis**

**Antifungal evaluation**

**Test organism:** The Clinical pathogenic yeast and mold used in the study; *Candida guielemondi*, *Candida albicans*, *Candida stelletoidea*, *Candida parapsilosis*, *Candida tropicalis*, *Candida pseudotropicalis*, *Candida krusei*, *Trichophyton tonsurans* and *Microsporum canis* were obtained from the Department of Medical Microbiology Ahmadu Bello University Teaching Hospital (ABUTH) Shika Zaria. All the Isolates were checked for purity and maintained in slants of Sabouraud Dextrose Agar (SDA).

**Antifungal assay:** The antifungal activity was carried out by utilizing the well-in-plate diffusion technique as reported by Karou *et al.* (2006). Pure culture of the organisms was inoculated on to Sabauroud Dextrose Agar (SDA) incubated for 24 h at 30°C. About 5 discrete colonies were aseptically transferred with a sterile wire loop into a tube containing sterile normal saline (0.85% NaCl) and were adjusted to a turbidity of 0.5 MacFarland standards. The suspension was used to streak on the surface of SDA plates with a sterile swap. A sterile 6 mm diameter cork borer was used to make holes into the set agar in the petri dishes containing the fungi culture. The wells were filled with $2.5\times10^{-3}$ μg $mL^{-1}$ concentrations of the compounds. Standard antifungal drugs *Fulconazole* ($2.5\times10^{-3}$ μg $mL^{-1}$), *Nystatin* ($2.5\times10^{-3}$ μg $mL^{-1}$) and *Fulcin* ($2.5\times10^{-3}$ μg $mL^{-1}$) were used as positive control. The plates were incubated for 1-7 days at 30°C. All the

tests were performed in triplicate and the antifungal activities were expressed as mean diameter of inhibition zones (mm) produced by the compounds and the standard drugs as shown in Table 2.

**Determination of minimum inhibitory concentration (MIC):** The Minimum Inhibitory Concentration (MIC) was carried out using the micro broth dilution technique in accordance with National Committee for Clinical Laboratory Standard (2006). Serial dilution of the concentration of the compounds and drug that showed activity was prepared using test tubes containing 9 mL of double strength broth. The tests tubes were inoculated with the suspension of the standardized inoculur and incubated at 30°C for 7 days. MIC was recorded as the lowest concentration of the compounds and drug showing no visible growth of the broth (Table 3, 4).

**Determination of minimum fungicidal concentration (MFC):** The Minimum Fungicidal Concentration (MFC) was determined by aseptically inoculating aliquots of culture from MIC tubes that showed no growth, on sterile nutrient agar plates and incubated at 30°C for 7 days. The MFC was recorded as the lowest concentration of the compounds and drug showing no colony growth as shown in Table 3, 4.

## RESULTS AND DISCUSSION

**Oleanolic acid (JH16):** White amorphous powder which was re-crystallized in MeOH, the melting point was recorded as 295-298°C; the Proton decoupled $^{13}C$ -NMR spectrum shows 30 Carbon atom (Table 1, Appendix 1), DEPT-90 (Appendix 1.1) subspectrum indicates five methine (CH) Carbon while DEPT-135 suggest 10 methylene ($CH_2$, negative), Seven methyl ($CH_3$, positive) groups. Quarternary carbon do not contained attached protons hence does not appear in the DEPT subspectrum, they may be identified as the signals which appear additionally in the proton broadband decoupled $^{13}C$ -NMR spectra. Therefore eight quaternary carbons were identified. The Electron impact mass spectroscopy (EI-MS, Appendix 1.2) showed a molecular ion $[M-Na]^+$ at m/z 479 which is in agreement with the formula mass 456.7 g $mol^{-1}$ for oleanolic acid. The Infra-Red (IR $V_{max}$, $cm^{-1}$, Appendix 1.3) Spectra showed absorption at 3384 this corresponds to (O-H) stretch for hydroxyl hydrogen bonded, the signal at 1686 is due to (C = O, acid) stretch of a carbonyl carbon this two support the Carboxylic acid (COOH) functional group at position C-28. The signal at 2937 is due to (C-H) stretch for an alkane this account for the high degree of saturation of the molecule. The signal at 1460 is due to ($CH_3$-, $CH_2$-). The assigned NMR spectra were in agreement with Literature value (Mahato and Kundu, 1994).

The results obtained from the well-in-plate diffusion method (Table 2) for the compound JH16 showed a marked antifungal activity as indicated by the inhibitory zone. *Candida stelletoidea* was the most sensitive organism with the largest inhibition zone (31±0.02) which was comparable with one of the positive control, *Fluoconazole* (30±0.33), *Nystatin* and *Fulcin* were resistant to the organism. The smallest inhibition zone was recorded for *Microsporum canis* (16±0.04) while for the rest of the organism the inhibition zone ranges from 19 to 23 mm as shown in Table 2. The result of the minimum inhibition concentration (MIC, Table 3) showed that a low concentration of $0.3125\times10^{-3}$ μg $mL^{-1}$ inhibited the growth of *Candida stelletoidea* while a concentration of $0.625\times10^{-3}$ μg $mL^{-1}$ inhibited the growth of *C. parapsilosis and C. pseudotropicalis* while $1.25\times10^{-3}$ μg $mL^{-1}$ inhibited the rest of the organism. The minimum fungicidal concentration (MFC, Table 3)

Table 1: NMR data of JH16 and JH23

| Carbon position | $\delta_c$ (ppm) JH16 | δc (ppm) JH23 |
|---|---|---|
| 1 | 38.37 | 39.27 |
| 2 | 27.09 | 23.52 |
| 3 | 78.61 | 80.94 |
| 4 | 38.69 | 37.69 |
| 5 | 55.16 | 55.28 |
| 6 | 18.28 | 17.17 |
| 7 | 33.08 | 33.78 |
| 8 | 39.25 | 38.05 |
| 9 | 47.55 | 47.54 |
| 10 | 36.96 | 36.99 |
| 11 | 22.96 | 23.39 |
| 12 | 122.06 | 122.55 |
| 13 | 144.00 | 143.61 |
| 14 | 41.65 | 40.90 |
| 15 | 28.10 | 25.90 |
| 16 | 23.29 | 32.44 |
| 17 | 45.99 | 45.83 |
| 18 | 40.31 | 41.54 |
| 19 | 46.15 | 46.54 |
| 20 | 30.63 | 32.51 |
| 21 | 33.85 | 33.06 |
| 22 | 32.41 | 28.04 |
| 23 | 28.10 | 22.86 |
| 24 | 15.64 | 18.17 |
| 25 | 15.27 | 15.34 |
| 26 | 17.00 | 16.65 |
| 27 | 25.85 | 23.57 |
| 28 | 180.44 | 184.10 |
| 29 | 33.08 | 30.66 |
| 30 | 23.56 | 27.66 |
| 1’ | - | 171.07 |
| 2’ | - | 21.31 |

Table 2: Zone of inhibition of the compounds against the fungi (mm)

| Test organism | JH16 | JH23 | FZ | NY | FL |
|---|---|---|---|---|---|
| *C. guielemondi* | 22±0.03 | 23±0.04 | 20 ±0.20 | R | R |
| *C. stelletoidea* | 31±0.02 | 24±0.01 | 30±0.33 | R | R |
| *C. albicans* | 21± 0.12 | 29±0.05 | 20±0.50 | 17±0.025 | R |
| *C. Krusei* | 23±0.01 | 26±0.04 | 21±0.05 | 14±0.05 | R |
| *C. parapsilosis* | 20±0.05 | 19±0.01 | 24±1.5 | 20±0.25 | 17±0.33 |
| *C. Tropicalis* | 23±0.01 | 30±0.02 | 21±0.80 | R | R |
| *C. pseudotropicalis* | 27±0.05 | 22±0.03 | 21±0.035 | R | R |
| *T. tonsurans* | 19±0.02 | 29±0.03 | 14±0.90 | 17±0.90 | 23±0.50 |
| *M. canis* | 16±0.04 | 30±0.01 | R | R | 24±0.05 |

FZ: Fluoconazole, NY: Nystatin, FL: Fulcin (FZ, NY, FL: Positive control), R: Resistant

Table 3: Minimum inhibition and fungicidal concentration (MFC and MIC) of JH16

| | Concentration ($\times10^{-3}$ µg mL$^{-1}$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | | | | | MFC | | | | |
| Test organism | 2.50 | 1.25 | 0.625 | 0.312 | 0.156 | 2.50 | 1.25 | 0.625 | 0.312 | 0.156 |
| *C. guielemondi* | - | ** | + | ++ | +++ | * | + | ++ | ND | ND |
| *C. stelletoidea* | - | - | - | ** | + | * | + | ++ | ND | ND |
| *C. albicans* | - | ** | + | ++ | +++ | - | * | + | ND | ND |
| *C. Krusei* | - | ** | + | ++ | +++ | * | + | ++ | ND | ND |
| *C. parapsilosis* | - | - | ** | + | +++ | * | + | ++ | ND | ND |
| *C. tropicalis* | - | - | ** | + | ++ | * | + | ++ | ND | ND |
| *C. pseudotropicalis* | - | - | ** | + | ++ | - | * | + | ND | ND |
| *T. tonsurans* | - | ** | + | ++ | +++ | * | + | ++ | ND | ND |
| *M. canis* | - | ** | + | ++ | +++ | * | + | ++ | ND | ND |

ND: Not determine, **: MIC, -: No growth, *: MFC, +: Slight growth, ++: Dense growth, +++: Very dense growth

Table 4: Minimum inhibition and fungicidal concentration (MIC and MFC) of JH23

| | Concentration ($\times10^{-3}$ µg mL$^{-1}$) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MIC | | | | | MFC | | | | |
| Test organism | 2.50 | 1.25 | 0.625 | 0.312 | 0.156 | 2.50 | 1.25 | 0.625 | 0.312 | 0.156 |
| *C. guielemondi* | - | - | ** | + | ++ | * | + | ++ | +++ | ND |
| *C. stelletoidea* | - | - | ** | + | ++ | * | + | ++ | +++ | ND |
| *C. albicans* | - | ** | + | ++ | +++ | * | + | ++ | +++ | ND |
| *C. Krusei* | - | ** | + | ++ | +++ | * | + | ++ | +++ | ND |
| *C. parapsilosis* | - | - | ** | + | ++ | * | + | ++ | +++ | ND |
| *C. tropicalis* | - | * | + | ++ | +++ | * | + | ++ | +++ | ND |
| *C. pseudotropicalis* | - | - | ** | + | ++ | * | + | ++ | +++ | ND |
| *T. tonsurans* | - | - | ** | + | ++ | - | * | + | ++ | ND |
| *M. canis* | - | ** | + | ++ | +++ | * | + | ++ | +++ | ND |

-: No growth, *: Mic, +: Slight growth, ++: Dense growth, +++: Very dense growth

results showed that JH16 did not only inhibited the growth of the test organism it was also able to kill them; a low concentration of $0.3125\times10^{-3}$ µg mL$^{-1}$ of JH16 was able to completely kill *Candida stelletoidea*, a concentration of $0.625\times10^{-3}$ µg mL$^{-1}$ was able to kill *C. parapsilosis* and *C. pseudotropicalis*. The rest of the organisms were completely kill at a concentration of $1.25\times10^{-3}$ µg mL$^{-1}$.

**3-acetyl-oleanolic acid (JH23):** White amorphous powder re-crystallized in Hexane, the melting point was recorded as 230-233°C. The Proton decoupled $^{13}$C -NMR spectrum shows 32 Carbon atoms (Table 1, Appendix 2.0), DEPT-90 (Appendix 2.1) subspectrum showed five methine (CH) Carbon while DEPT-135 showed 10 methylene ($CH_2$, negative) and eight methyl ($CH_3$, positive) groups. Nine quaternary carbons were identified in the signals which appear additionally in the proton broadband decoupled $^{13}$C -NMR spectra. The electron impact mass spectroscopy (EI-MS) gave a molecular ion $[M+Na]^+$ for $C_{32}H_{50}O_4Na$ at m/z 521.2 (Appendix 2.2). Hence the molecular mass was determined as 498.2 which were in agreement with the formula mass 498 gmol$^{-1}$. The IR-spectra ($IR_{Vmax}$ Cm$^{-1}$; Appendix 2.3) showed absorption at 3562 which corresponds to (O-H) stretch for hydroxyl hydrogen bonded, the signals at 1767 is due to a Carbonyl carbon (C = O, ester) while 1729 is due to (C = O, acid). The signal at 2921 is due to (C-H) stretch for an alkane

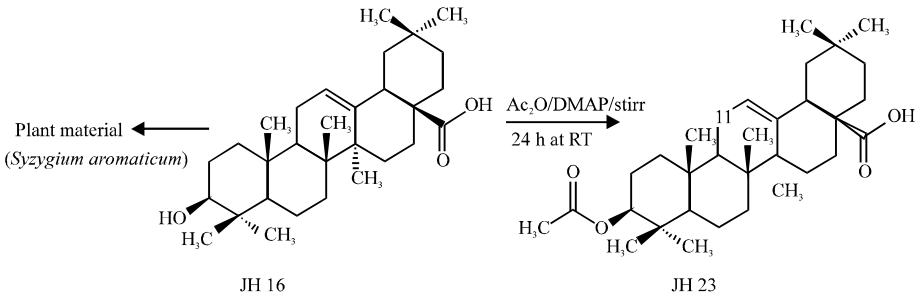



Fig. 1: Isolation of oleanolic and preparation of 3β-acetoxy-oleanolic acid

Table 5: Summary of MIC and MFC results ($\times 10^{-3}$ μg mL$^{-1}$)

| | MIC | | | MFC | | |
|---|---|---|---|---|---|---|
| Test organism | FZ | JH16 | JH23 | FZ | JH16 | JH23 |
| *C. guielemondi* | 1.25 | 1.25 | 0.63 | 1.25 | 2.50 | 2.50 |
| *C. stelletoidea* | 0.63 | 0.31 | 0.63 | 1.25 | 2.50 | 1.25 |
| *C. albicans* | 2.50 | 1.25 | 1.25 | 2.50 | 1.25 | 2.50 |
| *C. Krusei* | 2.50 | 1.25 | 1.25 | 1.25 | 2.50 | 2.50 |
| *C. parapsilosis* | 1.25 | 0.63 | 0.31 | 0.63 | 2.50 | 0.63 |
| *C. Tropicalis* | 0.63 | 0.63 | 1.25 | 0.63 | 0.63 | 1.25 |
| *C. pseudotropicalis.* | 1.25 | 0.63 | 1.25 | 0.63 | 2.50 | 2.50 |
| *T. tonsurans* | 2.50 | 1.25 | 0.63 | 2.50 | 2.50 | 1.25 |
| *M. canis* | ND | 1.25 | 0.63 | ND | 2.50 | 2.50 |

ND: Not determined, FZ: Fluoconazole (standard drug)

which account for the high degree of saturation of the molecule. The signal at 1453 is due to ($CH_3$-, $CH_2$-), 1630 is due to (C = C) and 1006 corresponds to (C-O).

The sensitivity test results (Table 2) showed that *C. tropicalis* and *M. canis* were the most sensitive organism with the largest zone of inhibition (30±0.01 and 30±0.02, respectively) which were observed to be greater than the standard, Fluoconazole (21±0.8) against *C. tropicalis* while it shows resistance for *M. canis*. The smallest inhibition zone was recorded for *C. parapsilosis* (19±0.01) while for the same organism the standard drugs had higher values; *Fuoconazole* (24±1.5), *Nystatin* (20±0.25) and *fulcin* (17±0.33). The MIC and MFC result (Table 3, 4) showed that *C. parapsilosis* was inhibited and completely killed by a low concentration of 0.3125×10 and $0.625\times10^{-3}$ μg mL$^{-1}$, respectively. A concentration of $0.625\times10^{-3}$ μg mL$^{-1}$ inhibited the growth of *C. stelletoidea* and *T. tonsurans* and complete kill the organism at $2.5\times10^{-3}$ μg mL$^{-1}$. *M. canis*. and *C. guielemondi* were inhibited at a concentration of $0.625\times10^{-3}$ μg mL$^{-1}$ and completely killed at $2.5\times10^{-3}$ μg mL$^{-1}$ while the rest of the organisms were inhibited at a concentration of $1.25\times10^{-3}$ μg mL$^{-1}$ and killed at a concentration of $2.5\times10^{-P3}$ μg mL$^{-1}$ (Table 5). The result of this investigation clearly shows that modification of oleanolic acid on the C-3 position led to enhanced biologically active compound (Fig. 1).

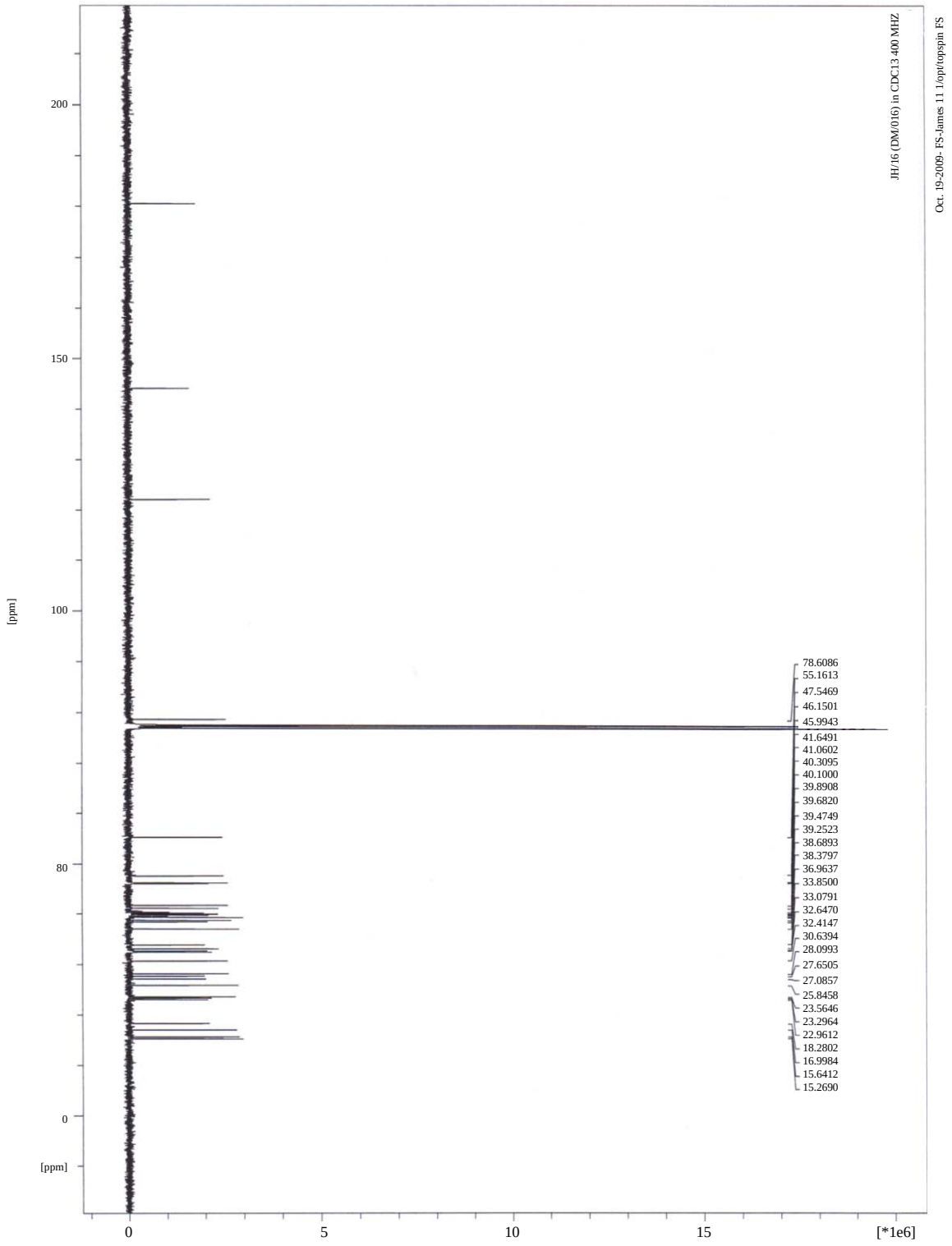
## CONCLUSION

The introduction of an acetyl group on the C-3 position of oleanolic acid led to an enhanced biological activity against most of the test organism. The inhibition zone determination showed that compound JH23 had larger values as compared to JH16 the parent compound; *C. guielemondi* (JH23, 23±0.04; JH16, 22±0.03), *C. albicans* (JH23, 29±0.05; JH16, 21±0.12), *C. krusei* (JH23, 26±0.04; JH16, 23±0.001), *C. tropicalis* (JH23, 30±0.02; JH16, 23±0.01), *T. tonsurans* (JH23, 29±0.03; JH16, 19±0.02) and *M. canis* (30±0.01; JH16, 16±0.04). The MIC and MFC determination results showed that JH23 did not only inhibited the test organism but was able to completely kill them at a concentration range of $0.31\times10$ to $2.5\times10^{-3}$ μg mL$^{-1}$ as compared to JH16 and the standard drug "Fluoconazole" used as positive control. Hence, has possibility that could be explore in the development of lead compound.

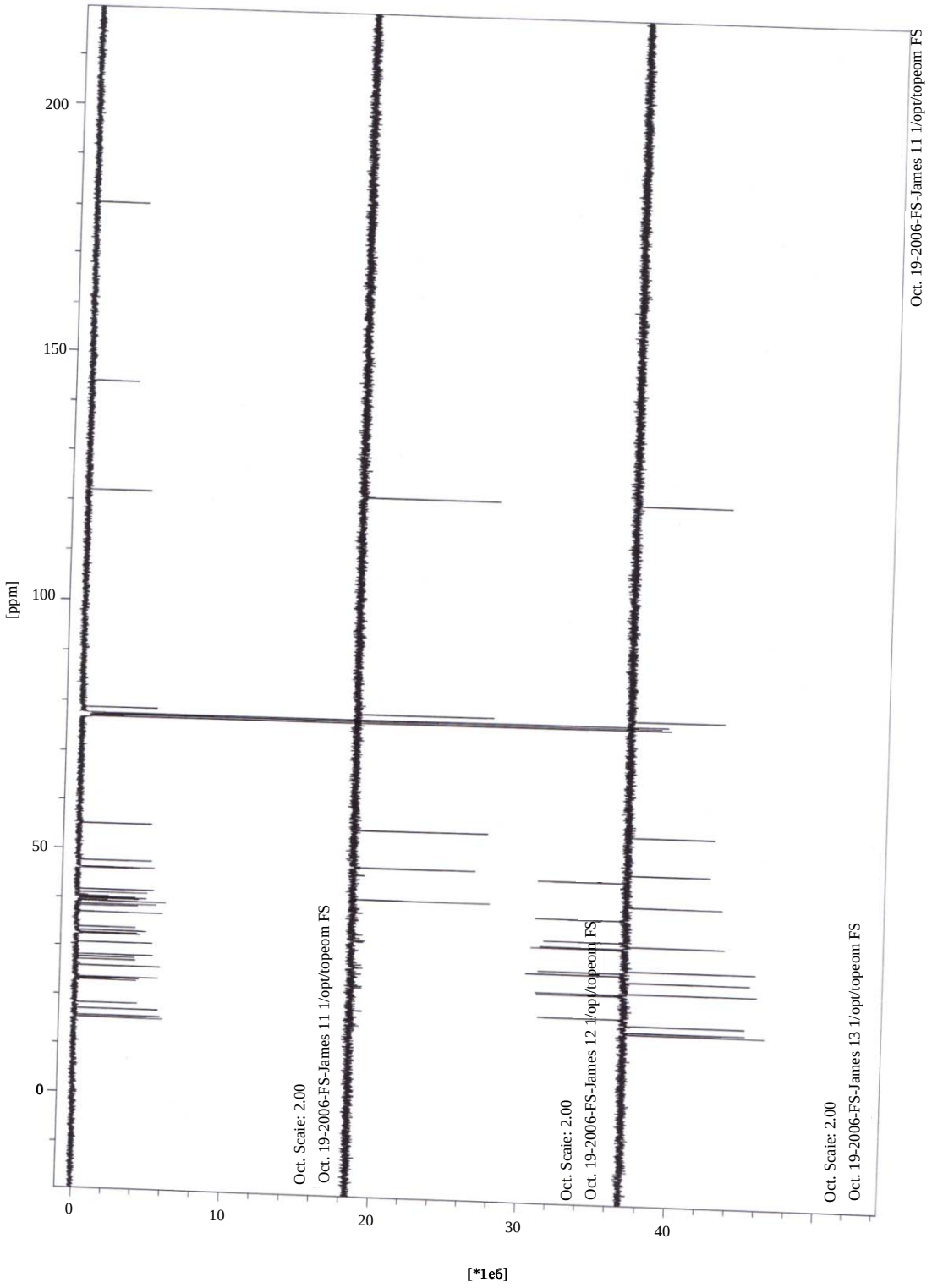
## ACKNOWLEDGMENT

We acknowledge the financial contribution of University of Kwazulu-Natal Durban, Westville campus, South Africa and Ahmadu Bello University Zaria, Nigeria and for providing facilities to carry out this research.

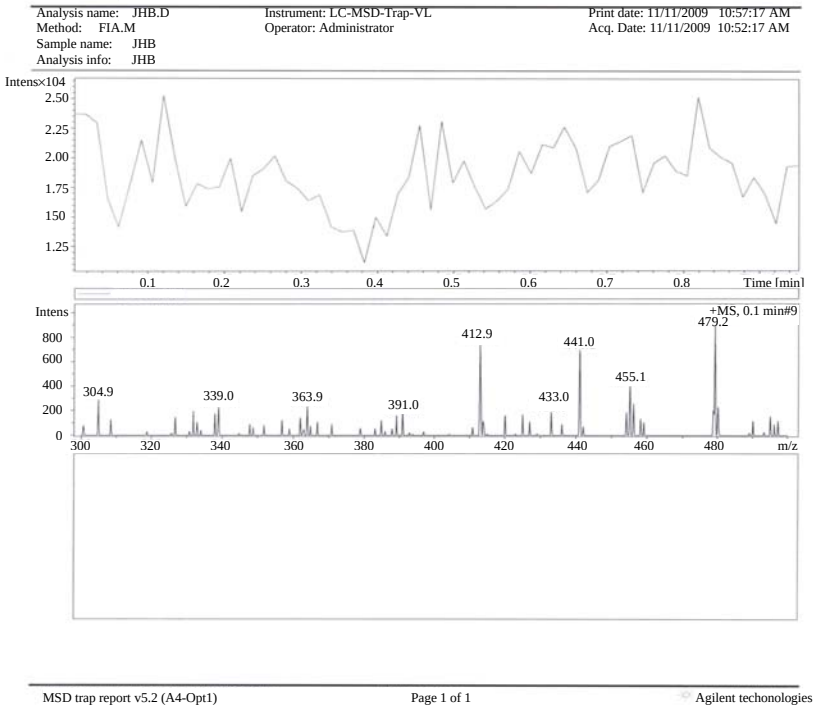
## APPENDIXES 1: $^{13}$C-NMR OF JH16



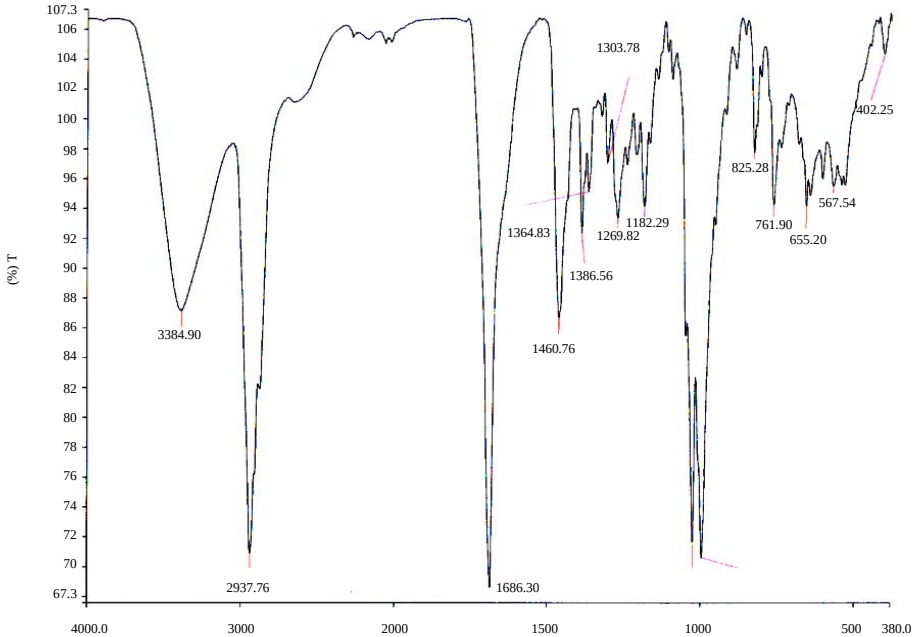
Appendix 1.1: Dept experiment of JH16



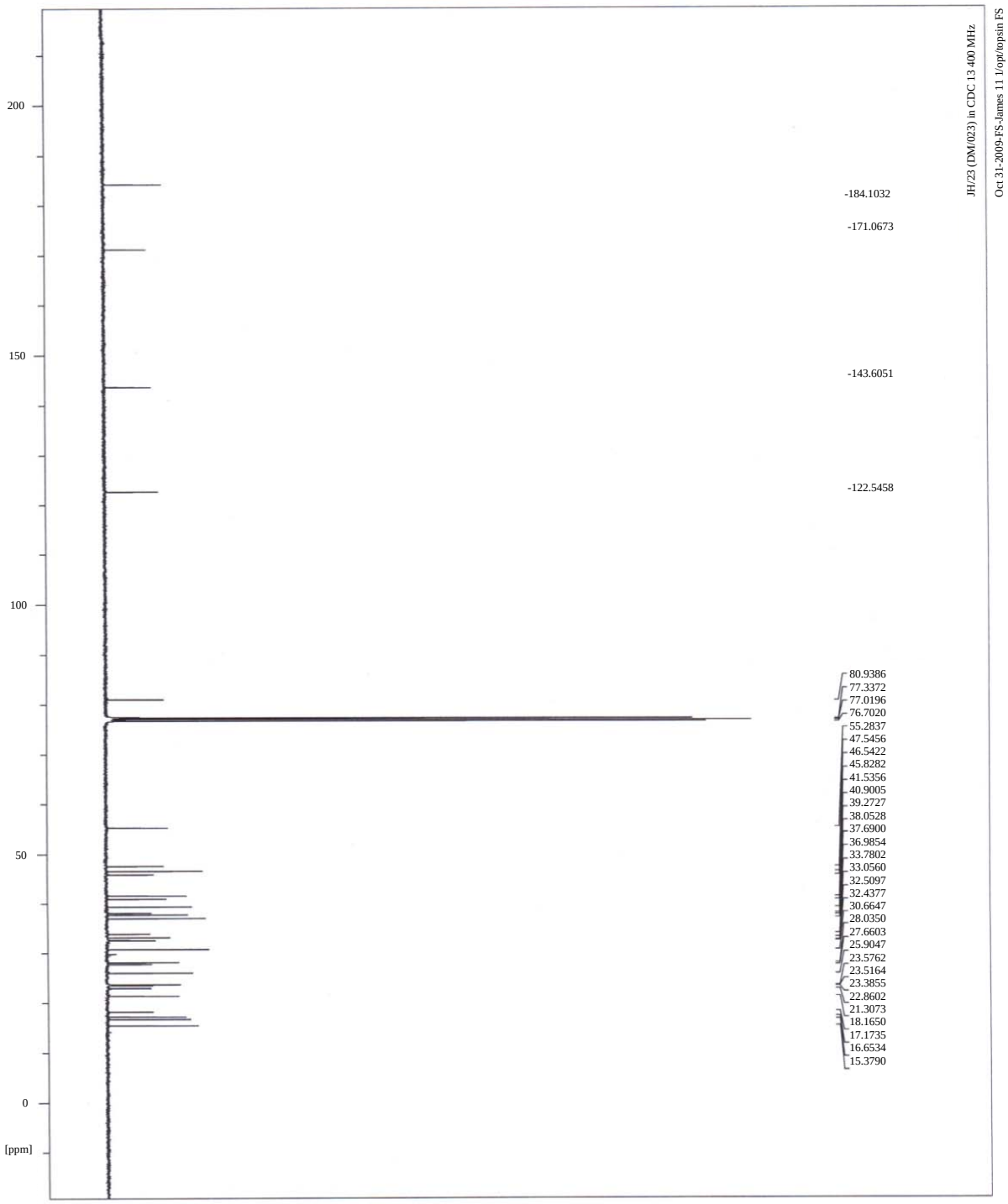
Appendix 1.2: LC-MS of JH16



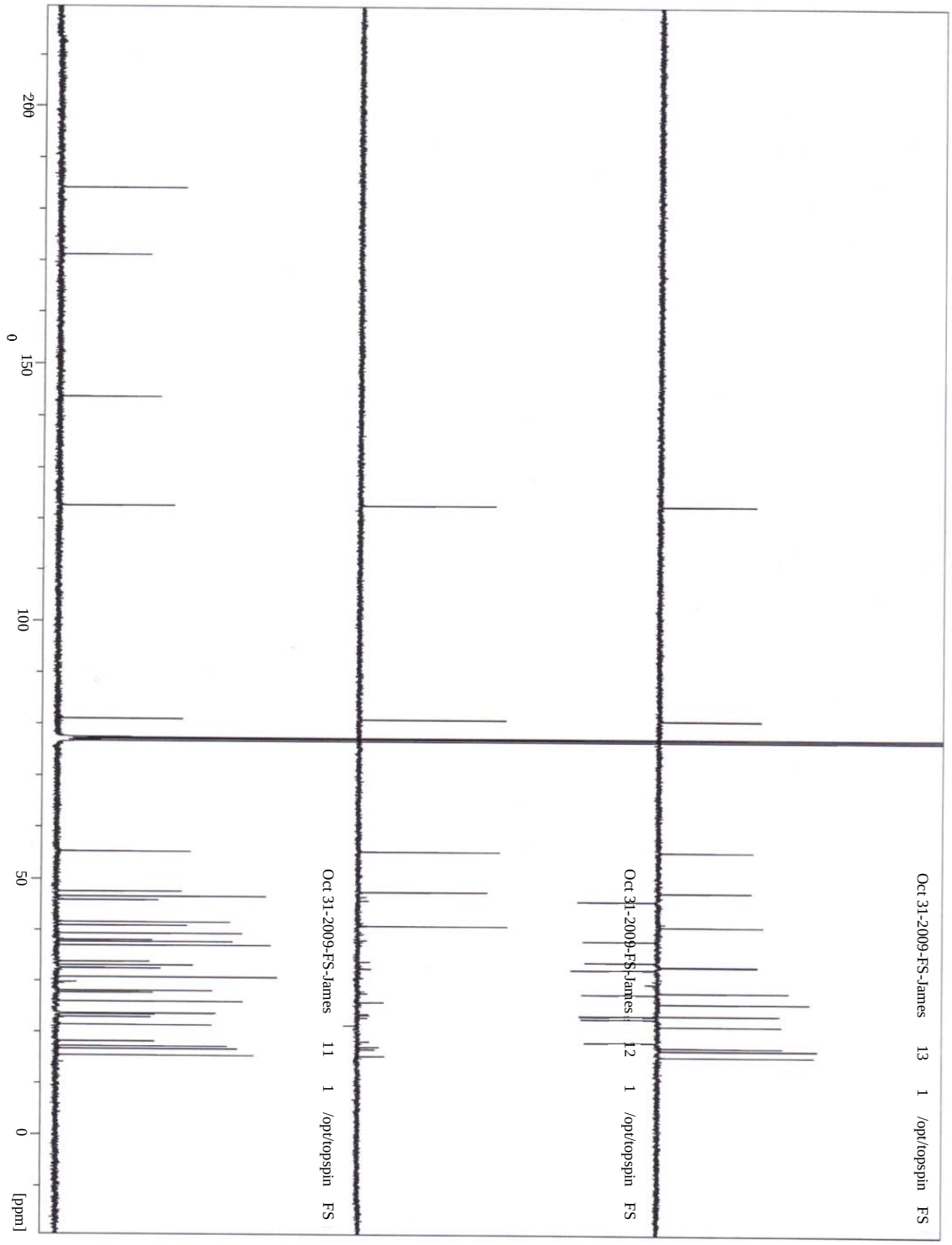

Appendix 1.3: IR-Spectra of JH16



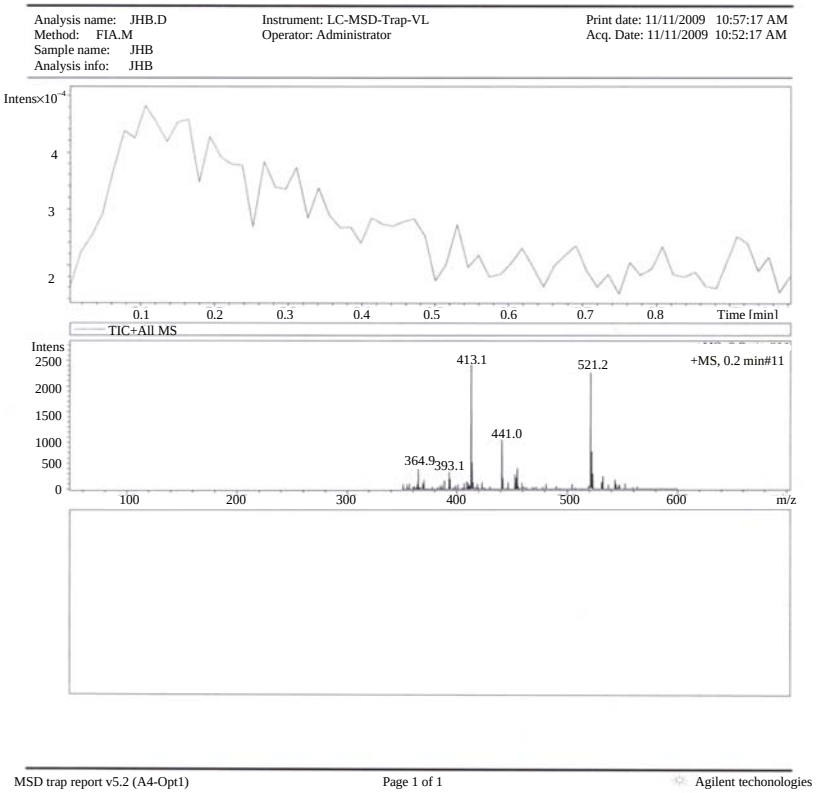
Appendix 2: $^{13}$C-NMR of JH23



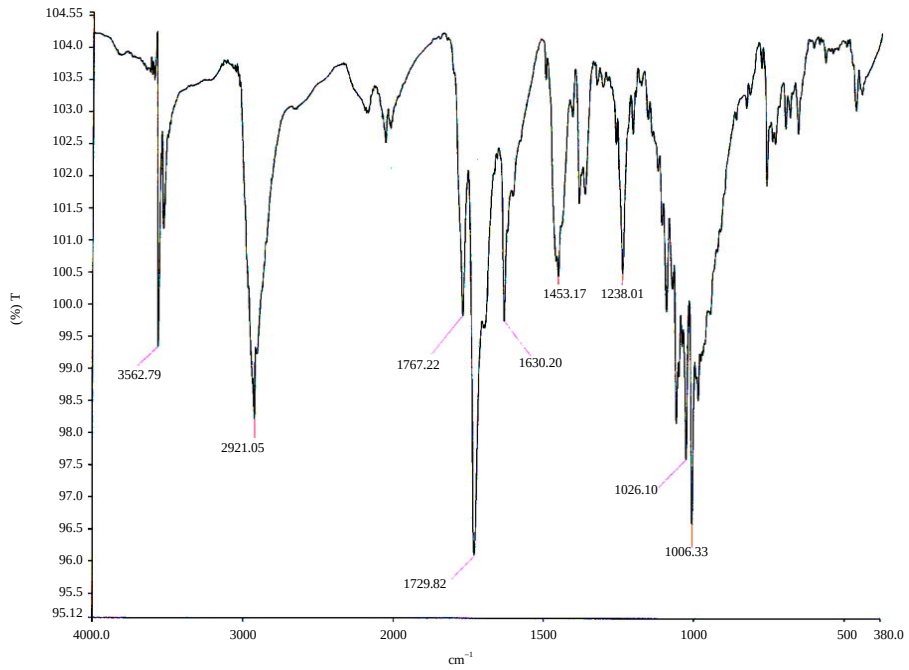
Appendix 2.1: Dept experiment of JH23



Appendix 2.2: LC-MS of JH23




Appendix 2.3: IR-spectra of JH23



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