ISOLATION, STRUCTURE ELUCIDATION, IDENTIFICATION AND QUANTITATIVE ANALYSIS OF 1'-ACETOXYCHAVICOL (ACA) FROM THE ROOTS OF *CHLOROPHYTUM BORIVILIUANUM (SAFED MUSLI)*

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Abstract

Chlorophytum borivilianum (safed musli) is a medicinally important plant. Its roots are being employed in folk medicine. Presently, the crude extract of *C. borivilianum* has been consumed for the treatment such as anti-diabetic, anti-aging, anti-oxidant, anti-ulcer and anti-inflammatory and previous studies have been carried out to further confirm these remarkable bioactivities of *C. borivilianum*. In this research, 1'-acetoxychavicol acetate (ACA) was isolated from the roots of *C. borivilianum*. The structure of ACA was elucidated based on the spectral data of ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMBC, HMQC and also based on the comparison with the previous literature data. ACA was isolated in an isocratic elution that eluted with hexane and ethyl acetate in the ratio of 10:0.25. In the HPLC analysis, the separation of the crude methanol extract was completed within 20 min and the retention time of ACA in the sample was 7.31 min. The regression equation of the calibration curve was developed and the correlation coefficient was found to be 0.991. This is the first report regarding the presence of ACA in *C. borivilianum* as well as its genus. For the first time, a high performance liquid chromatographic (HPLC) method with photodiode array detection was developed for the quantitative determination and identification of ACA.

Keywords: *Chlorophytum borivilianum*, Isolation, 1'-acetoxychavicol acetate (ACA), Structure elucidation, Quantification.

Nomenclatures

d	Doublet
J	Coupling constant
m	Multiplet
q	Quartet
R^2	Correlation coefficient
s	Singlet
t	triplet

Greek Symbols

δ	Chemical shift.

Abbreviations

1D, 2D	One-Dimensional, Two-Dimensional
ACA	1'-acetoxychavicol acetate
^{1}H NMR	Proton nuclear magnetic resonance
NMR	Nuclear Magnetic Resonance
CBA	Aqueous extract
CBC	Chloroform extract
CBH	Hexane extract
CD_3OD	Deuterated methanol
^{13}C NMR	Carbon nuclear magnetic resonance
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum correlation
HPLC	High performance liquid chromatography
maU	Milliabsorbance units
PDA	Photodiode array
ppm	Parts per million
PVDF	Polyvinylidene difluoride
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultra violet spectroscopy

1. Introduction

The plant *Chlorophytum borivilianum* (safed musli) is a medicinal plant belonging to family Liliaceae. *C. borivilianum* holds an important place in the traditional medicinal system due to its therapeutic importance. The economic part of the herb is its roots. Its roots are powdered and widely used in traditional folk medicines over past decades. *C. borivilianum* is a native Indian plant which is a part of an important class of ayurvedic herbs which are known as Rasayana. Rasayana constitutes of herbs with immunostimulatory and adaptogenic properties [1-3]. It is widely growing due to its versatile therapeutic uses and it is a chief ingredient in Ayurvedic, Unani, Homeopathic and Allopathic systems of medicine, where root of the plant holds principal place [2, 4, 5].

It was reported that the extracts of *C. borivilianum* and compounds isolated from the corresponding plant possess antioxidant [6], immunomodulatory [7], anti-

diabetic [8], anti-ulcer [9], analgesic [8], anti-microbial [10], anti-stress [11], anti-bacterial [12] and anti-arthritic [13] effects.

Previous studies were carried out regarding the isolation of the chemical constituents from the plant *C. borivilianum* due to its remarkable pharmacological activities. The studies revealed that the isolated chemical constituents are mainly saponins [13-16]. Interest is increasing to exploit this herbal plant for the development of therapeutics as it was reported that the plant was proven to possess aphrodisiac [6, 17] and anti-cancer activities [18]. Therefore, the extracts of *C. borivilianum* could be potentially used as sexual stimulant for impotence and to prevent or treat cancers. There are extensive studies reported on the biological activities of the crude extract of the roots of *C. borivilianum* in the past and there were only few studies had been done on the isolation of chemical constituents from the corresponding plant other than saponins.

The objectives of this paper are to isolate, elucidate, identify and quantify ACA from the roots of *C. borivilianum*. For this purpose, firstly, the isolation method of 1'-acetoxychavicol acetate (ACA) from *C. borivilianum* roots is established and this is the first report regarding the presence and isolation of ACA in the *Chlorophytum* genus. Secondly, an efficient HPLC method is first developed for the quantitative determination of ACA in the crude extract using the isolated ACA as standard marker and the quantitative analysis of ACA has not been previously reported. It could be a starting point to pave a way to isolate and quantify other pure compounds from this herbaceous plant in order to use it as a tool for quality control and also for the future development of other therapeutic applications.

2. Experimental Section

2.1. Plant material

Fresh roots of *C. borivilianum* (safed musli) were procured from University Teknologi Malaysia (UTM), Malaysia in January, 2013. The roots were dried in a drying oven (Model UFE-800, Memmert, Germany) at 40°C. The dried roots were then ground and sieved to pass through 60-mesh sieve.

2.2. Chemicals

Methanol, hexane and chloroform used were of analytical grade. Compound was visualized with 10% sulphuric acid reagent. Deuterated methanol was used for NMR experiments. HPLC grade acetonitrile, methanol and formic acid were purchased from Merck, Germany. Water was deionized (M) using a Milli-Q water purification system (Millipore, Bedford, MA). Solvents were filtered through a 0.45 μm Millipore membrane syringe filter (diameter: 17 mm, porosity: 0.45 μm, PVDF membrane, Whatman, USA). Stock solution (1 mg/mL) was freshly prepared in methanol. Standard solutions at seven concentrations (2, 4, 6, 8, 10, 12, 14 ppm) were prepared by a series of dilutions with methanol at a final volume of 1 mL.

2.3. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectroscopy was performed using a JEOL (Japan Electronic Optics Laboratory Co. Ltd., Tokyo, Japan) ECX 500MHz Fourier transform NMR spectrometer system (500 MHz) operating at 500MHz for ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMBC and HMQC experiments at Chemistry Department, Faculty of Science, Universiti Putra Malaysia. One-dimensional (1D) and two-dimensional (2D) experiments were performed with Delta NMR Processing and Control Software (V4.3.6, JEOL USA, Inc.). Isolated compound was dissolved in deuterated methanol (CD$_3$OD) and TMS was used as internal standard. The chemical shifts are reported in δ (ppm) and are referenced to the solvent peaks CD$_3$OD at δ_H 3.21, 4.79 and δ_C 49.3 ppm for ^{1}H and ^{13}C, respectively. HPLC analysis was performed with a system of HPLC equipped with Waters 600E system controller, Waters 996 sintered glass Büchner filter funnel, Waters online degasser, Waters 717 plus auto-sampler and column oven. The chemical compounds that pass through HPLC column was detected by Water 996 photodiode array (PDA) detector and the data was sent to the attached computer for analysis. Chromatographic separation was performed on a Phenomenex Luna C$_{18}$ 100A column (250 mm x 4.6 mm, 5 μm particle size, USA) as stationary phase with 0.1% formic acid and acetonitrile in a ratio of 40:60 (v/v) as isocratic mobile phase at a flow rate of 1 mL/min. The injection volume was 10 μL and the detection wavelength was 200 nm.

2.4. Extraction, partition and isolation of ACA

Solid-liquid extraction was employed as first step in the recovery and purification of ACA from the roots of *C. borivillianum*. 250 g of coarsely ground roots of *C. borivilianum* was macerated with 2.5L of fresh methanol and left to stand at room temperature for a period of three days. The mixture was agitated occasionally with a stirring rod. The resulting extract was filtered and concentrated to dryness under reduced pressure at a constant temperature of 40°C in a rotary evaporator to remove methanol while the residue was subjected to the same procedure thrice for proper extraction and the combined methanol extract was dried in a desiccators and yielded 24 g of crude methanol extract. The methanol extract was partitioned sequentially with water, hexane and chloroform and this afforded three partition fractions which were encoded as CBA, CBH and CBC for the extracts of aqueous layer, hexane layer and chloroform layer, respectively. The hexane extract (CBH) was chromatographed over a silica gel column (200-400 mesh, Merck, Germany) utilizing hexane and ethyl acetate as a mobile phase. The column was first eluted with hexane and then eluted sequentially with the mixture of hexane and ethyl acetate in the ratio of 10:0.10, 10:0.15, 10:0.20, and 10:0.25, v/v, affording 16 fractions (CBH1-CBH16). Thereafter, CBH1 was subjected to silica gel column chromatography eluted with hexane and ethyl acetate in an isocratic elution in the ratio of 10:0.25, v/v to afford 7.6 mg of ACA. The structure of the isolated ACA was identified by the comparison of NMR spectral data with those in the literature. The purity of isolated ACA was determined to be above 98% by HPLC analysis.

2.5. TLC analysis

Thin-layer chromatography was performed on the TLC precoated silica gel F$_{254}$ plates (0.2 mm thick, Merck, Germany) 5cm x 5 cm, using hexane/ ethyl acetate

in ratio 10:1 as mobile phase. The visualization method of TLC involved UV light at 254 and 365 nm, after spraying with 10% sulphuric acid, and then the spots were visualized in daylight after heating on a hot plate for 5 min.

2.6. HPLC analysis

The previously isolated ACA from the roots of *C. borivilianum* was used as the standard ACA. The stock solution of standard ACA was prepared in methanol at a concentration of 10 mg/mL. The working standard solution was prepared by further diluting 50 μL of stock solution with 950 μL of methanol to give a final concentration of 500 ppm. Solution used for calibration curve was prepared by dilution of the stock solution. For method development, standard working solutions were prepared by a series of dilutions (2, 4, 6, 8, 10, 12 and 14 ppm) with methanol at a final volume of 1 mL. A seven-points standard calibration curve of standard ACA with linear relationship between the peak area at the Y-axis and the concentration of standard ACA injected (ppm) at the X-axis was generated in the concentration range of 2 to 14 ppm of standard ACA. The injection volume of the filtrate was 10 μL, each with three replicates and the flowrate was 1.0 mL/min for a total run time of 20 min. The UV wavelength was scanned over the range of 190-400 nm for the presence of ACA.

3. Results and Discussion

The main aim in this study was to isolate, elucidate and quantify ACA from the roots of *C. borivilianum*. The purification of the hexane extract yielded a pure compound and the structural elucidation was carried out by detailed interpretation of 1D and 2D NMR spectroscopic data. Based on NMR spectroscopy study, , the isolated compound was identified as 1'-acetoxychavicol acetate (ACA) and it was isolated from the *Chlorophytum* genus for the first time. The analytical data of ^{1}H NMR and ^{13}C NMR were comparable with those reported earlier in the literature review for ACA [19-21]. The purity of isolated ACA was confirmed by TLC and HPLC analysis. ACA exhibited 97% purity by HPLC. The structure of ACA is shown is Fig. 1. The complete spectroscopic data (^{1}H NMR, ^{13}C NMR, COSY, DEPT, HMQC, HMBC) for ACA are shown in Table A-1 and the respective spectrum are shown in Figs. A-1 to A-6 (*Appendix A*).
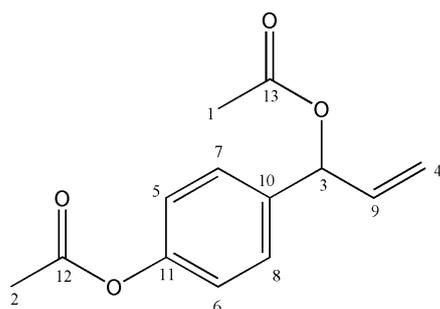

Fig. 1. Structure of 1'-acetoxychavicol acetate (ACA).

In the HPLC analysis, the isolated ACA was used as a standard marker for developing a quantitative analysis of ACA in the crude extract of *C. borivilianum*

roots. This was performed with a reversed phase HPLC system using the optimized HPLC conditions. In HPLC, different parameters such as solvent system, wavelength and flow rate were optimized in order to achieve good separation and resolution. A binary mobile phase composed of 0.1% formic acid as solvent A and acetonitrile as solvent B in a ratio of 40:60 (v/v) was employed after several trials with various elution systems and pushed through the column in an isocratic mode. A small amount of formic acid was added to enhance the resolution and eliminate the peak tailing. This mobile phase combination was selected through optimization for better separation of compounds and shorter analysis time. Isocratic HPLC elution was chosen because it showed satisfactory resolution of adjacent peaks. The separation of the crude methanol extract was completed within 20 min. ACA in the sample had a retention time of 7.31 min. The HPLC chromatogram of the crude extract recorded at 200 nm (Fig. 2) indicated that there were five components in crude extract at retention time range of 1-15 min.
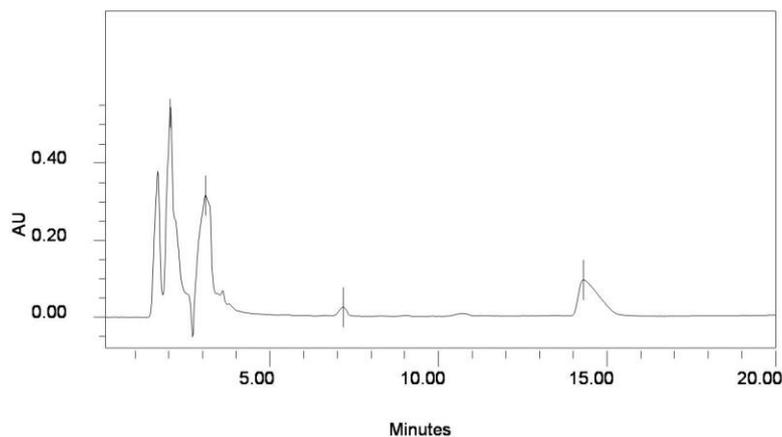

Fig. 2. HPLC chromatogram of methanolic extract of *C. borivilianum.*

After purification through partitioning and column chromatography, the purified ACA extract at retention time of 7.171 min from the roots of *C. borivilianum* recorded at 200 nm is shown in Fig. 3.
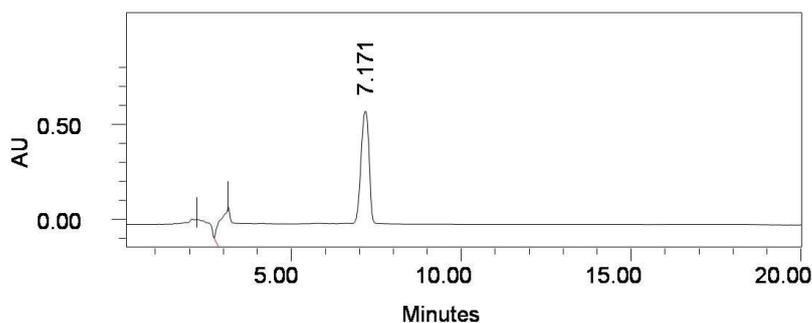

Fig. 3. HPLC chromatogram of standard ACA.

The developed method was validated for linearity. The linearity was evaluated using standard solution at six calibration points in the concentration of 2 to 14

ppm. Figure 4 presents the calibration curve of standard ACA. Calibration curve of standard ACA provided a linear relationship between the peak area (Y) in milliabsorbance units (maU) and the concentrations of standard ACA injected (X) in ppm.
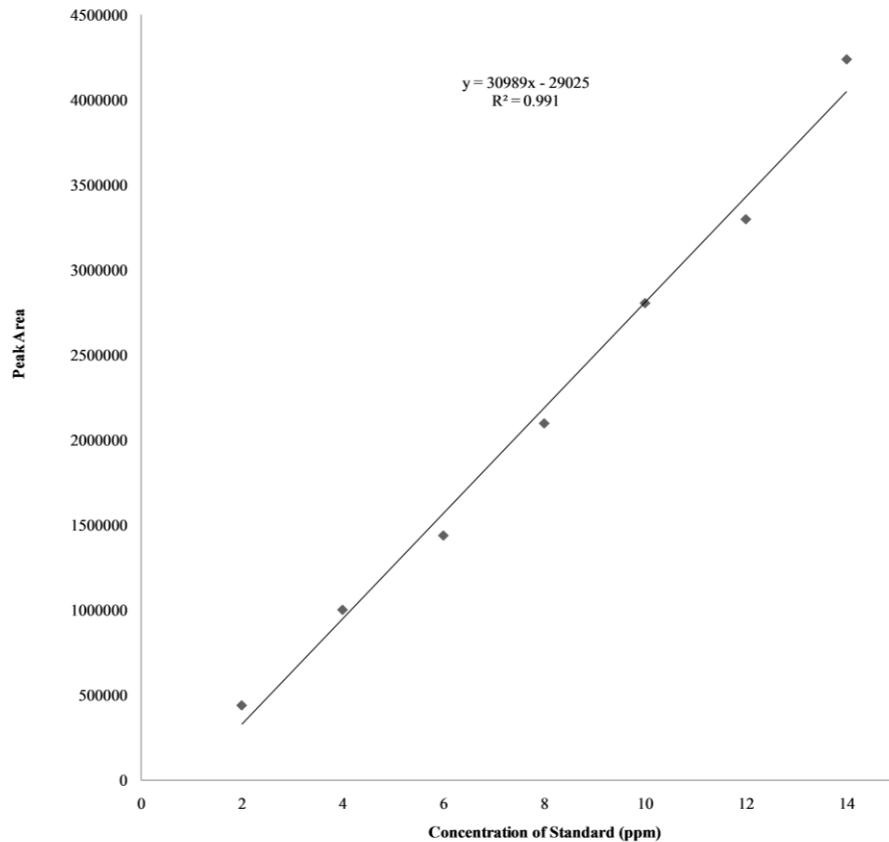

Fig. 4. Calibration curve of ACA

The regression equation of the calibration curve was linear and developed as shown in Eq. (1). The correlation coefficient (R^2) was found to be 0.991 for ACA calibration curve.

$Y = 30989X\text{-}29025$ (1)

4. Conclusions

The developed isolation method involved a simple solid-solvent extraction technique and further purification by partitioning and column chromatography. The developed isolation method was reported to be efficient at laboratory scale for the isolation of ACA. Since this extraction method is simple and rapid, it can be further optimized for future commercial extraction. Then, an isocratic HPLC method was developed for identification and quantitative analysis of ACA

presented in the roots of *C. borivilianum*. The developed HPLC method was found to be simple and it can be further validated for the standardisation of plant material containing ACA.

References

1. Thakur, M.; Connellan, P.; Deseo, M.A.; Morris, C.; and Dixit, V.K. (2011). Immunomodulatory polysaccharide from Chlorophytum borivilianum roots. *Evidence-Based Complementary and Alternative Medicine.*

2. Khanam, Z.; Singh, O.; Singh, R.; and Bhat, I. U.H. (2013). Safed musli (Chlorophytum borivilianum): A review of its botany, ethnopharmacology and phytochemistry. *Journal of Ethnopharmacology*, *150* (2), 421-441.

3. Singh, D.; Pokhriyal, B.; Joshi, Y.; and Kadam, V. (2012). Phytopharmacological aspects of Chlorophytum borivilianum (safed musli): A review. *International Journal of Pharmaceutical Chemistry Research,* 2(3), 853-859.

4. Satija, J.; and Singh, G. (2005). Safed musli: The golden root. *Indian Journal of Pharmaceutical Education*, 39(4), 180-185.

5. Thakur, G.S.; Bag, M.; Sanodiya, B.S.; Debnath, M.; Zacharia, A.; Bhadauriya, P.; Prasad, G.B.K.S.; and Bisen, P.S. (2009). Chlorophytum borivilianum: A white gold for biopharmaceuticals and neutraceuticals. *Current Pharmaceutical Biotechnology*, 10(7), 650-666.

6. Govindarajan, R.; Sreevidya, N.; Vijayakumar, M.; Thakur, M.; Dixit, V.K.; Mehrotra, S.; and Pushpangadan, P. (2005). In vitro antioxidant activity of ethanolic extract of Chlorophytum borivilianum. *Natural Product Sciences*, 11(3), 165-169.

7. Thakur, M.; Connellan, P.; Deseo, M.A.; Morris, C.; and Dixit, V.K. (2011). Immunomodulatory polysaccharide from Chlorophytum borivilianum roots. *Evidence-Based Complementary and Alternative Medicine.*

8. Panda, S.K.; Si, S.C.; and Bhatnagar, S.P. (2007). Studies on hypoglycaemic and analgesic activities of Chlorophytum borivilianum Sant & Ferz. *Journal of Natural Remedies*, 7(1), 31-36.

9. Panda, S.K.; Das, D.; and Tripathy, N.K. (2011). Studies on anti-ulcer activity of root tubers of Chlorophytum borivilianum santapau & fernandes. *International Journal of Pharmaceutical Sciences Review and Research*, 9(2), 65-68.

10. Chakraborthy, G.S.; and Aeri, V. (2009). Phytochemical and antimicrobial studies of Chlorophytum borivilianum. *International Journal of Pharmaceutical Sciences and Drug Research*, 1, 110-112.

11. Deore, S.; and Khadabadi, S. (2009). Screening of antistress properties of Chlorophytum borivilianum tuber. *Pharmacologyonline*, 1, 320-328.

12. Sundaram, S.; Dwivedi, P.; and Purwar, S. (2011). Antibacterial activities of crude extracts of Chlorophytum borivilianum to bacterial pathogens. *Research Journal of Medicinal Plant*, 5(3), 343-347.

13. Deore, S. L.; and Khadabadi, S.S. (2010). Isolation and characterization of phytoconstituents from Chlorophytum borivilianum. *Pharmacognosy Research*, 2(6), 343-349.

14. Acharya, D.; Mitaine-Offer, A.C.; Kaushik, N.; Miyamoto, T.; Paululat, T.; and Lacaille-Dubois, M.A. (2008). Steroidal saponins from the roots of Chlorophytum borivilianum. *Planta Medica*, 74(09), 177-181.

15. Acharya, D.; Mitaine-Offer, A.C.; Kaushik, N.; Miyamoto, T.; Paululat, T.; and Lacaille-Dubois, M.A. (2008). Furostane-Type Steroidal Saponins from the Roots of Chlorophytum borivilianum. *Helvetica Chimica Acta*, 91(12), 2262-2269.

16. Acharya, D.; Mitaine-Offer, A.C.; Kaushik, N.; Miyamoto, T.; Paululat, T.; Mirjolet, J.F.; Duchamp, O.; and Lacaille-Dubois, M.A. (2009). Cytotoxic spirostane-type saponins from the roots of Chlorophytum borivilianum. *Journal of Natural Products*, 72(1), 177-181.

17. Thakur, M.; Bhargava, S.; Praznik, W.; Loeppert, R.; and Dixit, V.K. (2009). Effect of Chlorophytum Borivilianum Santapau and Fernandes on sexual dysfunction in hyperglycemic male rats. *Chinese Journal of Integrative Medicine*, 15(6), 448-453.

18. Kumar, M.; Meena, P.; Verma, S.; and Kumar, A. (2010). Anti-tumour, anti-mutagenic and chemomodulatory potential of chlorophytum borivilianum. *Asian Pacific Journal of Cancer Prevention*, 11(2), 327-334.

19. Seo, J.W.; Cho, S.C.; Park, S.J.; Lee, E.J.; Lee, J.H.; Han, S.S.; Pyo, B.S.; Park, D.H.; and Kim, B.H. (2013). 1′-Acetoxychavicol acetate isolated from alpinia galanga ameliorates ovalbumin-induced asthma in mice. *PLOS ONE*, 8(2), e56447.

20. Awang, K.; Nurul Azmi, M.; Lian Aun, L.I.; Nazif Aziz, A.; Ibrahim, H.; and Hasima Nagoor, N. (2010). The apoptotic effect of 1'S-1'-Acetoxychavicol acetate from alpinia conchigera on human cancer cells. *Molecules*, 15(11), 8048-8059.

21. Lee, S.J.; and Ando, T. (2001). Optically active 1'-acetoxychavicol acetate and its positional isomers: Synthesis and repellent effect against adzuki bean weevil. *Journal of Pesticide Science*, 26(1), 76-81.

Appendix A

Spectroscopic Data and the Respective Spectrum of ACA (^{1}H NMR, ^{13}C NMR, COSY, DEPT, HMQC, HMBC)

Table A-1 shows the complete spectroscopic data (^{1}H NMR, ^{13}C NMR, COSY, DEPT, HMQC, HMBC) for ACA and Figs. A-1 to A-6 show the respective spectrum

Table A-1. 1D and 2D Spectral Data Corellations for ACA Recorded at 126 and 500 MHz.

No.	δ_{H} (mult, J in Hz)	δ_{C}, DEPT	HMQC	^{1}H-^{1}H COSY	HMBC
1	1.99 (s)	21.2 CH$_3$	C$_1$-H$_1$		H$_1$→C$_{13}$
2	2.17 (s)	21.3 CH$_3$	C$_2$-H$_2$		H$_2$→C$_{12}$
3	6.14 (d, 6.9)	77.4 CH	C$_3$-H$_3$		H$_3$→C$_{4, 7, 8, 9, 10, 13}$
4	5.19 (d, 17.2), 5.14 (d, 10.3)	117.6 CH$_2$	C$_4$-H$_4$	H$_4$-H$_9$	H$_4$→C$_3$
5	6.99 (d, 8.0)	123.2 CH	C$_5$-H$_5$	H$_5$-H$_7$	H$_5$→C$_{7, 8, 10, 11}$
6	6.99 (d, 8.0)	123.2 CH	C$_6$-H$_6$	H$_6$-H$_8$	H$_6$→C$_{7, 8, 10, 11}$
7	7.29 (d, 8.0)	129.6 CH	C$_7$-H$_7$		H$_7$→C$_{3, 5, 6, 11}$
8	7.29 (d, 8.0)	129.6 CH	C$_8$-H$_8$		H$_8$→C$_{5, 6, 11}$
9	5.93 (ddd, 17.2, 10.3, 6.9)	137.9 CH	C$_9$-H$_9$	H$_9$-H$_3$	H$_9$→C$_3$
10		138.4 C			
11		152.3 C			
12		171.4 C			
13		171.9 C			
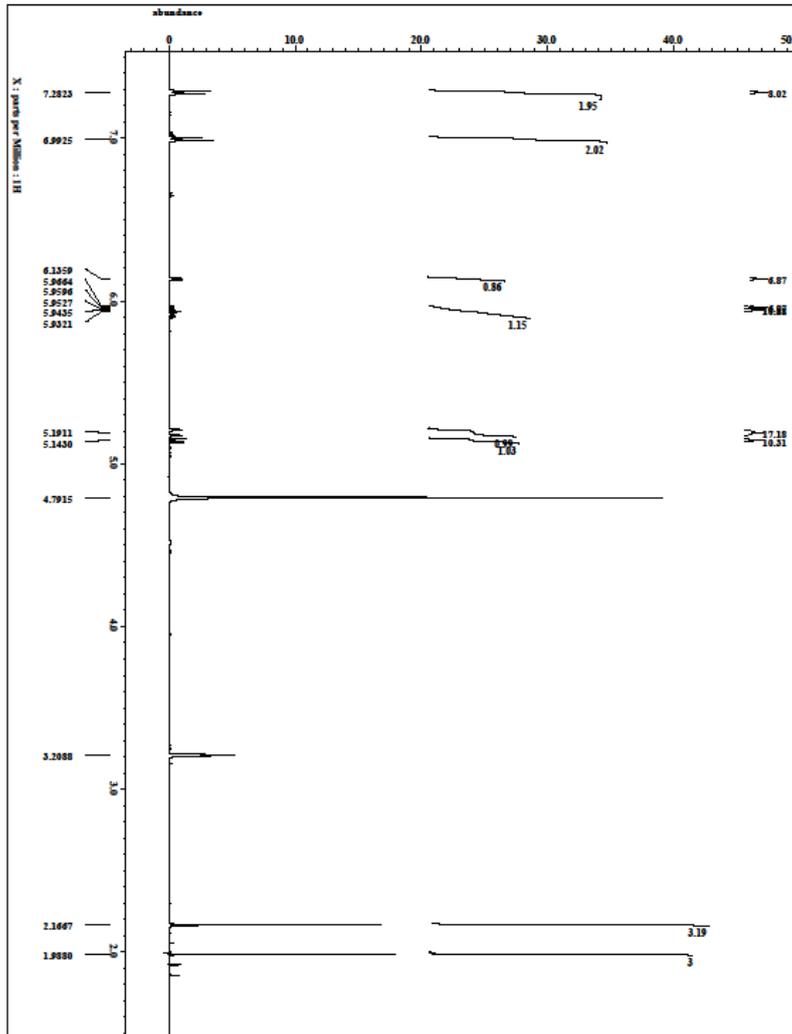
Fig. A-1. ¹H NMR spectrum of ACA.

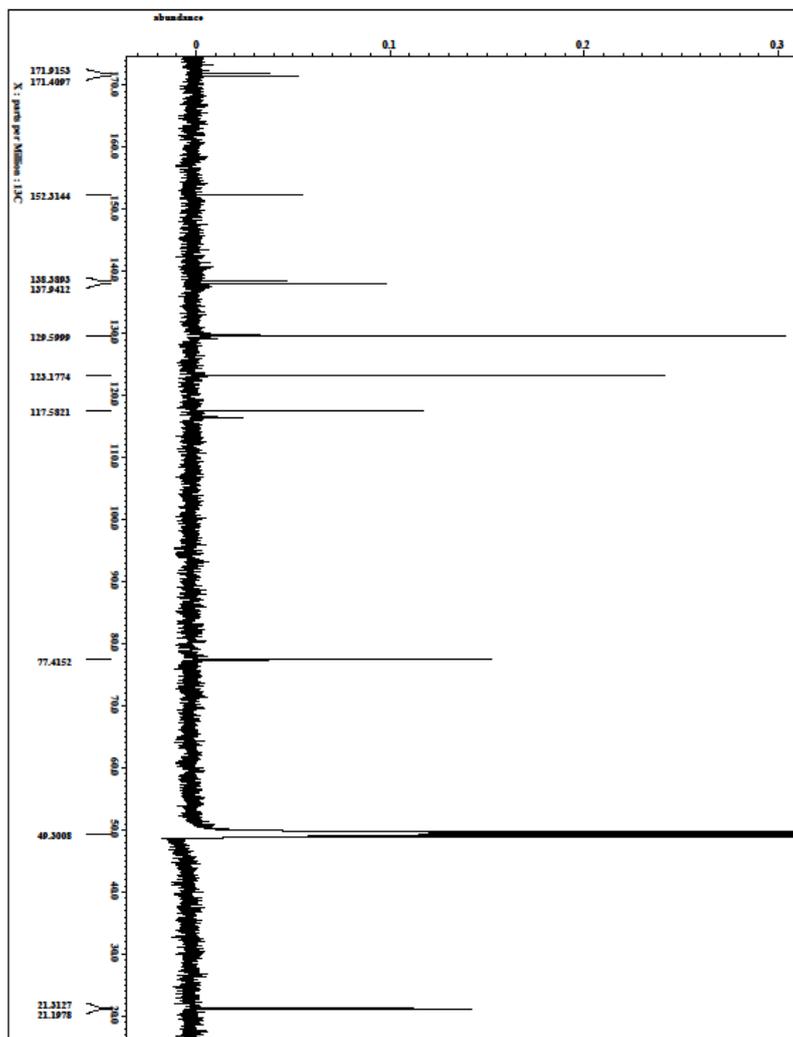**Fig. A-2. ^{13}C NMR spectrum of ACA.**
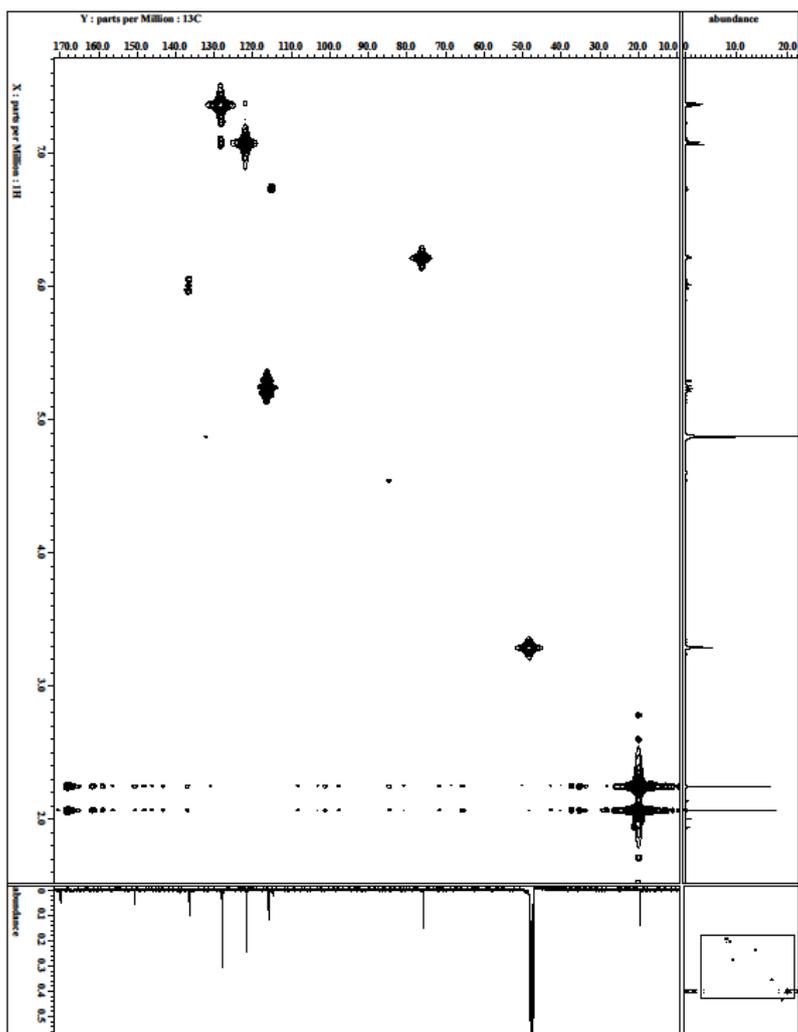
Fig. A-3. HMQC spectrum of ACA.
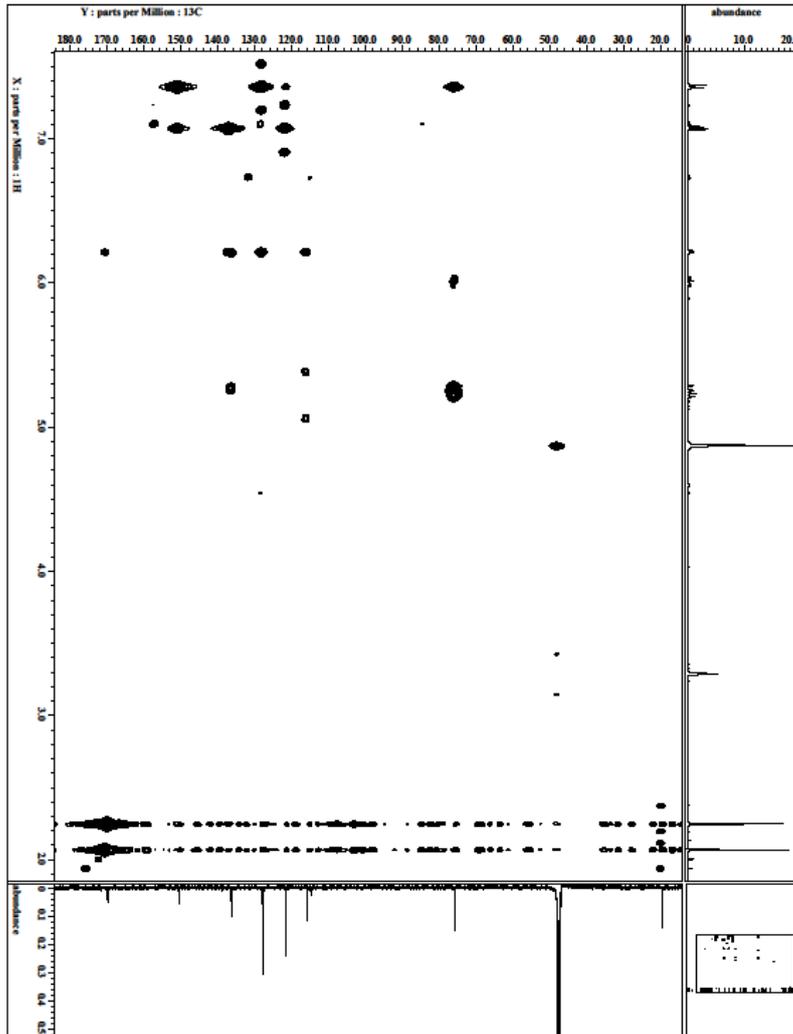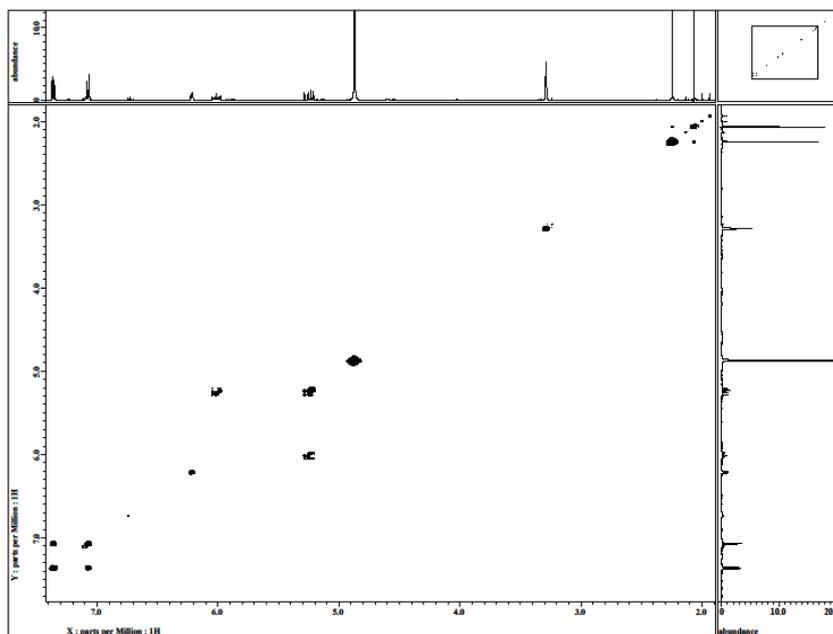
Fig. A-4. DEPT spectrum of ACA.

Fig. A-5. HMBC spectrum of ACA.

Fig. A-6. COSY spectrum of ACA.