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Comparative assessment of safety and quality of tinospora species-tinospora crispa and tinospora sinensis

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COMPARATIVE ASSESSMENT OF SAFETY AND QUALITY OF *TINOSPORA* SPECIES-*TINOSPORA CRISPA* AND *TINOSPORA SINENSIS*

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ABSTRACT

Tinospora crispa Miers ex Hook.f. & Thomson and *Tinospora sinensis* (Lour.) Merr. (Menispermaceae) are two species that have morphological similarities which makes distinction between the two of critical importance because *T. crispa* has been reported to cause hepatotoxicity. Comparative morpho-anatomy of the leaves and stems of *Tinospora* species, indigenous to South and Southeast Asia, was undertaken to elaborate their distinguishing pharmacognostic features. Chemical characterization of the secondary metabolites of the two species may aid in elucidating their chemical diversity and hence help to resolve safety and quality issues. Phytochemical investigation of the stems led to the isolation of nineteen compounds including one new compound and three new source compounds from *T. crispa* and fifteen phytoconstituents from *T. sinensis* including two new compounds and five new source compounds. Chemical structures were elucidated by 1D and 2D NMR spectroscopy and confirmed by HRESIMS. The effect of *Tinospora* plants and borapetosides B, C and F on liver was assessed in a 21-day short term and acute study in healthy and LPS induced health compromised mice. No hepatotoxicity was observed for any of the crude extracts (1 g/kg b.wt./day oral) or pure compounds (500 mg/kg b. wt./day oral) in either the 21 day or acute study. The hepatoprotective effect was assessed using the monocrotaline-LPS induced hepatotoxicity model. Pretreatment with the crude methanolic extracts of *T. crispa*, *T. sinensis*

(500 mg/kg b.wt./day oral) or combination BCF (250 mg/kg/day) for seven days did not counter the monocrotaline-LPS induced hepatotoxic insult. Elevated ALT levels and alteration in liver histological morphology was observed. A rapid, sensitive and reproducible method was established using ultra-high-performance liquid chromatography coupled with photodiode array and single quadrupole electrospray mass spectrometry detectors to achieve decisiveness in not only identifying but also differentiating *T. crispa* from *T. sinensis* and other closely related *Tinospora* species. A chemical fingerprint was developed with a flavonoid, two alkaloids, an amide and six diterpenoids. Thirty-four *Tinospora* plant samples and dietary supplements claiming to contain *T. crispa*, *T. sinensis* and *T. cordifolia* were analyzed. The newly developed and validated method successfully resulted in the conclusive identification of two dietary supplements that were mislabeled.

DEDICATION

This work is dedicated to Madame Helene Berton, “Be positive you are doing good work”.

Her unrelenting support gave me the courage and inspiration to keep going against all odds.

LIST OF ABBREVIATIONS

HPTLC	High Performance Thin Layer Chromatography
BCF	Combination of borapetosides B, C and F
LPS	Lipopolysaccharide
TNF-α	Tumor necrosis factor alpha
IL-1	Interleukin 1
MCT	Monocrotaline
EDTA	Ethylene diamine tetra-acetic acid
AST	Aspartate transaminase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ALB	Albumin
TBIL	Total bilirubin
AMY	Amylase
BUN	Blood Urea Nitrogen
PHOS	Phosphorus
CRE	Creatinine
TP	Total Protein

WBC	White Blood Cell
GLOB	Globulin
MON	Monocyte
GRA	Granulocyte
LY%	Lymphocyte
HGB	Hemoglobin
HCT	Hematocrit
MCV	Mean Corpuscular Hemoglobin
PLT	Total Platelet Count
LYM	Lymphocyte
MO%	Monocyte
GRA%	Granulocyte
RBC	Red blood cell
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
RDWc	Red blood cell Distribution Width
IP	Intra peritoneal
Rf	Retention factor
CC	Column chromatography
RSD	Relative standard deviation

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Chapter 1

COMPARATIVE MORPHO-ANATOMY AND HPTLC ANALYSIS OF *TINOSPORA* SPECIES

1.1. Introduction

Species of *Tinospora* (Menispermaceae) are a group of large, deciduous plants. The genus consists of over 34 species distributed in the tropical and subtropical parts of Australia, Africa and Asia (Chi et al. 2016). The plants are twinning, woody climbers and possess therapeutic properties, particularly anti-diabetic. Overlapping geographical occurrence, history of traditional use, confusion in species identification and morphological resemblances among various species **(Fig. 1.1a-d)** are some considerations that necessitate qualitative analysis for efficient quality control. *T. crispa* Miers ex Hook.f. & Thomson, *Tinospora sinensis* (Lour.) Merr *and T. cordifolia* (Willd.) Hook.f. & Thomson are three common species most likely to be confused and thus adulterated or substituted with one another (Raghunāthan, K., & Mitra 1982). *Tinospora sinensis* is usually substituted for *T. cordifolia* (Seetha Chandran, Harisha CR, 2016), but the likelihood of substitution of either species with *T. crispa* could render the unsuspecting consumer to adverse effects such as hepatotoxicity; since cases of hepatotoxicity from the use of *T. crispa* continues to be reported with the latest case, so far reported in 2018 (Cachet et al. 2018). The possibility of substitution also exists remarkably due to common names employed for the species in vernacular **(Table 1.1)**.

Table 1.1. Habitat and Vernacular names of the three *Tinospora* species

	Tinospora crispa	***Tinospora sinensis***	***Tinospora cordifolia***
Habitat	India, China, Bangladesh, Malaysia, Indonesia, Philippines, Thailand, Vietnam	Pakistan, India and China	India and China
Vernacular names	Heavenly elixir, Heartleaf moonseed (English) Akar patawali, Akar Seruntum (Malysia), Brotowali (Indonesia), Boraphet (Thailand), Gulonchi (Bangladesh), Makabuhay(Phillipines), Da ye ruanjinteng (China)	Heavenly elixir, Heartleaf moonseed Chinese tinospora, Giloy, Gulancha, Gurch, Kattu amrita Malabar gulbel Kuan-Jin-Teng(China)	Heavenly elixir, Heartleaf moonseed, Guduchi Gilo Guluncho Amrita

T. baenzigeri Forman another closely related *Tinospora* species is known in Thailand as Ching cha chali in vernacular (Katib et al. 2017).

Figure 1.1. Photographs of (a) *T. crispa*, (b) *T. sinensis*, (c) *T. cordifolia* and (d) *T. baenzigeri*

The phytochemical profile of the plant species may vary either qualitatively or quantitatively thereby affecting the quality and degree of biological effect exerted. In this context, recognizing the need to differentiate the three species in terms of morphology, microscopy and macroscopic study is of considerable value and thus the aim of research showcased in this chapter in order to contribute to the quality control of herbal dietary supplements containing *Tinospora* plants. An HPTLC method was developed for acquisition of fingerprints for the *Tinospora* plants with qualified chemical markers, since no such chromatographic study including different species of *Tinospora* has been initiated. The method was also applied for the analysis of commercially available dietary supplements of *Tinospora.*

1.2. Experimental Section

1.2.1. Chemicals

Basic fuchsin and Astra blue, safranin, and glycerol were purchased from Fischer Scientific (Fair Lawn, NJ, USA).

1.2.2. Botanical materials

Fresh materials of leaves, petioles and stems of *T. sinensis* (NCNPR # 20876), *T. crispa* (NCNPR # 20877) and *T. cordifolia* (NCNPR # 20875) were acquired from India for morphological studies. Seventeen plant samples which included four species T. *crispa, T. sinensis, T. cordifolia* and *T. baenzigeri* were obtained from various authentic and commercial sources. These included accession numbers for *T. crispa* [NCNPR#17091 (2), #20869 (3) and #16849 (4)), *T. sinensis* (NCNPR#17003 (5), #3104 (6), #16885 (7), #20863 (8)), *T. cordifolia* (NCNPR#20769 (9), #5799 (10), #5212 (11), #17303 (12), #8069 (13), #10107 (14), # 20865 (15), #20866 (16), # 20867 (17)) and *T. baenzigeri* (#20870 (18)]. *T. crispa, T. sinensis, T.*

cordifolia are sold as dietary supplements. Seventeen dietary supplements of *Tinospora* species were purchased through online sources after thorough search, and included accession numbers, *T. crispa* (NCNPR#20754, 20756, 20760, 20762, 20798, 20799), *T. sinensis* (NCNPR#20766), *T. cordifolia* (NCNPR#20753, 20758, 20761, 20759, 20763, 20764, 20765, 20767, 20768, 20864). All the samples were deposited at the botanical repository of NCNPR, University of Mississippi.

1.2.3. Mixture of standards used for HPTLC

Standards, for HPTLC, included borapetosides B and C, columbin, baenzigeride A, N-trans feruloyl tyramine and tinosineside A. All standards were isolated from *T. crispa* except tinosineside A which was isolated from. *T. sinensis* at the NCNPR, University, MS. Stock solutions of 1 mg/mL were prepared for each standard and a standard mix of 0.1 mg/mL was prepared as reference.

1.2.4. Preparation of samples for light microscopy

Fixation of the leaf materials was achieved by placing in formalin-acetic acid-alcohol (FAA) solution for seven days (Johansen 1940) followed by washing in distilled water and storage in 70% ethanol (v/v) (Berlyn, G.P., Miksche, 1976). The transverse sections of the leaf blade were made using razor blades manually. Sections were hydrated and double-stained with basic fuchsin and astra blue (Roeser 1972). Mounts were prepared on glass slides with 50% glycerol solution. The epidermal characters of the leaf specimens were analyzed by immersion in commercial bleach (2.5% sodium hypochlorite) solution until translucent. The translucent specimens were washed with water thrice and stained in safranin (Fuchs, 1963). Photomicrographs were prepared using Nikon E600 microscope equipped with Nikon DSFiv

camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo, Japan). Fluorescent microscope (Nikon E600) was used to study the fluorescent components. Calcium oxalate crystals were observed using a polarized microscope camera systems and Nikon Elements imaging software (Nikon Inc., Tokyo). The stomatal index (SI) was calculated by using the following formula, SI =SE+S× 100, wherein S = number of stomata per unit area, and E = number of epidermal cells in the same unit area.

1.2.5. Micrometrics

The stomatal index, stomatal size and cell size of starch was measured by taking ten to twenty cells from multiple sections for each species. The measurements were averaged and analyzed using Nikon Elements imaging software.

1.2.6. Preparation of plant and dietary samples for HPTLC

The dried stems of samples were ground into fine powders, using a MixerMillMM400 (Retsch, Haan, Germany). 1.0 g powder of each of the 34 samples, consisting of *Tinospora* plant samples and dietary supplements, was extracted successively with 10 mL methanol. The extraction was performed by sonication in an ultrasonic bath sonicator at room temperature for 15 min, followed by centrifugation for 5 min at 3000 rpm. The supernatant was filtered through a 0.45 μm PTFE membrane filter, evaporated in a speed vacuum (Savant SC250DDA SpeedVac Plus vacuum; Thermo Scientific, Waltham, MA, USA). 20 mg of dried extract was dissolved in 1 mL methanol for application in HPTLC analysis.

1.2.7. Instrumentation and chromatographic conditions for HPTLC

The HPTLC system (Camag, Muttenz, Switzerland) consist of a DigiStore2 digital system along with winCATS software ver.1.4.3. (CAMAG, Switzerland); automatic developing chamber CAMAG ADC2 and a Linomat 5 sample applicator with 100 µL syringes, nano-silica HPTLC plates (silica gel 60 F UV254, glass-backed, 200 µm, 20 × 10 cm) (Millipore Sigma, MA, USA) were used for analysis. All samples were applied according to the following settings: 10 mm from the bottom of the plate, band width 8 mm. A constant application rate of 5 µL/s was employed. The images of the chromatograms were taken using a Reprostar 3 interfaced with winCATS 4 software ver. 1.3.2 (CAMAG, Switzerland). The plates were developed using chloroform-ethyl acetate-ethanol- 0.1% formic acid: 10-15-5-0.5(v/v/v/v/v). Plates were developed in a Camag automatic developing chamber ADC2 presaturated with 25 mL of mobile phase at room temperature (23 ± 2 °C) at a relative humidity of 39 ± 5% and 10 mL of mobile phase for development of plates at the same temperature and relative humidity. After development, the plates were then derivatized by dipping into vanillin–sulfuric acid using the Immersion Device (dipping time 0 s, dipping speed 5 cm/s), followed by heating at 100°C for 3 min, and then images were recorded again under UV 366 nm and white light, respectively. Vision CATS software ver. 2.0 was used for device controlling and image processing.

1.3. Results

The differences in morphological features of the leaves of *T. crispa*, *T. sinensis* and *T. cordifolia* provide clues to correctly distinguish these three species of *Tinospora*. The features are listed in **Table 1.2**. The leaves of *Tinospora* species are cordate, simple, with a long and slender petiole. The margin is entire and venation is reticulate. Fresh green leaves have a dark

green color on the adaxial side than on the abaxial side, which displays a lighter complexion. Venation is more conspicuous on the abaxial side than on the adaxial side. The stems of the three *Tinospora* species have protuberances which are more conspicuous on the stems of *T. crispa* (Katib et al. 2017). The stems of *T. sinensis* have a smooth surface between tubercles. A diagnostically distinguishing feature is that the leaf of *T. sinensis* is pubescent and has soft texture. *T crispa* and *T. cordifolia* are glabrous and smooth in texture.

Microscopic Characters of *Tinospora* stems

The transverse section of the stems of *T. crispa* **(Fig. 1.2a)**, *T. sinensis* **(Fig. 1.2b)** and *T. cordifolia* **(Fig. 1.2c)** can be differentiated into five main zones, cork, cortex, pericyclic fiber, vascular bundle and pith (Gokarn et al. 2014). As can be seen from the transverse section, all species show similarity in the basic arrangement of the tissues **(Fig. 1.2)**.

Cork: The outermost layer observed in the transverse section of the stem of the three *Tinospora* species is a brownish cork made up of compressed single layered thick walled cells.

Cortex: The cortex differentiates into an outer, uniform 5-6 layered, hypodermis composed of tangentially arranged elongated cells. The inner cortex in *T. cordifolia* is composed of 4-8 and in *T. sinensis*, 3-9 layers of polygonal shaped parenchymatous cells. In *T. crispa*, the inner cortex is composed radially arranged polygonal parenchymatous cells of varying sizes reaching up to over 20 layers. Scattered starch grains are observed in the cortex region in *T. cordifolia.* Secretory cells were observed as reported in *T. cordifolia* and *T. sinensis.*

Pericycle: The pericycle forms a complete ring of 5-9 in *T. cordifolia*, 4-8 in *T. sinensis* and 4-6 layers in *T. crispa* of lignified cells arranged in an arch like structure.

Vascular bundle: Vascular bundles lie beneath each arch of the pericyclic fiber. Phloem made up of sieve tube, companion cells and phloem parenchyma. Xylem consists of vessels, tracheids, parenchyma and fibers.

Pith: The central part of the stem is shown to consist of large, thin walled cells, containing less starch in *T. cordifolia*, no starch in *T. sinensis* and abundant starch in *T. crispa*.

1.3.2. Microscopic Characters of *Tinospora* leaves

The transverse section of the leaf of *T. crispa* **(Fig. 1.3a)** showed 2-3 layer of palisade and 6-13 layers of spongy mesophyll. Glandular trichomes were present. The leaves of *T. sinensis* **(Fig. 1.3b)** showed wavy, sinuous, anticlinal epidermal cell walls on the adaxial side. Subsidiary cells are same as observed on the abaxial side. Thin walled, non-glandular, multicellular, and uniseriate trichomes composed of up to 6 cells were observed. Several trichomes were observed on the adaxial side contrary to the abaxial side. The non-glandular trichomes arise from the base of rosette of cells. Crystals of calcium oxalate observed were in the form of prismatic and styloid crystals.

The transverse section of *T. cordifolia* leaf **(Fig. 1.3c)** showed a thin striated cuticle above the uniseriate epidermis. Trichomes were observed either on the lower epidermis. The upper epidermis is followed by a layer of palisade mesophyll and 4-7 layers of spongy mesophyll. A single large vascular bundle is present in the center surrounded by thin parenchymatous cells of the midrib. *T. cordifolia* showed glandular trichomes, straight anticlinal epidermal walls on the abaxial side. The trichomes are rare on the adaxial side. The adaxial side showed the presence of prismatic crystals and styloids in the epidermal cells.

The three *Tinospora* species have anomocytic stomata. Length and width of the palisade varied between the plant species between 30-40 mm in length and 12-16 mm in width. Cross section of the midrib was biconvex with varying convexity on adaxial side and on abaxial side. In lamina portion, minor vascular bundles were present in both *T. crispa* and *T. sinensis* plant species.

1.3.3 Microscopic Characters of *Tinospora* petiole

The transverse section of *T. crispa* **(Fig. 1.3d)** shows an epidermal layer followed by a 10-12 layered cortical parenchyma. Lignified pericyclic arch like structure, 3-5 layered encloses 8-12 bicollateral vascular bundles, which lie beneath each crest of the uninterrupted pericycle.

The transverse section of the petiole of *T. sinensis* **(Fig. 1.3e)** shows numerous trichomes, a single layer of epidermal cells, followed by 4-6 layers of polygonal cells forming the hypodermis, followed by cortical parenchyma which contains 13 bicollateral vascular bundles

Transverse section of the petiole of *T. cordifolia* **(Fig. 1.3f)** shows an outline, more or less circular (N. Choudhary, Siddiqui, and Khatoon, 2014) consisting of a layer of thick walled epidermis. Hypodermis, 3-5 layered, is followed by 3-6 layers of cortical parenchyma containing numerous tannin containing cells. A non-lignified wavy line encloses 8-9 bicollateral vascular bundles and central pith composed of parenchymatous cells.

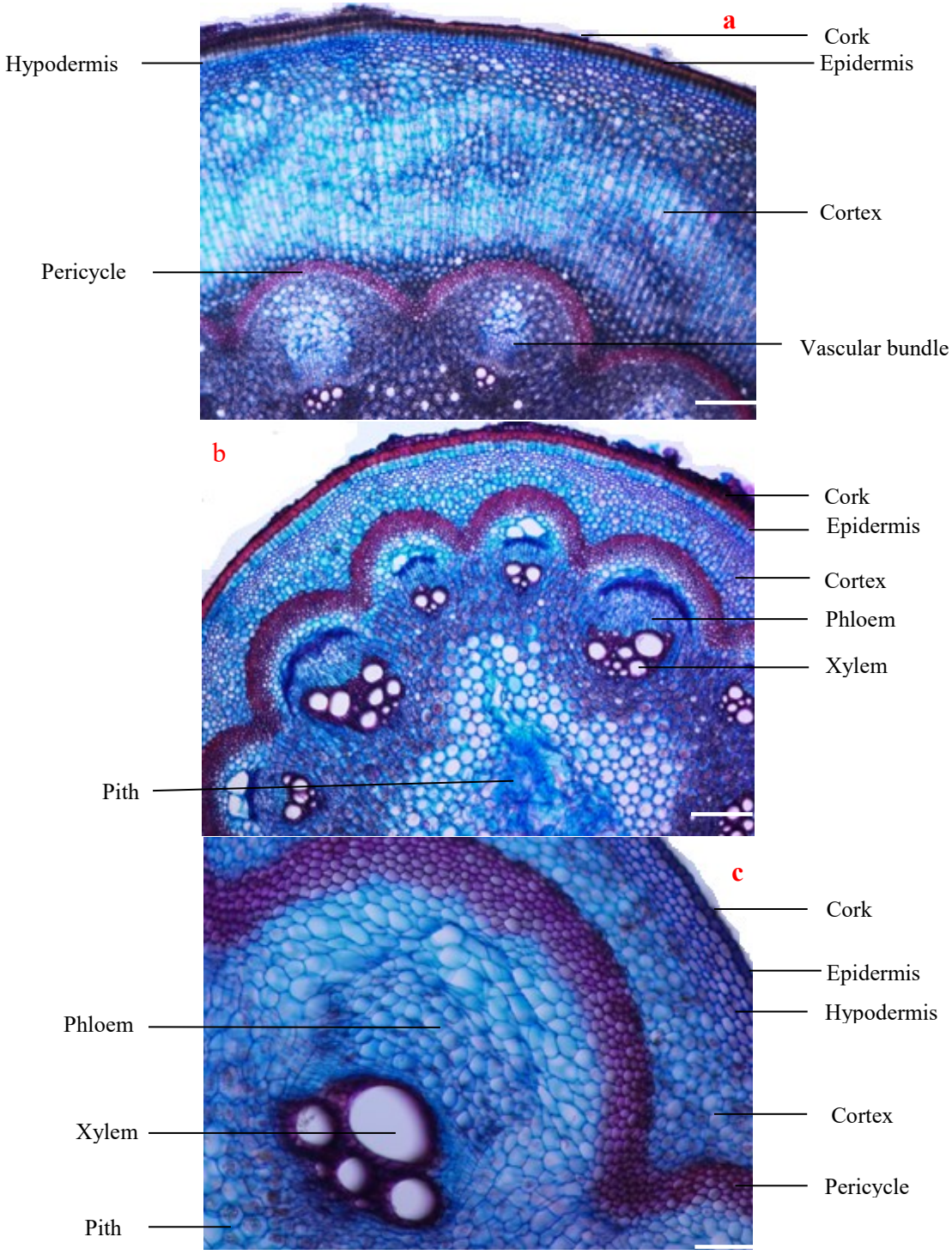


Figure 1.2. Stem anatomy of *T. crispa*, *T. sinensis* and *T. cordifolia*.

Bars: a -c = 200 µm

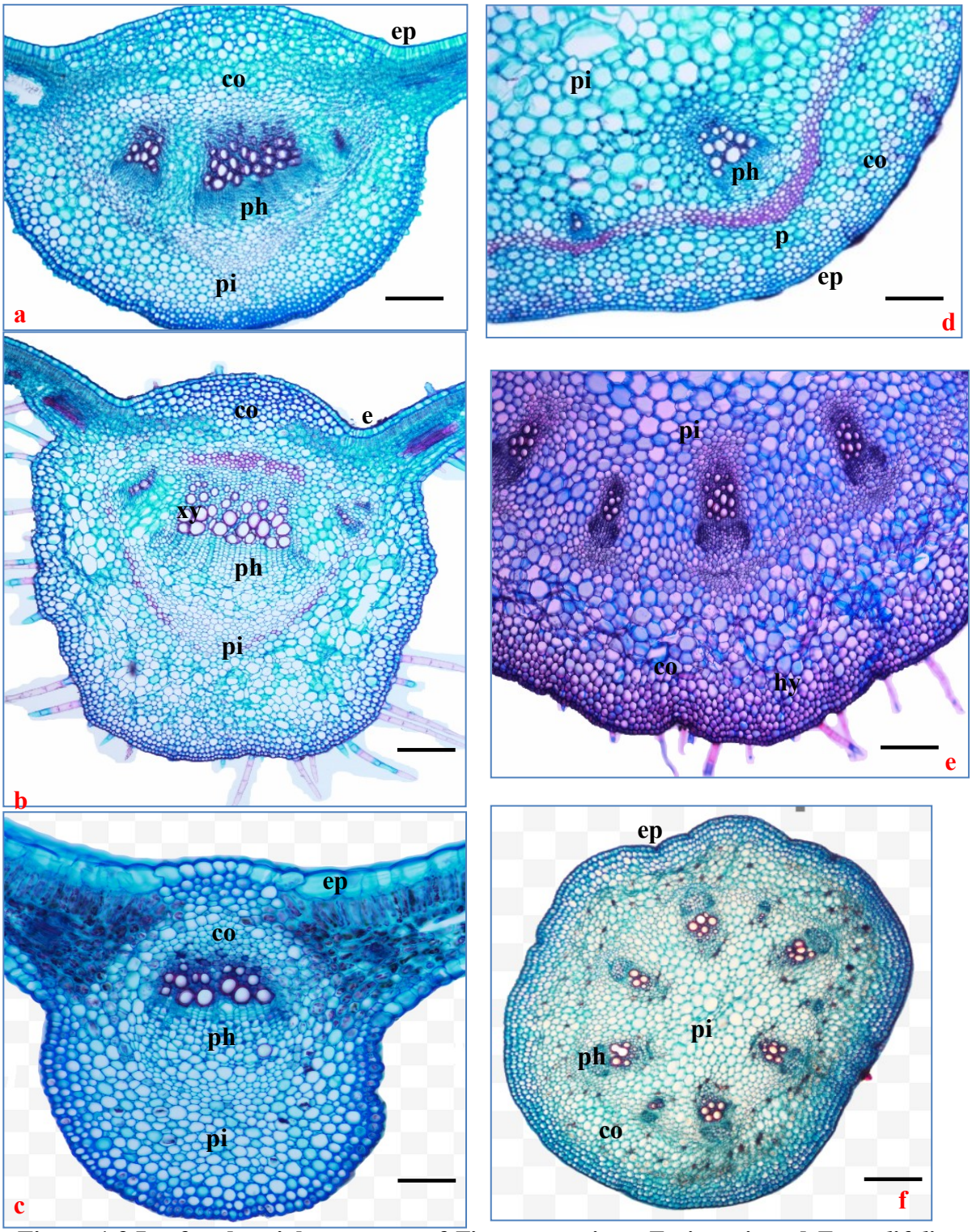


Figure 1.3.Leaf and petiole anatomy of *Tinospora crispa*, *T. sinensis* and *T. cordifolia*
ep-epidermis, **tr**-trichome, **hy**-hypodermis, **co**-cortex, **xy**-xylem, **ph**-phloem, **vb**-vascular bundle, **p**- pericycle, **pi**-pith. Bars: a, b, d-f=200 µm; c = 400 µm

Table 1.2.Macroscopic and microscopic features of *Tinospora* species

	T.crispa	***T. sinensis***	***T. cordifolia***
Adaxial lamina			
Abaxial lamina			
Stomatal size (average length x width in µm)	25.59±4.28 × 19.83± 3.02	21.45±1.75 × 16.91±1.23	23.60±2.45 × 19.85±1.75
Palisade cell size L x W(Leaf)	66.51±8.29 × 12.19±3.33	44.78±4.76 × 13.81±3.47	47.03±6.24 × 12.03±3.11
Epidermal cell size (Leaf)	41.99±11.89 x 57.76±11.55	20.61±7.32 x 20.75±6.51	31.34±9.33 x 32.60±12.19
Number of Vascular bundles in stem	18	13	14
Number of Vascular bundles in petiole	12	13	8-9
Starch diameter in leaf (mm)	74.86±51.75 × 70.07±35.96	16.40±6.40 × 25.05±10.80	-
Starch diameter in stem (mm)	26.52±18.12 × 20.38±12.77	14.28±3.30 × 8.56± 1.89	-
Starch diameter petiole (mm)	27.25±16.03 × 27.51±12.85	Absent	16.83 ±6.98 × 17.00 ±3.72
Crystals type – calcium oxalate,	Druses, styloids, prisms	Styloids, prisms	Styloids, prisms , needle shaped
Trichomes-frequency (250 µm)	2	Numerous	1-2
Trichome type and shape	Glandular, ellipsoid	Non glandular, uniseriate	Glandular, ellipsoid or elongated
Trichome size L x W (µm)	37.84± 6.72 × 38.83±7.29	-	53.30 ± 4.77× 28.30±3.12
Stomatal Index	14	30	9

1.3.4. Development of HPTLC method

High Performance Thin Layer Chromatographic fingerprint for *Tinospora* plant samples consisting of four species was obtained with a reference standard mix, consisting of six compounds. The mobile phase consisting of chloroform-ethyl acetate-ethanol- 0.1% formic acid: 10-15-5-0.5(v/v/v/v/v) produced good resolution for all six standards. Rf for the reference compounds in the standard mixture is given as follows.

Table 1.3. Rf, UV active (254 nm) and color after derivatiziation of the compounds

	Reference Standards	Rf	UV active (254nm)	Color
1	Tinosineside A	0.13	-	Pink
2	Borapetoside B	0.20	-	Pink
3	Borapetoside C	0.40	UV active	Pink
4	N-trans feruloyl tyramine	0.73	UV active	-
5	Baenzigeride A	0.75	-	Pink
6	Columbin	0.79	-	Bluish grey

Two HPTLC chromatograms were developed for the qualitative analysis of *Tinospora* species. The chromatogram was developed with the voucher and commercially obtained plant samples. A second chromatogram was developed with the dietary supplements available for the *Tinospora* species.

1.3.4.1. HPTLC for *Tinospora* plant samples

The value of High performance thin layer chromatography (HPTLC), as a tool for qualitative and quantitative analysis lies in it being simple in performance and the results obtained are accurate and precise. The qualitative analysis of *Tinospora* species yielded satisfactory results. Comparison of commercial plant samples with voucher samples (3, 5, 9, 11, and 18) clearly lays out the qualitative differences between samples **(Fig. 1.5).** *T. crispa* samples (2-4) showed the presence of the two major bands corresponding to borapetosides b and c, as two pink spots (Rf 0.2 and 0.4). These bands are useful chemical markers to distinguish *T. crispa* from other closely related species. *T. cordifolia* showed numerous bands, among which a brownish yellow band (Rf 0.26) was a feature differentiating *T. cordifolia* from the rest of the species. The presence of two closely lying bands colored bluish gray and yellow (Rf 0.15, 0.13) were the chief difference observed and useful in distinguishing T. *baenzigeri* from *T. crispa*, *T. sinensis* and *T. cordifolia*. The marker compound for *T. sinensis* added to the reference mixture was tinosineside A, and appeared as a lighter band in the tracks for *T. sinensis*, but the absence of the distinguishing bands of other three species can also be an important indication for qualitative analysis. N-trans-feruloyl tyramine, visible under UV 254 nm, was present in all species and observed as a blue band (Rf 0.73) **(Fig. 1.5a)**. No significant difference in profile was observed within each specie group except for three commercial samples of *T. cordifolia* (Tracks-12, 15 and 17).

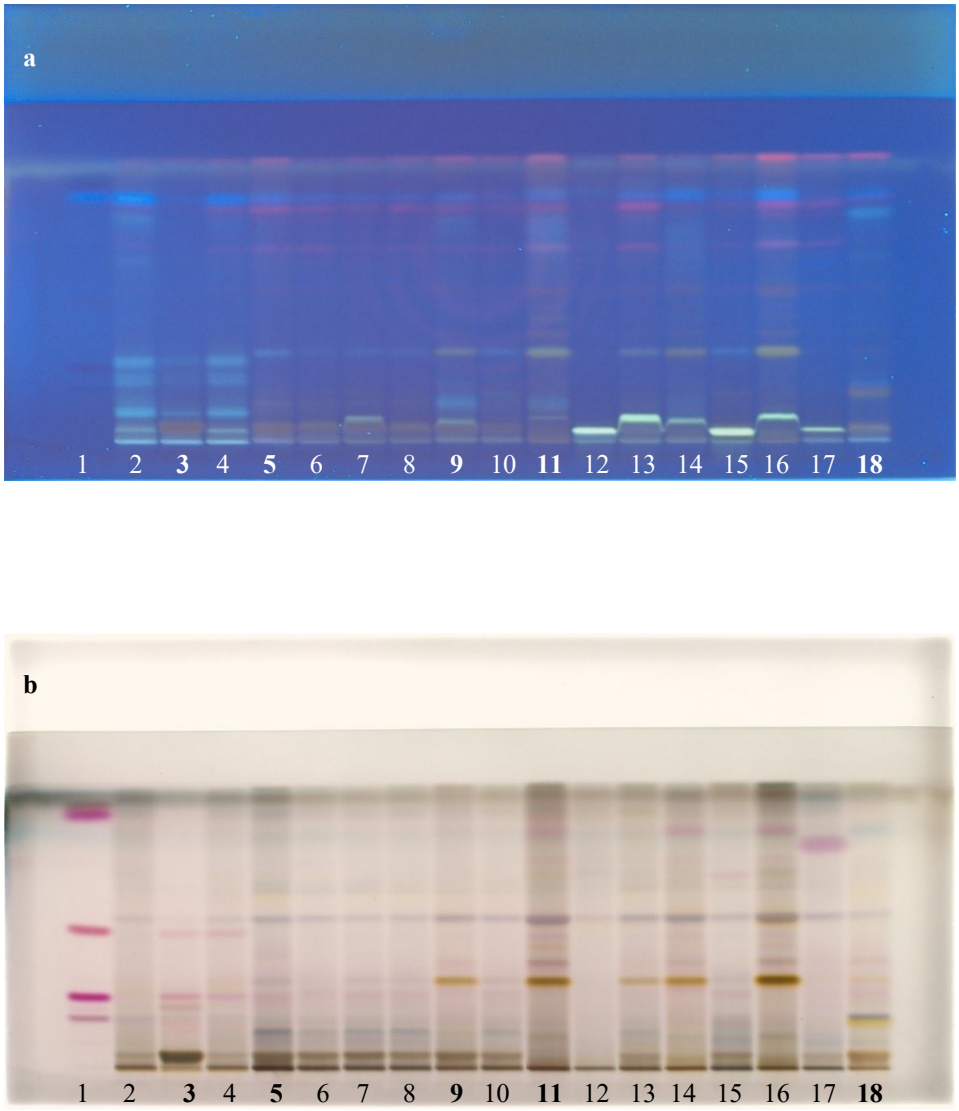


Figure 1.4. HPTLC under UV (366 nm) prior to derivatization (a) and white light after derivatization (b).

Track 1: Reference standards: **1**. Tinosineside A, **2**. borapetoside B, **3**. borapetoside C, **4**. N-trans feruloyl tyramine, **5**. baenzigeride A, **6**. columbin; stem samples, 2-4 (*T. crispa*), 5-8 (*T. sinensis*), 9–17 (*T. cordifolia*) and 18 (*T. baenzigeri*).
Numbers in bold represent voucher samples.

1.3.4.2. HPTLC for *Tinospora* dietary supplements

The results obtained for the dietary supplements **(Fig. 1.6)** were consistent with the results obtained for the plant samples **(Fig. 1.5)**. Dietary supplements claiming *T. crispa* (1-7) was easily distinguishable from those claiming *T. sinensis* and *T. cordifolia*. Two pink bands (Rf 0.2, 0.4) corresponded to the reference compounds borapetosides B and C. The bands are more conspicuous in tracks 4, 5 and 6 whereas lighter in color in tracks 2, 3 and 7 giving qualitative insight. The dietary supplement claiming *T. sinensis* (8) showed a pink band (Rf 0.13) which corresponded to tinosineside A, a chemical marker for *T. sinensis*. The track profiles for dietary supplements claiming *T. cordifolia* (9-18) were similar to those observed for the *T. cordifolia* plant samples **(Fig. 1.5.)** with the visible brownish yellow band (Rf 0.26). A blue band, Rf 0.73, **(Fig. 1.6.a)** corresponding to N-trans-feruloyl tyramine was visibly present in all *Tinospora* dietary supplements except in tracks 8 and 9.

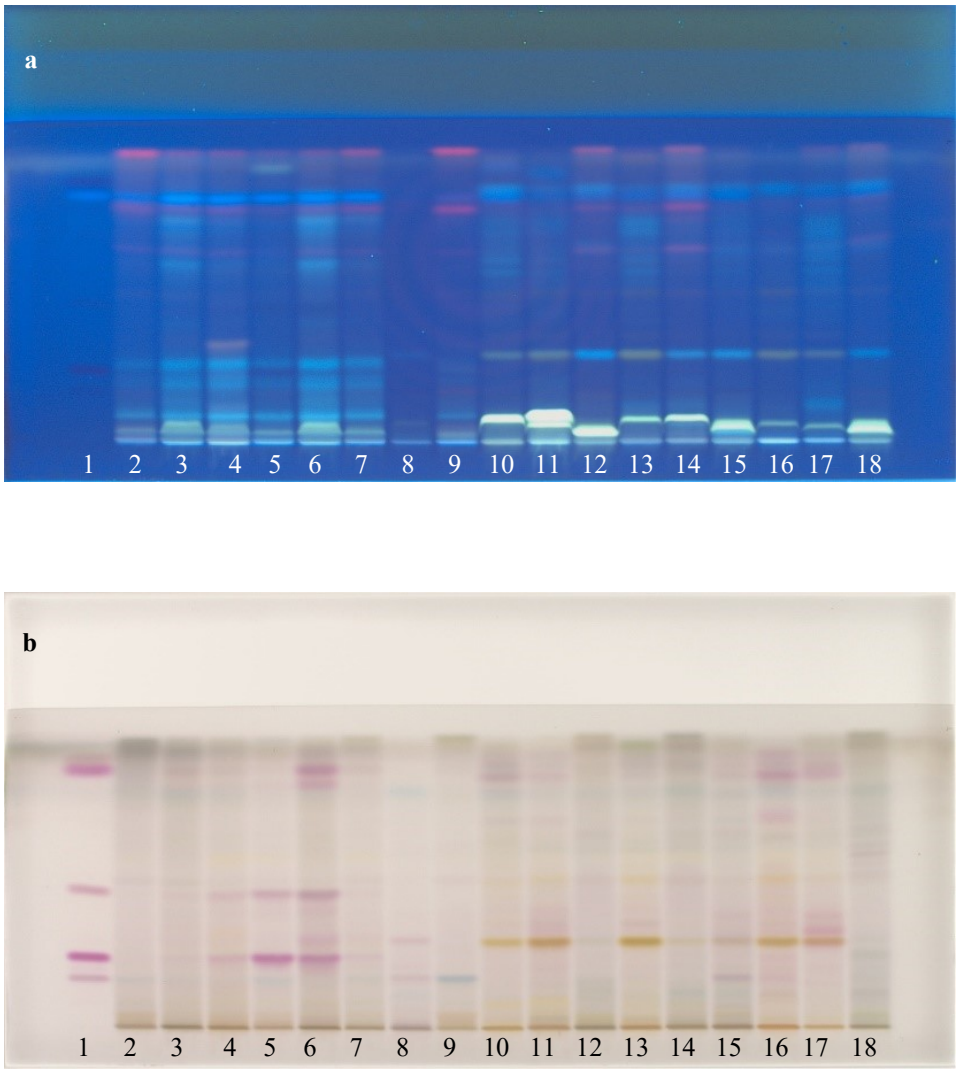


Figure 1.5.HPTLC under UV (366 nm) prior to derivitization (a) and white light after derivatization (b).

Track 1: Reference standards: **1**. Tinosineside A, **2**. borapetoside B, **3**. borapetoside C, **4**. N-trans feruloyl tyramine, **5**. baenzigeride A, **6**. columbin; dietary supplements, 2-7 (*T. crispa*), 8 (*T. sinensis*), and 9-18 (*T. cordifolia*)

1.4. Discussion

The micro-anatomical results of the stems of the *Tinospora* species evidently show that similarity of basic arrangement of vessels present a challenge to decipher the specie type. A closer analysis of the trichomes, starch grains and crystals in the leaf, stem and petiole could be useful in identifying the species. The leaves and petiole of *T. sinensis* bear numerous non glandular trichomes which can aid in differentiating it. The presence of starch grains in copious amount in the cortex of *T.sinensis* and *T cordifolia* was in agreement with published literature.(Seetha Chandran, Harisha CR 2016). *T. crispa* and *T. cordifolia* bear greater similarity in that both have glandular trichomes. However, for efficient quality control, the utility of HPTLC in differentiating *Tinospora* species has been demonstrated in the current investigation. The simplicity and accuracy of chromatographic technique resulted in chromatographic profile track for each species sample. Visualization of the bands after derivatization with vanillin sulphuric acid facilitated interspecies comparison between the plant samples studied as well as dietary supplements claiming various *Tinospora* species. Quality control for *Tinospora* species is critical since substitution is practiced for *T. crispa*, *T. sinensis* and *T. cordifolia.*

For plant samples (Fig 1.5.), three samples (Track 12, 15 and 17) showed different profile from the voucher samples of *T. cordifolia* (Track 9, 11). Track 12 did not show any significant band which may be suggestive of an exhausted sample with poor quality profile. Track 15 showed a profile that did not match with *T. cordifolia* instead it showed similarity with the profiles for *T. sinensis*. Track 17 demonstrated a profile that could not be related to any of the four species and therefore was questionable quality-wise. The HPTLC chromatogram after

derivatization showed the reliability of the method for quality control of dietary supplements as well.

The profiles obtained for supplements claiming *T. crispa* and *T. sinensis* showed bands that corresponded to the reference marker compounds for each species. However, the supplements claiming *T. cordifolia* (9-18), showed two tracks (9 and 15) that were inconsistent. The bands present in sample 9 were not in concord with the other tracks in the group of supplements claiming *T. cordifolia* (10-18). A closer look at the profile of track 9 (Fig 1.6a) clearly highlights the difference in profile. Comparison with other species shows a similarity of its profile with those of the *T. crispa* claiming supplements (2-7). The profile for the track 15 showed a band (Rf 0.13) that corresponded with tinosineside A, a chemical marker of *T. sinensis*, which disputes the quality of the dietary supplement as a genuine *T. cordifolia* supplement.

1.4. Conclusion

The differences in the morpho-anatomy of *Tinospora* species are subtle. Botanical description and analysis may be applied to plant materials but extracts require qualitative assessment with qualified reference standards to distinguish them conclusively. The developed HPTLC method showed accurate results for both plant samples and the dietary supplements, and can prove useful for the quality control of plant samples and dietary supplements of *Tinospora* species.

Chapter 2

EXTRACTION, ISOLATION AND CHEMICAL CHARACTERIZATION OF THE PHYTOCONSTITUENTS FROM *TINOSPORA CRISPA*

2.1. Introduction

T. crispa is popular for its medicinal value in south Asian countries including Malaysia, Philippines Thailand, Indonesia, Bangladesh and Vietnam (Ahmad, Jantan, and Bukhari 2016). In Thailand, a decoction of the stems of *T. crispa* is used to maintain good health, treat internal inflammations, enhance appetite and quench thirst (Kongsaktrakoon et al., 1984; Dweck and Cavin, 2006). Infusions of stems possess worm expulsion qualities; syphilitic sores are treated with decoctions and the crushed leaves are used as antipruritic dressings and applied on wounds (Dweck and Cavin, 2006). *T. crispa* has been used conventionally against a wide variety of health ailments such as bruises, septicemia, fever, fracture, scabies, and other tropical ulcer-related disorders by the Yao communities of China. (Li et al., 2006). In Malaysia, *T. crispa* is employed for numerous therapeutic purposes like diabetes, hypertension, appetizer and insect repellent (Gimlette and Burkill, 1930). Moreover, it is used as an anti-parasitic agent in both humans and domestic animals; local traditional medicine practitioners recognize it as a general tonic (Noor et al., 1989). In Bangladesh, the stem juice is used in the treatment of intestinal disorders, (Rahmatullah et al., 2011) jaundice, rheumatism, body pain, paralysis, skin disease,

and leprosy (Kadir et al., 2011). The leaves and stems are used as antiflatulent, antirheumatic, antidiarrheal and for dyspepsia by traditional healers in the Philippines. Arthritis is treated with poultice of *T.crispa* with coconut oil (Quisumbing 1951). *Tinospora crispa* has been reported to possess antidiabetic (Banerjee et al., 2017), anti-inflammatory (Ibrahim Abdelwahab et al. 2012), immunomodulatory (Haque et al., 2017), cytotoxic (Abu et al., 2013), insecticidal (Elanchezhiyan, et.al., 2015), anticholinesterase (Yusoff et al., 2014) and cardio-tonic activity (Praman et al. 2011).

Several phytoconstituents have been isolated from *T. crispa*, and about seventy-five compounds have been reported. Clerodane-type furanoditerpenoid glycosides known as borapetosides are the major compounds present in *T. crispa*. Among these isolated compounds, some representative examples of flavonoids (genkwanin, apigenin), alkaloids (jatrorrhizine, magnoflorine, N-formylanonaine), clerodane diterpenes (borapetoside A), lignans (secoisolariciresinol) and nucleosides (adenosine) are given in **Figure 2.1**.

Although *T. crispa* has been used traditionally and has been reported to possess pharmacological potential, reports of hepatotoxicity from this species continue to trigger the interest of the scientific community. Phytochemical isolation was undertaken to gain access and insight into the diverse chemical spectrum of *T. crispa* in an attempt to elucidate and resolve safety and quality issues surrounding the *Tinospora* species.

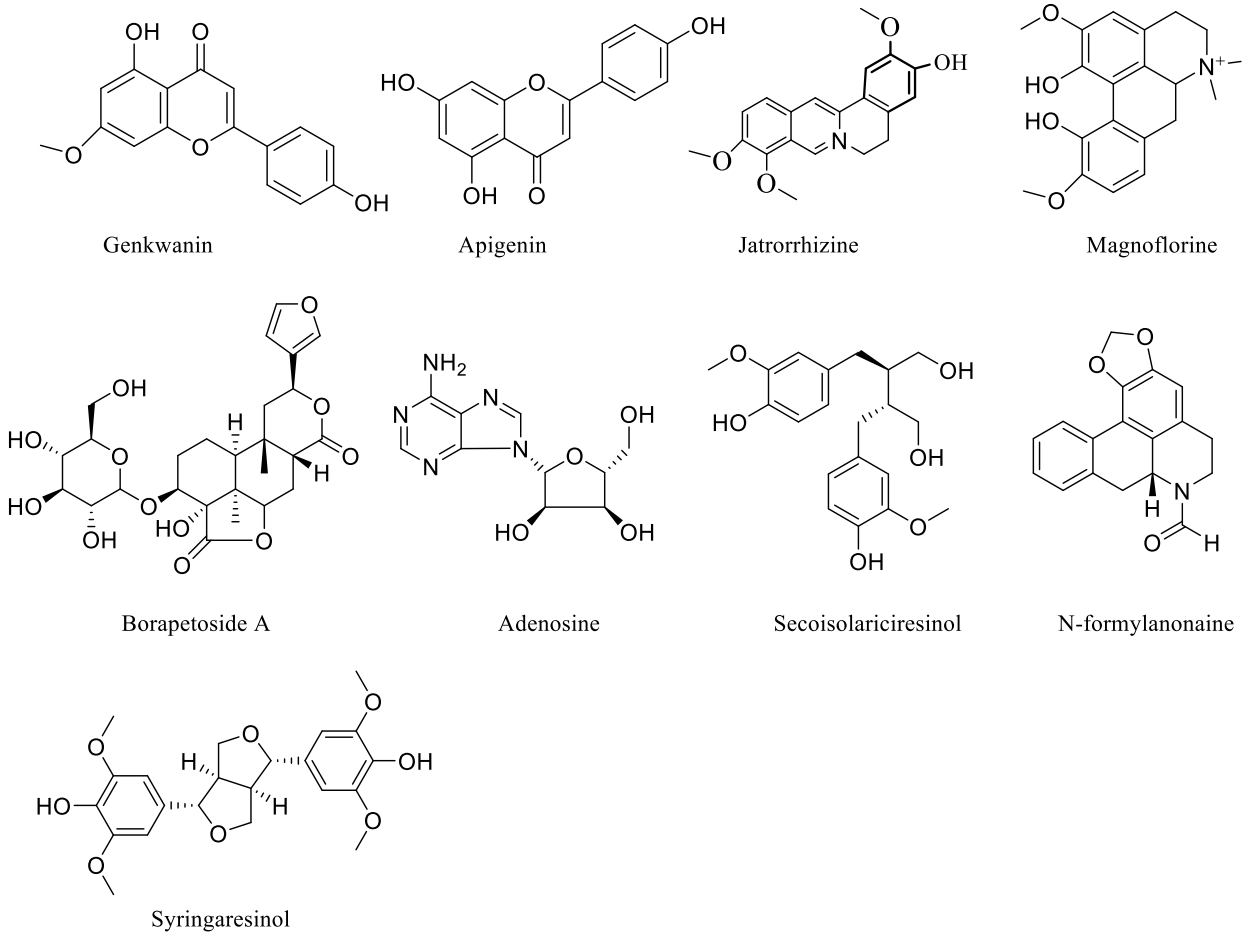


Figure 2.1 Representative examples of chemical constituents reported from *T. crispa*

2.2. Experimental section

2.2.1. General methods

NMR spectra were obtained on Bruker AU III 400 or 500 MHz NMR spectrometers using $CDCl_3$ or CD_3OD solvents. The UV spectra were recorded on a Thermo Scientific UV-Visible Spectrophotometer Evolution 200 Series. Optical rotations were measured on Rudolph Research Analytical Autopol IV automatic polarimeter. Agilent Technologies Cary 630 FTIR was used to obtain the IR spectra. The mass spectra were recorded on an Agilent technology 6200 series mass spectrometer. Thin layer chromatography was performed using aluminum-

backed, TLC plates, pre-coated with silica gel F254 (200 μm, 60 Å, Merck), flash silica gel (40 μm, 60 Å, J. T. Baker), sephadex LH-20 (Sigma) and reversed phase RP-C_{18} silica (Polarbond, J.T. Baker). Detection methods included UV at 254 nm and 1% vanillin-sulphuric acid spray reagent followed by heating. Preparative-TLC plates (20 cm × 20 cm, 500 μm, Analtech) were used for final purification. Preparative HPLC separation was performed on Waters separation module (2795) with photodiode array detector. Separation was achieved by a Phenomenex Gemini C_{18} 110 Å column (5μm, 250 × 21.2 mm) with mobile phase consisting of methanol and water (containing 0.1% formic acid).

2.2.2. Plant material

Stem samples of *Tinospora crispa* were obtained from Thailand (NCNPR# 17091) and identified by co-TLC with authentic sample of *T. crispa* (NCNPR# 17320) deposited at the Botanical Repository in the National Center for Natural Products Research, University of Mississippi, University, MS, United States.

2.2.3. Extraction and Isolation of Constituents

The extraction of *T. crispa* was performed in two different ways. Method I involved separation of the non-polar and polar fractions by percolation **(Scheme 1)** of the powdered stems (2.0 kg) with dichloromethane until no more color was observed to obtain a yield of 83.0 g. The material exhausted of its non-polar fraction was then sonicated with methanol [3 L] to obtain a yield of 35.0 g. The dichloromethane extract was fractionated by VLC [silica gel (93 cm × 15 cm), hexanes: chloroform, 100-0:0-100; chloroform: methanol, 9.5:0.5-9:1 and acetone] to obtain six nonpolar fractions 1.1-1.8. **Cycloeucalenol** (7.0 mg) was obtained from the fraction 1.5 (36.0 g) by repeated column chromatography [silica gel (55 cm × 7.5 cm),

hexanes:chloroform 3:7, (2 L); silica gel (91 cm × 3.8 cm) hexanes:chloroform, 50:50-0:100; silica gel (32 cm × 2.5cm), hexanes: EtOAc 9.5:0.5]. **Cycloeucalenone** (13.0 mg) was isolated from Fr. 1.6 (1.4.0 g) by repeated column chromatography [Sep-Pak cartridge (10.0 g) water:methanol 1:9; silica gel (38 cm × 2.5 cm), hexanes:chloroform, 6:4]. The methanolic fraction (33.0 g) was fractionated by column chromatography using gradient elution [silica gel, (55 cm × 7 cm), ethyl acetate: chloroform:methanol:water, 15:8:2:0.5 (5 L), 15:8:4:1 (8.4 L) and 6:4:4:1 (6 L)] to obtain twenty fractions, Fr.2.1-2.20. Fraction 2.2 (89.0 mg) was chromatographed [silica gel (24 cm × 1.5 cm), chloroform (250 mL); silica gel (70 cm × 1 cm), hexane:ethyl acetate 7:3 (800 mL)] to obtain **naringenin** (17.0 mg). **Syringin** (71.0 mg) was obtained from fraction 2.15 (1.0 g) by column chromatography over [RP C_{18} silica gel (55 cm × 2.5 cm), methanol:water 1:9-4:6 (13 L); silica gel (33 cm × 2.5 cm), chloroform:methanol:water 8:2:0.25 (500 mL)]. Fraction 2.17 (700.0 mg) was subjected to column chromatography [Sephadex LH-20 (60 cm × 2.5 cm), methanol (500 mL); silica gel (30 cm × 1 cm), chloroform:methanol: water 9:1:0.1 (1.5 L)] to obtain **palmatine** (4.0 mg). **Steponine** (52.0 mg) was obtained from fraction 2.19 (1.5.0 g) by repeated chromatography [LH-20 (60 cm × 2.5 cm), MeOH (400 mL); silica gel (25 cm × 2 cm), chloroform:methanol:water 8.5:1.5:0.5 (800 mL); sephadex LH-20 (30 cm × 2.5cm), MeOH (150 mL)]. **Borapetoside B** (15.0 mg) was purified from fraction 2.14 (4.0 gm) by column chromatography [silica gel (40 cm × 2.5 cm), chloroform:methanol:water, 8.5:1.5:0.5; RP C-18 (25 cm × 2 cm), methanol:water 6:4 (800 mL); Sephadex LH-20 (51 cm × 3 cm) methanol:chloroform 1:1 (800 mL); silica gel (109 cm × 2 cm), chloroform:methanol:water 8.5:1.5:0.5]. **Borapetoside F** (40.0 mg, RP C_{18}, 5 mL/min, t_R= 32.6

min) and **borapetoside C** (100.0 mg, RP_{18}, 5 mL/min, t_R= 34 min) were purified using preparative HPLC.

A second method of extraction **(Scheme. 2)** was carried out in order to optimize the extraction procedure and maximize the yield of the borapetosides. The method involved sonication of the dried powdered stems of *T. crispa* (2.0 kg) with methanol [8 L] to obtain the methanolic extract (110.0 g). The methanolic extract was fractionated successively with hexanes, chloroform, ethyl acetate and n-butanol. Each fraction was concentrated separately under vacuum to afford 19.0 g (hexanes), 22.0 g (chloroform), 12.0 g (ethyl acetate) and 44.0 g (n-butanolic) fractions, respectively. The hexane fraction (19.0 g) CC over silica gel (92 cm × 5 cm) using gradient elution with hexanes:chloroform 100:0-0:100 followed by chloroform:methanol 100:0:95:5 (3 L) to obtain 58 sub-fractions. **(-)-Pinoresinol** (3.7 mg) and **lysicamine** (2.0 mg) were isolated from sub-fraction 21 by repeated CC over sephadex LH-20 (81 cm × 2.5 cm) using chloroform:methanol 5:5 (195 mL) ; silica gel (33 cm × 12 cm) hexanes:ethylacetate:acetone 4:5:1 (600 mL).

The chloroform fraction was chromatographed on RP C_{18} using gradient elution with methanol: water, 5:5-7.5:2.5 (3L) to obtain seven sub-fractions, C_1-C_7. **(6*S*,9*R*)-vomifoliol** (5 mg) was isolated from sub-fraction C_1 (2.0 g) by CC over silica gel (35 cm × 2.5 cm) with gradient elution using chloroform: methanol 100:0-0:100 (6L) and purified by PTLC (20 × 20, 500µm) using ethyl acetate:chloroform:methanol 34:16:1 as the mobile phase. Subfraction C_2 (9.2 g) was chromatographed on silica gel (45 cm × 6 cm) by gradient elution using chloroform:methanol 100:0-9:1 to get 46 sub-fractions (C_21-C_246). **Borapetoside F** (60.3 mg, RP C_{18}, 5mL/min, t_R=32.6 min) and **borapetoside C** (287.4 mg, RP C_{18}, 5mL/min, t_R= 34 min)

were purified from $C_2$40 (319.0 mg) on preparative HPLC using a C_{18} bounded silica gel column as the stationary phase with water-methanol (90:10-100:0). **Columbin** (20.0 mg) was purified using CC over silica gel (34 cm × 2 cm) with hexanes:ethylacetate 7:3 (1.5 L) from fraction $C_2$10. **Baenzigeride A** (56.3 mg) and **tinocrispide** (3.3 mg) were purified from $C_2$18 (153 mg) by silica gel CC (78 cm × 2 cm) using hexanes: ethylacetate 6:4 (1L). **N-trans-feruloyl tyramine** (100.0 mg, RP C_{18}, 5mL/min, t_R= 25.5 min) was purified on preparative HPLC (RP C-18). **Borapetol A** (3.3 mg) was purified from $C_2$25 by CC silica gel (46 cm × 2.5 cm) with hexanes:ethylacetate 3:2 (700 mL). The ethyl acetate fraction (12.0 g) was subjected to CC over Sephadex LH-20 using methanol to obtain eight subfractions E_1-E_8. Subfraction E_2 (9.0 g) was eluted in order of increasing polarity with chloroform:methanol, 100:0-9.25:0.75 (1.6 L) by CC over silica gel (45 cm × 2.5 cm) to obtain nine subfractions, $E_2$1-$E_2$9. **(2*R*,5*R*,6*S*,9*S*,10*S*,12*S*)-15,16-Epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,7,13(16),14-tetraen-17,12-olid-18-oic acid methyl ester** (7.0 mg, RP C_{18}, 5mL/min, t_R= 23 min) was purified from $E_2$5 (95.0 mg) on preparative HPLC (RP C_{18}). **Borapetoside B** (353.0 mg) and **(2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*) -15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester** (38 mg) were purified from $E_2$8 over silica gel CC (45 cm × 2.5 cm) using chloroform:methanol:water 9:1:0.1 (1L). The chemical constituents isolated from *T. crispa* **(Fig.2.2)** are enumerated as follows

2.3.1. ***Tinocrispide*** (**1**): White amorphous powder, HRESIMS *m/z* 403.1408 $[M+HCO_2H]^-$ (calcd. for $[C_{20}H_{22}O_6$-H+$HCO_2H]$, (403.1393); ν_{max} 3416, 1710, 1742, 1647 cm^{-1}; $[\alpha]^{25}_D$: - 29 (c = 0.1, MeOH); ^{1}H NMR (400 MHz, chloroform-*d*) δ_H 7.46 (1H, dt, *J* = 1.7, 0.8 Hz, H-16), 7.43 (1H, t, *J* = 1.7 Hz, H-15), 7.06 (1H, dd, *J* = 7.3, 2.3 Hz, H-7), 6.40 (1H, dd, *J* = 1.7, 0.8 Hz, H-

14), 5.63 (1H, dd, *J* = 11.8, 4.5 Hz, H-12), 4.62 (1H, t, *J* = 9.1 Hz, H3α), 4.03 (1H, dd, *J* = 9.1, 4.0 Hz, H-3β), 2.92 (1H, dddd, *J* =9.9, 9.1, 4.0, ,2.0 Hz, H-2), 2.47 (1H, dd, *J* =18.7, 2.3 Hz, H-6β), 2.21 (1H, overlapped, H-1α), 2.19 (1H, dd, *J* = 13.6, 11.8 Hz, H-11α), 2.16 (1H, dd, *J* = 18.7, 7.3 Hz, H-6α), 1.96 (1H, dd, *J* =13.6, 4.5 Hz, H-11β), 1.81 (1H, overlapped, H-10), 1.79 (1H, ddd, *J* = 16.9, 7.0, 2.0 Hz, H-1β), 1.30 (3H, s, H_3-20), 1.18 (3H, s, H_3-19); ^{13}C NMR (101 MHz, chloroform-*d*) δ_C 177.9 (C-18), 165.9 (C-17), 143.8 (C-15), 139.5 (C-16), 137.6 (C-7), 133.2 (C-8), 125.2 (C-13), 108.4 (C-14), 86.5 (C-4), 73.2 (C-3), 71.3 (C-12), 52.3 (C-10), 47.9 (C-5), 43.0 (C-2), 42.6 (C-11), 35.3 (C-9), 33.5 (C-1), 30.5 (C-6), 23.1 (C-19), 21.5 (C-20).

2.3.2. ***Baenzigeride A*** (**2**) White amorphous powder, HRESIMS *m/z* 357.1840 $[M-H]^-$ (calcd. for $[C_{20}H_{22}O_6$ -H], (357.1338); ^{1}H and ^{13}C NMR data matched with the literature (Tuntiwachwuttikul,et al., 1999)

2.3.3. ***Columbin*** (**3**) White crystals; HRESIMS *m/z* 393.1126 $[M+Cl]^-$ (calcd. for $[C_{20}H_{22}O_6$+ $Cl]^-$, (393.1105); ^{1}H and ^{13}C NMR data matched with the literature (Lam et al., 2012)

2.3.4. ***Borapetol A*** (**4**): White amorphous solid; HRESIMS *m/z* 421.1517 $[M-H+HCO_2H]^-$ (calcd. for $[C_{20}H_{24}O_7$-H+$HCO_2H]^-$, (421.1499); ^{1}H NMR (400 MHz, chloroform-*d*) δ_H 7.51 (brs, 1H, H-16), 7.46 (t, *J* = 1.7 Hz, 1H, H-15), 6.40 (d, *J* = 1.7 Hz, 1H, H-14), 5.70 (dd, *J* = 12.6, 4.7 Hz, 1H, H-12), 5.06 (dd, *J* = 12.6, 4.7 Hz, 1H, H-6), 4.03 (t, *J* = 3.2, 1H, H-3), 2.73 (dd, *J* =12.6, 4.7 Hz, 1H, H-8), 2.43 (dt, *J* =12.6, 4.7 Hz, 1H, H-7β), 2.36 (dd, *J* =15.4, 4.7 Hz, 1H, H-11α), 2.21 (q like, 12.6 Hz, 1H, H-7α), 2.01 (dd *J* = 15.4, 12.6 Hz, 1H, H-11β), 1.95 (overlapped, 1H, H-2a), 1.92 (overlapped, 1H, H-10), 1.85 (overlapped, 1H, H-2b), 1.71 (overlapped, 2H, H_2-1), 1.25 (s, 3H, H_3-19), 1.18 (s, 3H, H_3-20); ^{13}C NMR (101 MHz, chloroform-*d*) δ_C 179.3 (C-18), 172.6 (C-17), 143.9 (C-16), 139.7 (C-15), 124.2 (C-13), 108.4 (C-14), 80.8 (C-4), 76.0 (C-6),

71.8 (C-3), 70.7 (C-12), 47.4 (C-10), 47.1 (C-8), 46.1 (C-5), 44.2 (C-11), 35.1 (C-9), 33.1 (C-20), 28.3 (C-2), 25.5 (C-7), 17.9 (C-1), 17.5 (C-19). (Fukuda et al., 1985)

2.3.5. ***Borapetoside B*** (**5**): White crystals; HRESIMS *m/z* 597.2203 $[M-H+HCO_2H]^-$ (calcd. for $[C_{27}H_{36}O_{12}-H+HCO_2H]$, (597.2183); ^1H and ^{13}C NMR data matched with the literature (M. I. Choudhary et al. 2010).

2.3.6. ***Borapetoside C*** (**6**): White crystals; HRESIMS *m/z* 581.2273 $[M-H+HCO_2H]^-$ (calcd. for $[C_{27}H_{36}O_{11}-H+HCO_2H]$, (581.2234); ^1H and ^{13}C NMR data matched with the literature (M. I. Choudhary et al. 2010)

2.3.7. ***Borapetoside F*** (**7**): White amorphous powder; HRESIMS *m/z* 579.2105 $[M-H+HCO_2H]^-$ (calcd. for $[C_{27}H_{34}O_{11}-H+HCO_2H]$, (579.2078); ^1H and ^{13}C NMR data matched with the literature (Martin et al., 1996)

2.3.8. ***(2R,5R,6R,8R,9S,10S,12S)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oic acid methyl ester*** (**8**): Amorphous, colorless solid; HRESIMS *m/z* 597.2203 $[M-H+HCO_2H]^-$ (calcd. for $[C_{27}H_{36}O_{12}-H+HCO_2H]$, (597.2183); ^1H and ^{13}C NMR data matched with the literature (M. I. Choudhary et al. 2010)

2.3.9. ***(2R,5R,6S,9S,10S,12S)-15,16-epoxy-2-hydroxy-6-O- (β-D-glucopypranosyl)-cleroda-3,7,13(16),14-tetraen-17,12-olid-18-oic acid methyl ester*** (**9**): Amorphous, colorless solid HRESIMS *m/z* 595.2058 $[M-H+HCO_2H]^-$ (calcd. for $[C_{27}H_{34}O_{12}-H+HCO_2H]$, (595.2027); ^1H and ^{13}C NMR data matched with the literature (M. I. Choudhary et al. 2010).

2.3.10. ***Cycloeucalenol*** (**10**): White amorphous solid; HRESIMS *m/z* 471.3879 $[M-H+HCOOH]^-$ (calcd. for $[C_{30}H_{50}O-H+HCOOH]$, (471.3838); ^1H and ^{13}C NMR data matched with the literature (Khuong-huu et al. 1975; Kongkathip et al. 2002).

2.3.11. *Cycloeucalenone* (**11**): White amorphous powder; HRESIMS *m/z* 447.3633[M+Na] $^{+}$ (calcd. for [$C_{30}H_{48}O$+Na], (447.3603); ^{1}H and ^{13}C NMR data matched with the literature (Khuong-huu et al., 1975; Kongkathip et al., 2002).

2.3.12. *(6S, 9R)-Vomifoliol* (**12**): Colorless crystals, $[\alpha]^{25}_{D}$: +240 (c=0.1, MeOH), HRESIMS *m/z* 269.1394 [M-H+HCO_2H]$^{-}$ (calcd. for [$C_{26}H_{32}O_{11}$-H+HCO_2H], (269.1389); ^{1}H and ^{13}C NMR data matched with the literature (Yamano and Ito 2005).

2.3.13. *(-)-Pinoresinol* (**13**): Brownish solid; $[\alpha]^{25}_{D}$: -39 (c=0.1, MeOH), HRESIMS *m/z* 403.1290 [M-H+HCOOH]$^{-}$ (calcd. for [$C_{20}H_{22}O_6$-H+HCOOH], (403.1393); ^{1}H and ^{13}C NMR data matched with the literature (Greca et al., 1998)

2.3.14. *Syringin* (**14**): White, crystals; HRESIMS *m/z* 395.1326 [M+Na]$^{+}$ (calcd. for [$C_{17}H_{24}O_9$+ Na], (395.1318); ^{1}H and ^{13}C NMR data matched with the literature (Wang et al., 2014).

2.3.15. *Lysicamine* (**15**): Amorphous powder HRESIMS *m/z* 314.0808 [M+Na]$^{+}$ (calcd. for [$C_{18}H_{13}NO_3$+ Na], (314.0793); ^{1}H and ^{13}C NMR data matched with the literature (Costa et al. 2011)(Xu et al. 2013).

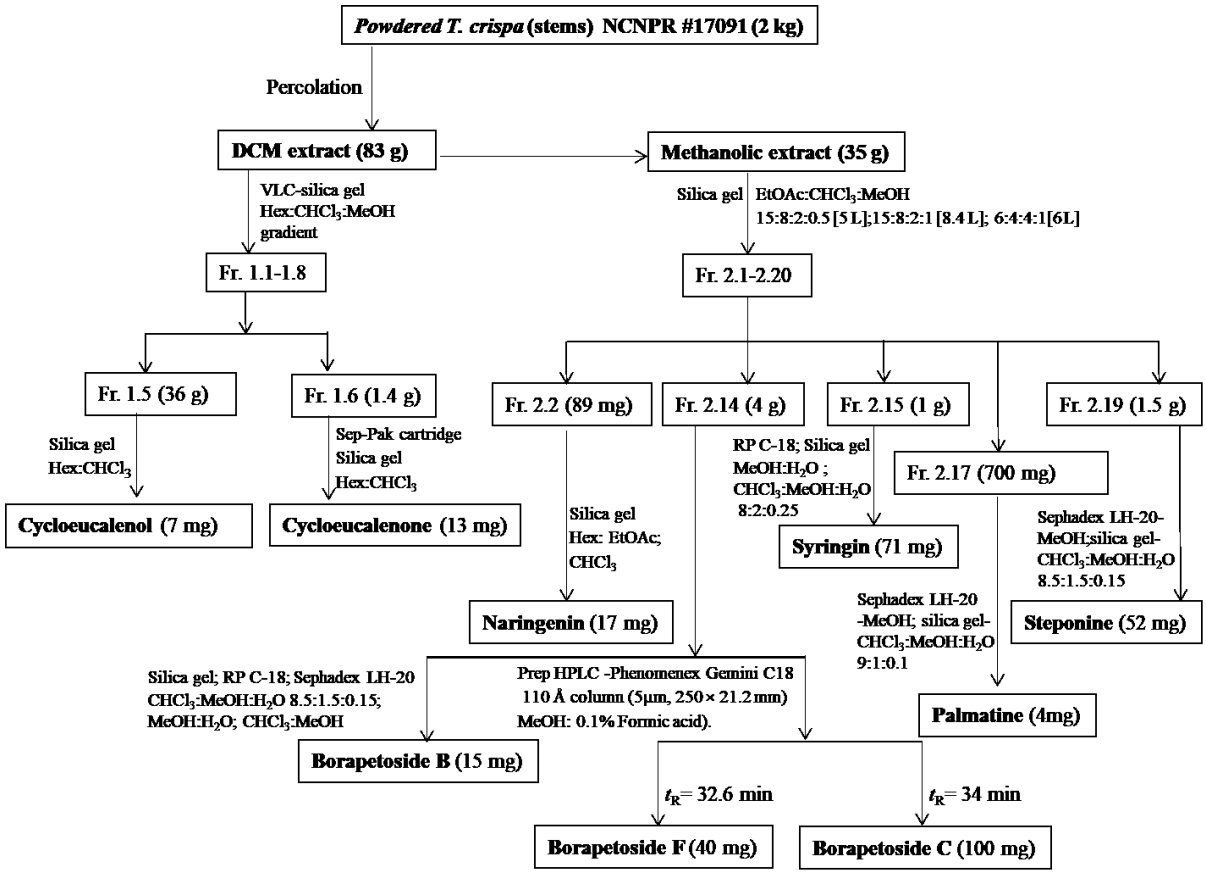
2.3.16. *Palmatine* (**16**): Yellowish solid; HRESIMS *m/z* 352.1560 [M+H]$^{+}$ (calcd. for [$C_{21}H_{22}NO_4$+H], (352.1549); ^{1}H and ^{13}C NMR data matched with the literature (Yu et al. 2014).

2.3.17. *Naringenin* (**17**): Yellowish brown crystals, HRESIMS *m/z* 271.0626 [M-H]$^{-}$ (calcd. for [$C_{15}H_{12}O_5$-H], (271.0606); ^{1}H and ^{13}C NMR data matched with the literature (Chen et al., 2010).

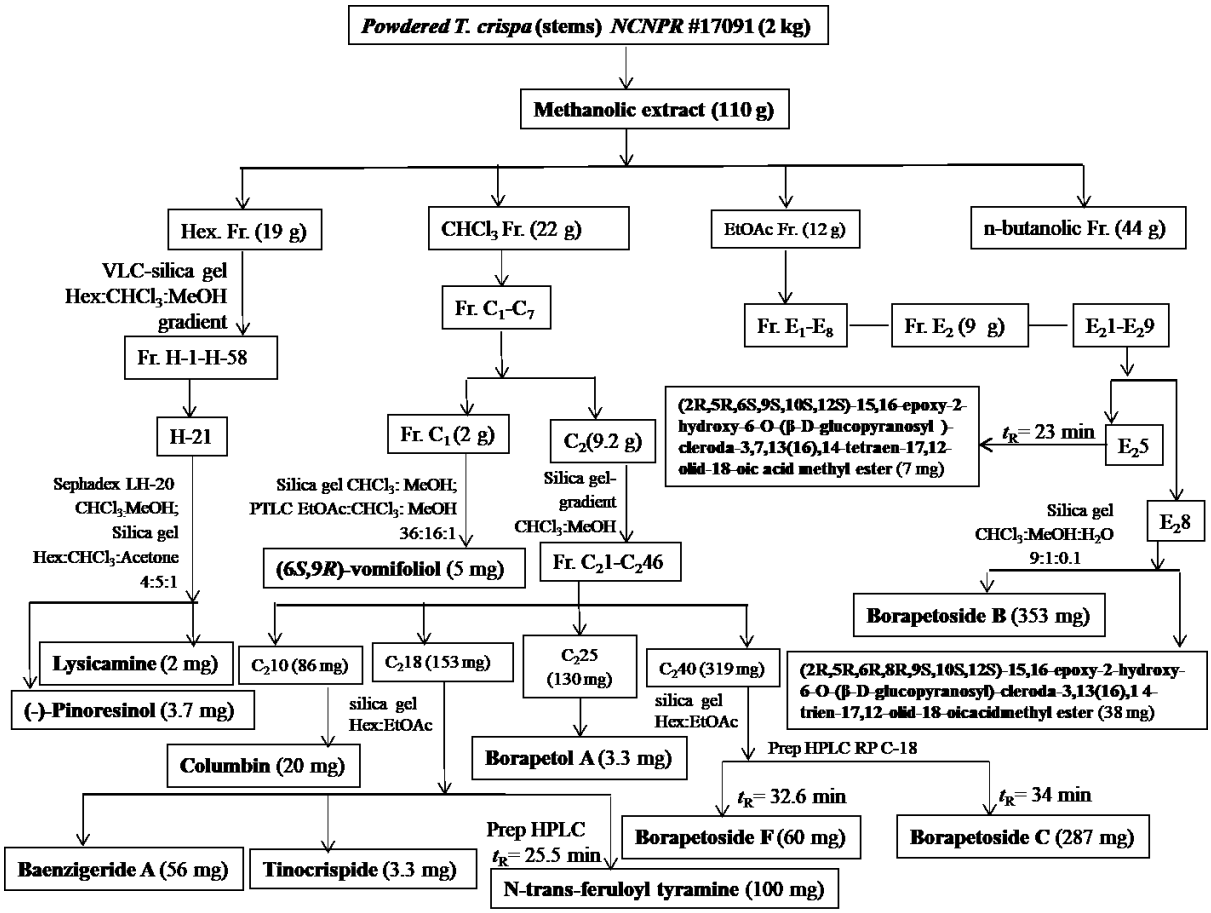
2.3.18. *N-trans feruloyl tyramine* (**18**): Colorless crystals; HRESIMS *m/z* 314.1381 [M+H]$^{+}$ (calcd. for [$C_{18}H_{19}NO_4$+H], (314.1392); ^{1}H and ^{13}C NMR data matched with the literature (Maurya and Handa, 1998)

2.3.19. *Steponine* (**19**): Yellowish red crystals, $[\alpha]^{25}_{D}$: -29 (c=0.1, MeOH), HRESIMS *m/z* 342.1701 $[M+H]^+$ (calcd. for $[C_{20}H_{24}NO^+_4+H]$, (342.1700); ^1H and ^{13}C NMR data matched with the literature (Tanahashi et al., 2000)

Scheme 1 First extraction and isolation scheme of *T. crispa*



Scheme 2 Second extraction and isolation scheme of *T. crispa*



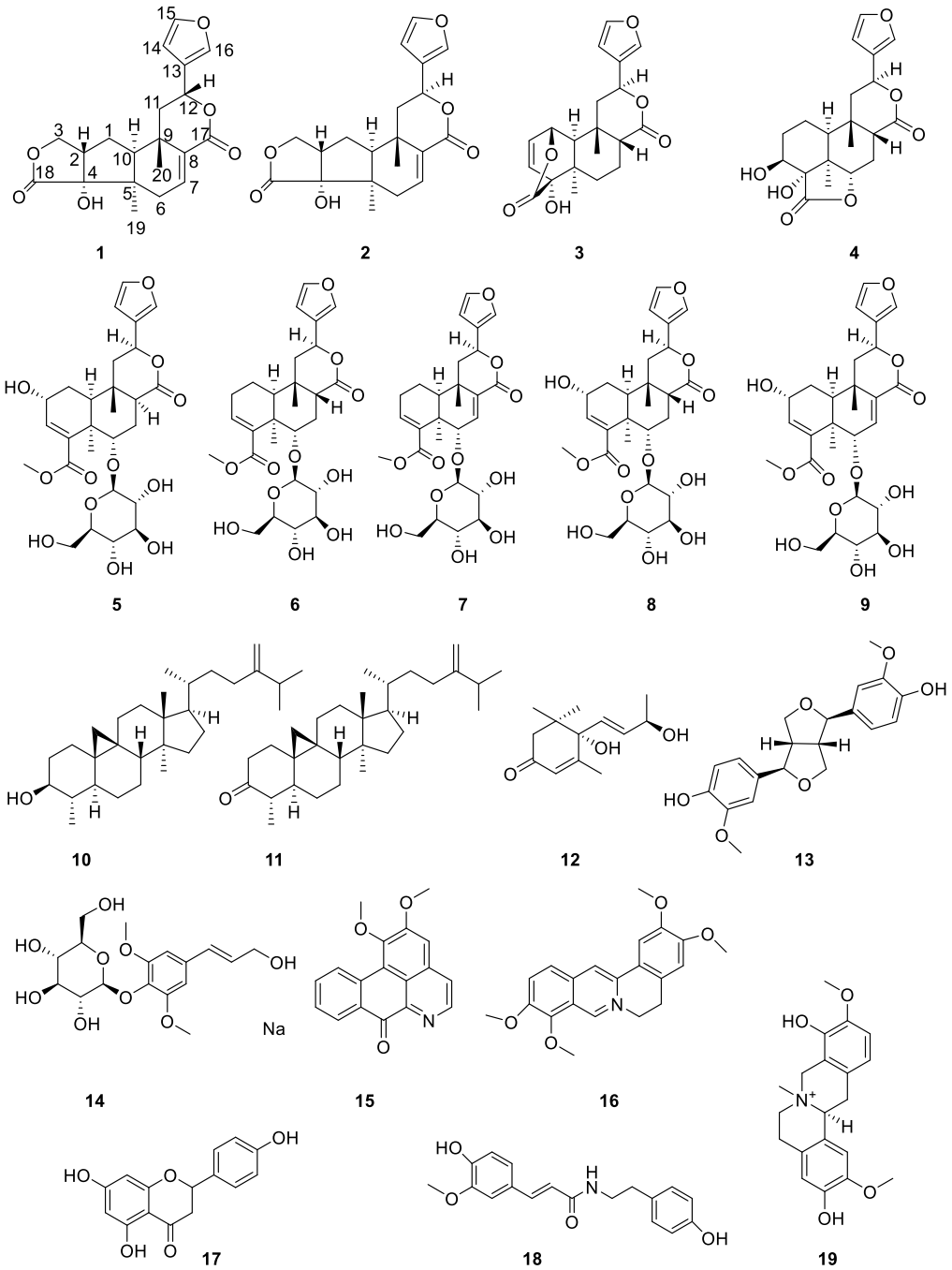


Figure 2.2. Structures of compounds isolated from *T. crispa*

2.3. Results and Discussion

The phytochemical investigation of *T crispa* was carried out utilizing two different isolation schemes. The first attempt led to the isolation of nine known compounds, namely borapetosides B, C and F, naringenin, syringin, palmatine, cycloeucalenol, cycloeucalenone and steponine. Due to the low yield of borapetosides, the isolation scheme was optimized to improve the yields as well as make the process time efficient. The second approach led to improved yields of borapetosides as well as the isolation of a new compound, named tinocrispide. Baenzigeride A, previously reported from *T. baenzigeri*, was isolated and is being reported for the first time from *T. crispa.* Thirteen compounds were isolated from the second attempt. The spectroscopic data of the known compounds were in agreement with the published data. The ^{13}C NMR data of borapetol A has been revised.

Tinocrispide (**1**) was obtained as white amorphous powder and HRESIMS showed [M-H+HCOOH]$^-$ deprotonated molecular ion adduct at *m/z* 403.1408, (calcd. for $C_{20}H_{22}O_6$-H+HCOOH, 403.1393). The absorptions at 3416, 1710, 1742 and 1647 cm^{-1} in the IR spectrum showed the presence of hydroxy, lactone carbonyl and olefin functions. The decoupled ^{13}C NMR spectrum showed twenty resonances which were differentiated by DEPT 135 NMR spectrum as two methyl, five methylene, six methine, and seven non-protonated carbons. The ^1H NMR spectrum exhibited singlets at δ_H 1.18 and 1.30 corresponding to the protons of the tertiary methyl groups [CH_3-19 and CH_3-20]. The resonances at δ_H 7.46 (dt, J = 1.7, 0.8 Hz, H-16), 7.43 (t, J = 1.7 Hz, H-15), 6.40 (dd, J = 1.7, 0.8 Hz, H-14) were assigned to the protons of a furan ring. The ^{13}C NMR showed three olefinic methine carbons (δ_C 143.8, 139.5 and 108.4), and an olefinic non-protonated carbon (δ_C 125.2) of the furan moiety in consistence with the ^1H NMR.

Further analysis of ^{1}H and ^{13}C NMR data **(Table 2.1)** revealed the presence of an olefinic bond [δ_H/δ_C 7.06 (dd, *J* = 7.3, 2.3 Hz, H-7)/137.6 and δ_C 133.2 (C-8)], an oxymethine [δ_H/δ_C 5.63 (dd, *J* = 11.8, 4.5 Hz, H-12)/71.3 (C-12)], an oxymethylene [δ_H/δ_C 4.62 (t, *J* = 9.1 Hz, H-3α) and 4.03 (dd, *J* = 9.1, 4.0 Hz, H-3β)/73.2 (C-3)], an oxygenated tertiary carbon [δ_C 86.5 (C-4)] in addition to three methylenes (CH_2-1, CH_2-6, CH_2-11) and two methines (CH-2 and CH-10). Moreover, the ^{13}C NMR spectrum of **1** displayed resonances for two carbonyl carbons at δ_C 165.9 (C-17) and δ_C 177.9 (C-18) and two quaternary carbons at δ_C 47.9 (C-5) and δ_C 35.3 (C-9). The position of a furan moiety was confirmed by the HMBC correlations of H-12 (δ_H 5.63) with C-13 (δ_C 125.2), C-14 (δ_C 108.4) and C-16 (δ_C 139.5). H-12 (δ_H 5.63) showed further correlation with carbonyl carbon at δ_C 165.9 (C-17) confirming the δ-valerolactone (six-member lactone) unit. Similarly, γ-butyrolactone (five membered lactone) was confirmed by HMBC correlations of C-3 methylene protons (δ_H 4.62 and 4.03) with carbonyl carbon at δ_C 177.9 (C-18). The location of CH_3-19 was assigned by the correlation of its protons (δ_H 1.18) with C-6 (δ_C 30.4), C-10 (δ_C 52.3) and C-4 (δ_C 86.5) and C-5 (δ_C 47.9), which ultimately confirmed the position of hydroxy group at C-4. Similarly, the HMBC correlations **(Fig.2.11)** of the methyl protons (CH_3-20, δ_H 1.30) were observed with C-11 (δ_C 42.6), C-10 (δ_C 52.3), C-9 (δ_C 35.2) and C-8 (δ_C 133.2) leading to the confirmation of its position at C-9. The relative configuration of compound 1 was derived from the NOESY correlations **(Fig.2.12)**. A biogenetically β oriented methyl group at C-9, (CH_3-20, δ_H 1.30) showed NOESY correlations with H-12 (δ_H 5.63) and H-2 (δ_H 2.92) confirming their β-orientations. The correlations shown by H-2 (δ_H 2.92) with the methyl protons at C-9 is only feasible when the hydroxy group at C-4 is α oriented, ultimately confirming α-orientation of the hydroxy group at C-4. The α orientation of H-10 (δ_H 1.81) was deduced from

its NOESY correlation with the biogenetically α oriented methyl protons (CH_3-19, δ_H 1.18) at C-5. Compounds **1** and **2**, both isolated in our study were found to be C-12 epimers. Their ^{13}C NMR data displayed 4-6 ppm difference for CH-7, C-17, and CH_3-20 **(Table 2.1, 2.2)**. H-12 showed NOESY correlation with a biogenetically α oriented methyl group at C-5 (CH_3-19 δ_H 1.25) in compound **2** in contrast to **1**, where a NOESY interaction was observed between H-12 and CH_3-20 **(Fig. 2.12)**. Furthermore, H-11β (δ_H 1.96 in **1** and δ_H 2.01 in **2**) displayed NOESY correlation with β oriented CH_3-20 in compounds **1** and **2** and appeared as a double doublet (dd) with coupling constant of $J_{11\alpha\text{-}11\beta}$=13.6 Hz and $J_{11\beta\text{-}12\beta}$=4.5 Hz in **1** and $J_{11\alpha\text{-}11\beta}$=14.0 Hz and $J_{11\beta\text{-}12\alpha}$=11.3 Hz in **2**. A relatively small vicinal coupling constant ($J_{11\beta\text{-}12\beta}$ =4.5 Hz in 1 and a larger value ($J_{11\beta\text{-}12\alpha}$=11.3 Hz) in **2** for H-11β confirmed its placement as equatorial in 1 and axial in 2. Moreover, in compound **1** the H-11α (δ_H 2.19, dd $J_{11\alpha\text{-}11\beta}$=13.6 Hz and $J_{11\alpha\text{-}12\beta}$ =11.8 Hz) showed NOESY correlation with α-oriented CH3-19 and a larger coupling constant with H-12 (11.8 Hz) confirming its axial orientation **(Fig. 2.12)**, thereby confirming the elucidation of tinocrispide (**1**) **(Fig. 2.2.)**

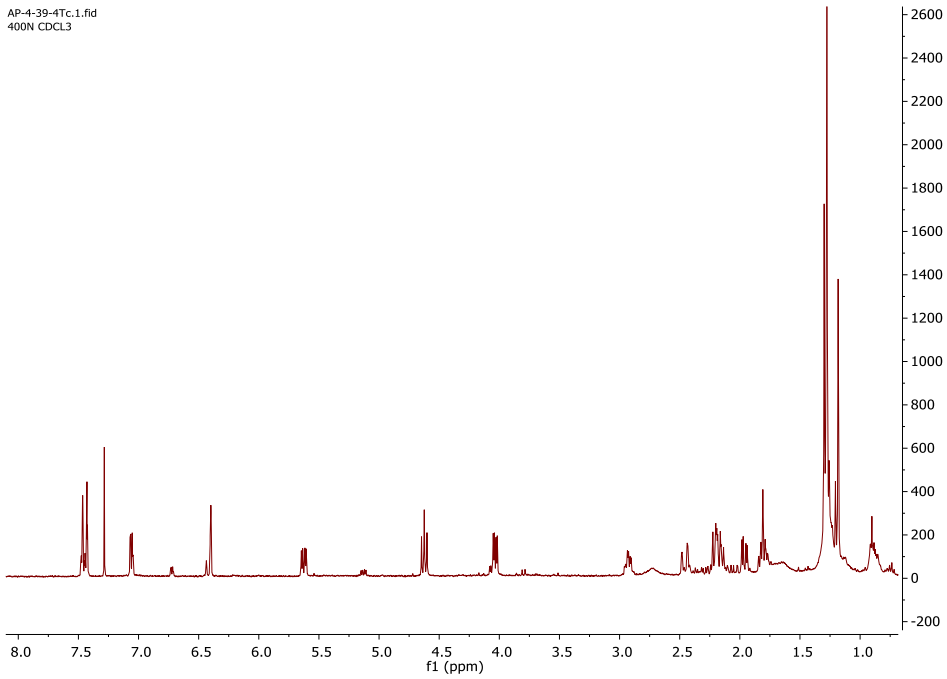


Figure 2.3. ^{1}H NMR spectrum of tinocrispide

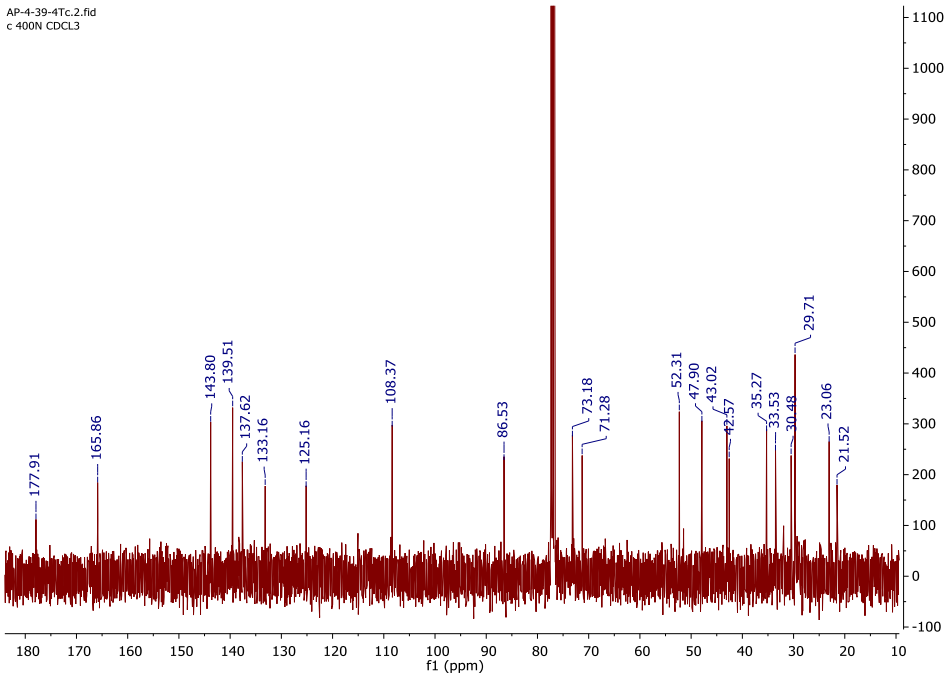


Figure 2.4. ^{13}C NMR spectrum of tinocrispide

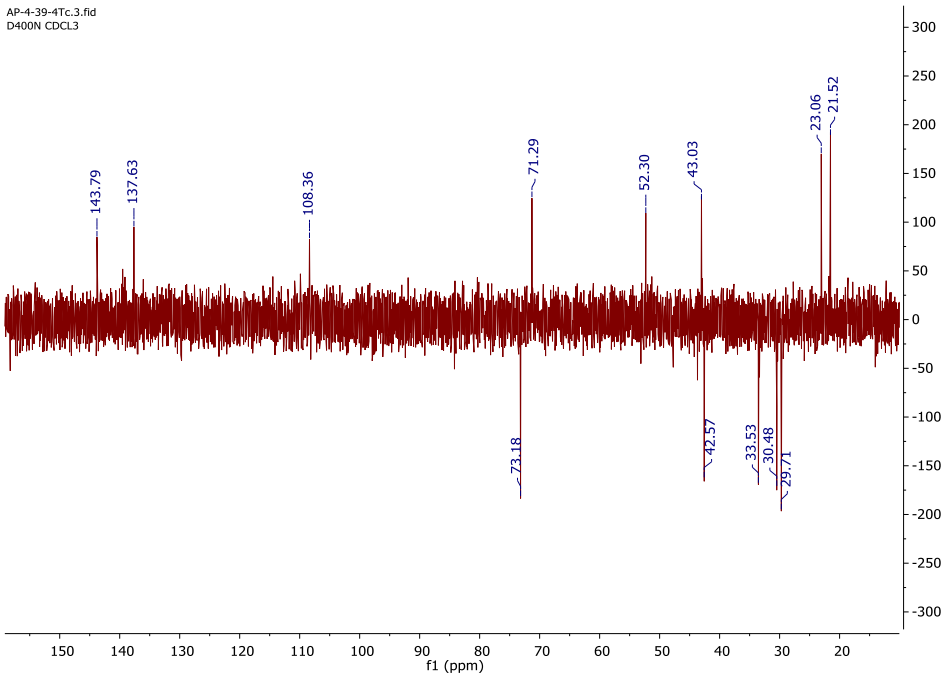


Figure 2.5. DEPT 135 NMR spectrum of tinocrispide

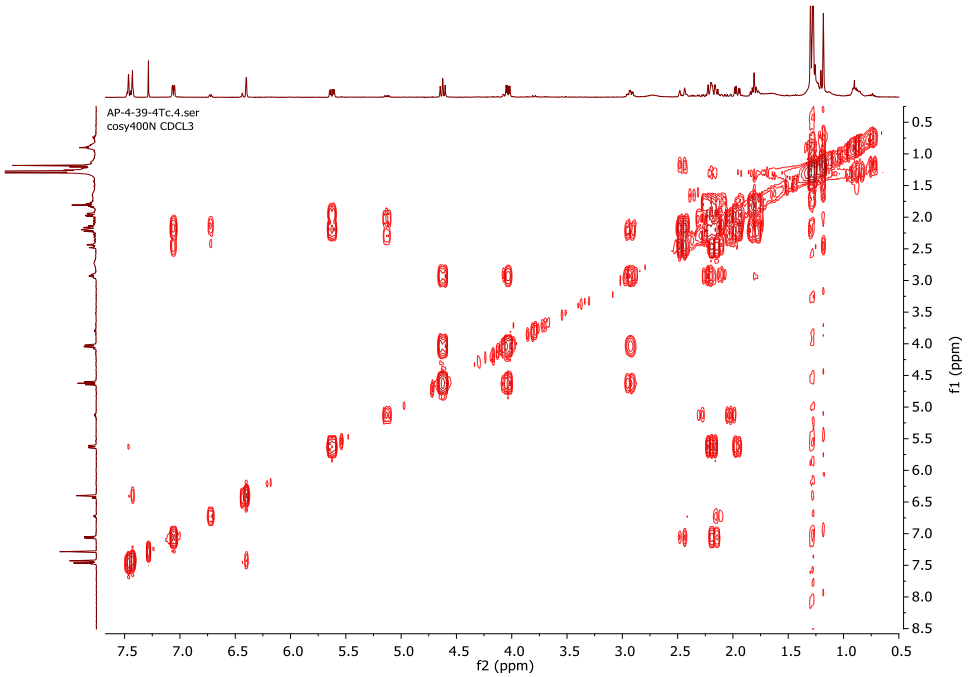


Figure 2.6. COSY spectrum of tinocrispide

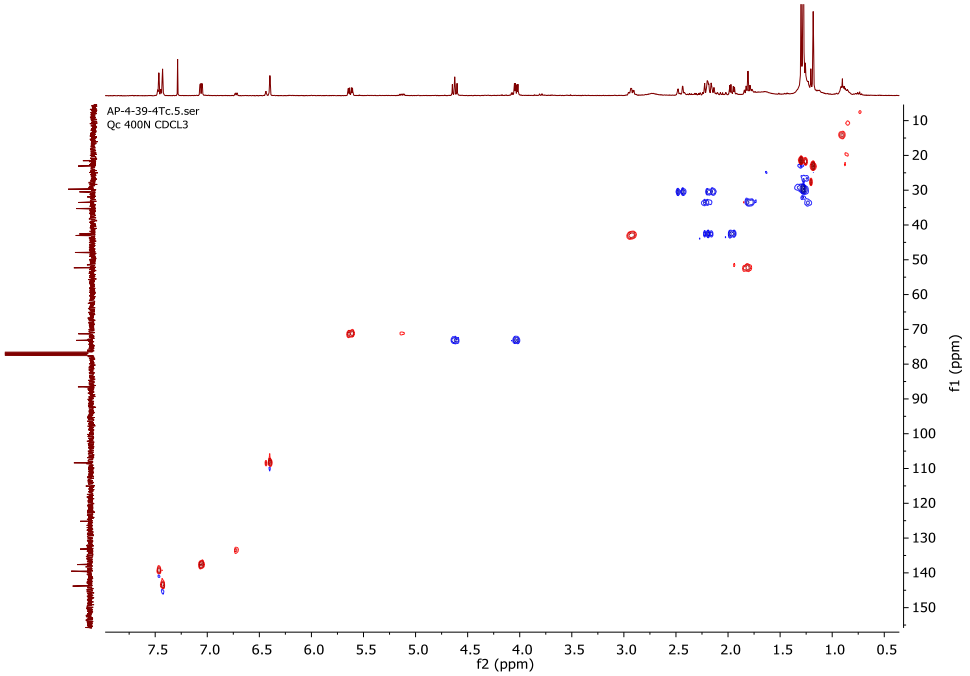


Figure 2.7 HSQC spectrum of tinocrispide

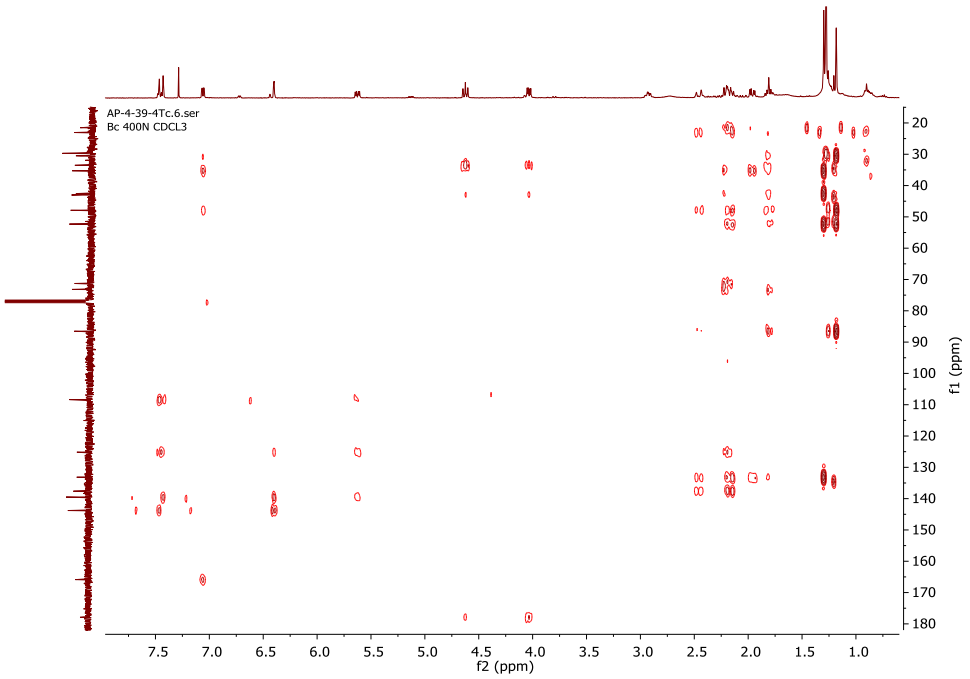


Figure 2.8. HMBC spectrum of tinocrispide

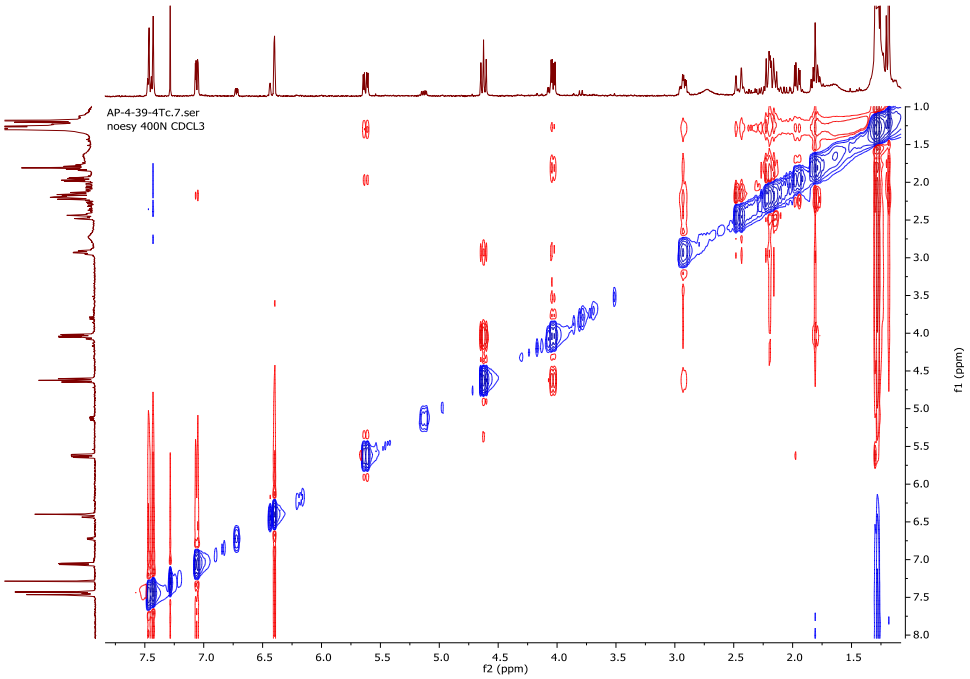


Figure 2.9. NOESY spectrum of tinocrispide

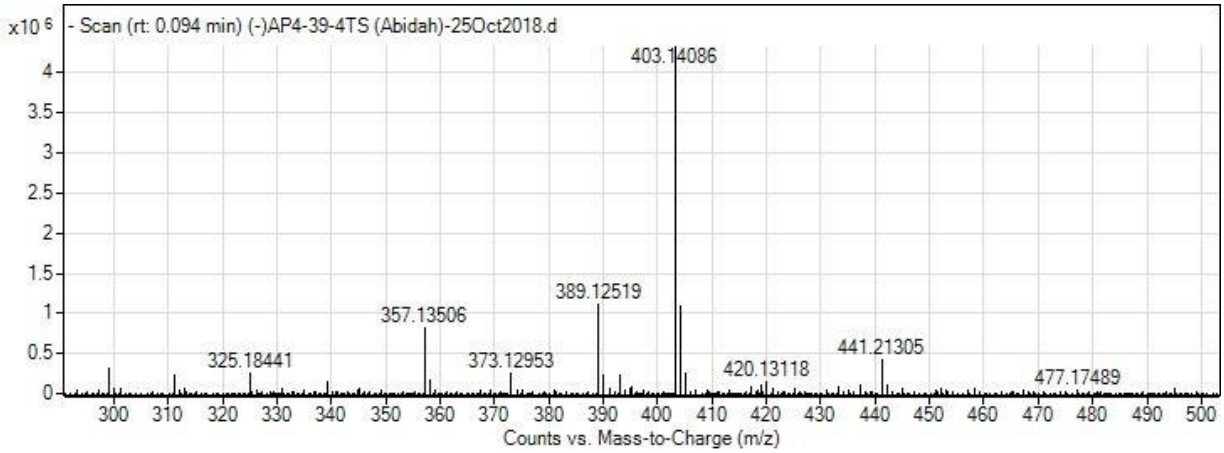


Figure 2.10. ESI mass spectrum of compound 1

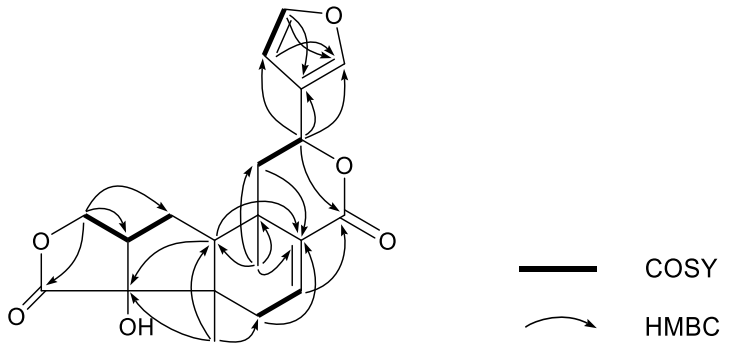


Figure 2.11.Key COSY and HMBC correlations for compound 1.

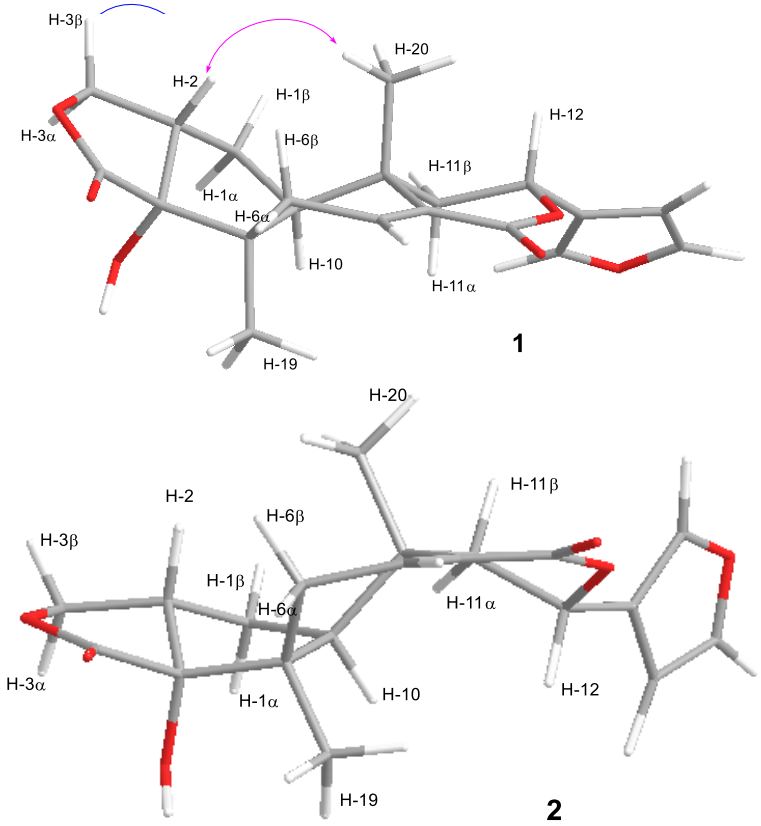


Figure 2.12. NOESY correlations for compounds 1 and 2

Table 2.1. $^{1}H^{a}$ NMR (400 MHz, chloroform-d) and13C NMR (101 MHz, chloroform-*d*) data of compound 1

Position	δ_C, Multiplicity	$\delta_H{}^{a}$ Multiplicity (*J* in Hz)
1	33.5, CH_2	1.79 ddd (16.9,7.0, 2.0), 2.2 (H-1β)
2	43.0, CH	2.92 dddd (9.9,9.1,4.0,2.0)
3	73.2, CH_2	4.03 dd (9.1,4.0, H-3α), 4.62 t (9.1, H-3β)
4	86.5, C	-
5	47.9, C	-
6	30.5, CH_2	2.16 dd (18.7, 7.3, H-6α), 2.47 dd (18.7, 2.3, H-6β)
7	137.6, CH	7.06 dd (7.3, 2.3)
8	133.2, C	-
9	35.3, C	-
10	52.3, CH	1.81
11	42.6, CH_2	1.96 dd (13.6, 4.5, H-11β),2.19 dd (13.6, 11.8, H-11α)
12	71.3, CH	5.63 dd (11.8, 4.5)
13	125.2, C	-
14	108.4, CH	6.40 dd (1.7, 0.8)
15	143.8, CH	7.43 t (1.7)
16	139.5, CH	7.46 dt (1.7, 0.8)
17	165.9, C	-
18	177.9, C	-
19	23.1, CH_3	1.18 s
20	21.5, CH_3	1.30 s

[a] Multiplicity is not clear for some signals due to overlapping

Table 2.2. $^{1}H^{a}$ NMR (400 MHz, chloroform-*d*) and ^{13}C NMR (101 MHz, chloroform-*d*) data of compound 2

Position	δ_C, Multiplicity	$\delta_H{}^{a}$ Multiplicity (*J* in Hz)
1	33.5, CH_2	1.76, ddd (13.3,7.0, 1.9, H-1β), 2.10 (H-1α)
2	43.0, CH	2.90 dddd (10.0,8.3,3.5,1.9)
3	73.4, CH_2	4.06 dd (9.5,3.5, H-3α), 4.62 dd (9.5, 8.3, H-3β)
4	86.6, C	-
5	47.4, C	-
6	30.5, CH_2	2.14 dd (18.7, 6.8, H-6α), 2.44 dd (18.7, 2.2, H-6β)
7	133.6, CH	6.72 dd (6.8, 2.2)
8	134.7, C	-
9	34.7, C	-
10	51.6, CH	1.93 dd (11.3,7.0)
11	43.8, CH_2	2.01 dd (14.0,11.3, H-11β), 2.29 dd (14, 5.2, H-11α)
12	71.3, CH	5.12 dd (11.3, 5.2)
13	124.0, C	-
14	108.8, CH	6.42 dd (1.7, 0.8)
15	143.9, CH	7.44 t (1.7)
16	139.8, CH	7.47 dt (1.7, 0.8)
17	169.9, C	-
18	177.9, C	-
19	21.9, CH_3	1.25 s
20	27.8, CH_3	1.20 s

[a] Multiplicity is not clear for some signals due to overlapping

Table 2.3. ^{1}H NMR (400 MHz, chloroform-*d*) and^{13}C NMR (101 MHz, chloroform-*d*) data of compound 4

Position	δ_C Mult.	δ_H Mult. (*J* in Hz)
1	17.9, CH2	1.71*
2	28.3, CH2	1.85*, 1.95*
3	71.8, CH	4.03, t (3.2)
4	80.8, C	-
5	46.1, C	-
6	76.0, CH	5.06 (dd 12.6, 4.7)
7	25.5, CH_2	2.43 (dt, 12.6, 4.7, H-7β), 2.21 (q like, 12.6, H-7α)
8	47.1, CH	2.73 dd (12.6, 4.7)
9	35.1, C	-
10	47.4, CH	1.92*
11	44.2, CH_2	2.36 (dd 15.4, 4.7, H-11α), 2.01 dd (15.4, 12.6, H-11β)
12	70.7, CH	5.70 dd (12.6, 4.7)
13	124.2, C	-
14	108.4, CH	6.40, d (1.7)
15	139.7, CH	7.46, t (1.7)
16	143.9, CH	7.51, brs
17	172.6, C	-
18	179.3, C	-
19	17.5, CH_3	1.25, s
20	33.1, CH_3	1.18, s

* Multiplicity is not clear for some signals due to overlapping.

2.4 Conclusion

The phytochemical investigation of *T. crispa* resulted in the isolation of nineteen compounds. The isolation methodology was optimized to improve the yield of the major furanoditerpenoids and make the process time efficient. To conclude, one new and eighteen known compounds were isolated from *T. crispa.* The assignment of chemical shift values for borapetol A (**4**) were revised. Baenzigeride A and steponine are being reported for the first time and tinocrispide was characterized as a new compound from *T. crispa*. The isolated borapetosides, borapetoside B, C and F were the furanoditerpenoids of interest to carry out the hepatotoxic evaluation as well as serve as important markers for analytical studies.

Chapter 3

PHYTOCHEMICAL CONSTITUENTS OF *TINOSPORA SINENSIS*

3.1. Introduction

Tinospora sinensis is of considerable importance to investigate in order to fully elucidate the quality control considerations associated with *Tinospora* plants. Indigenous to Southeast Asia, and widely recognized as a species commonly substituted for *T. cordifolia* (Raghunāthan, K., & Mitra 1982), *T. sinensis* is a herb of medicinal value in the Ayurvedic system of medicine. The phytochemical profile of *T. sinensis* is usually likened to *T. cordifolia*. However, the medicinal components of *T. sinensis* are comparatively less in amount. (G. V. Srinivasan et.al., 2008). Isolation of the phytoconstituents of *T. sinensis* was undertaken with the aim to elucidate the chemical diversity of *T. sinensis* in comparison to *T. crispa*.

The traditional use of *T. sinensis* is recorded in the Southeast Asian countries of China, India and Pakistan. In China, it is listed officially in the modern Jingzhu Bencao and in the Chinese Pharmacopoeia (Ed. 2015). Alleviation of pain and muscle rigidity, restoration of health and vigor, tranquility of mind and treatment of palpitation are among the attributes recorded in the Chinese Tibetan medicinal encyclopedia, Jingzhu Bencao and asserted by Chinese Dai Medicine. As the main ingredient in “Si Wei Zang Zang Mu Xiang” powder, a famous traditional formula, *T. sinensis* mitigates deficiency- heat syndrome and wind-heat disease (Chi

et al. 2016). The leaves and the stem are used for the treatment of rheumatism, gastritis, piles, ulcerated wounds and fractured bones (Hegde and Jayaraj 2016).

Previous studies on the stems of *T. sinensis* have validated its pharmacological potential as an anti-inflammatory (S.-H. Lam et al. 2018), anti-diabetic (Banerjee et al., 2017; Yonemitsu, Michiko, 1993), antileishmanial (Singh et al. 2008) and immunomodulatory (Manjrekar, Jolly, and Narayanan 2000). To date, over 90 phytoconstituents have been reported from *T. sinensis* **(Table 3.1)**.

Table 3.1: Summary of phytoconstituents isolated from *T. sinensis*

Compound Class	Reported compounds	References
Lignans (12)	(+)-pinoresinol, syringaresinol, medioresinol , (+)-epi-syringaresinol, (+)-pinoresinol monomethyl ether , (+)-glaberide I , sesamin, sesamolin, (+) pinoresinol O-β-D-glucopyranoside, (+)-pinoresinol monomethyl ether O-β-D-glucopyranoside, (+)-syringaresinol O-β-D-glucopyranoside, (-)-isolariciresinol 3α-O-β-D-glucopyranoside	(S.-H. Lam et al. 2018) (Chi et al. 2016) (Jiang, et.al., 2017) (Loi et. al., 2017)
Pyrrole alkaloids (4)	5-(hydroxymethyl)-1H-pyrrole-2-carbaldehyde, methyl 4-[formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoate , methyl 4-[formyl- 5-(methoxymethyl)-1H-pyrrol-1-yl] butanoate, 4-[formyl-5-(methoxymethyl)-1H-pyrrol-1-yl] butanoic acid ,4-[formyl-5-(hydroxymethyl)-1H-pyrrol-1-yl] butanoic acid	
Benzenoids (17)	rhodiolate, methyl ferulate , β-hydroxypropiovanillone, syringin, 2-methyl-4,5-dimethoxybenzoic acid, vanillic acid, salidroside, p-hydroxyl phenethanol, tachioside, icariside D2 , icariside D1, cordifolioside A, p-hydroxybenzoic acid, isovanillic acid , 4-(2-hydroxyethyl)benzoic acid, syringic acid-4-O-α-L-rhamnoside and syringic acid	
Terpenoids (10)	loliolide, abscisic acid , 3(17)-phytene 1,2-diol, malabarolide , lupeol , 3-O-acetyloleanolic acid , cycloeucalenol , cycloabyssinone , cycloartane-3β,25-diol , cycloart-22-ene-3β,25-diol	
Steroids (8)	stigmasterol, 7α-hydroxy-sitosterol , 7α-hydroxystigmasterol , 6β-hydroxystigmast-4-en-3-one , 6β-hydroxystigmasta-4,22-dien-3-one, 7-ketositosterol,3β-hydroxy-stigmasta-5,22-dien-7-one, cycloeuphordenol, β-sitosterol	
Amides (4)	5,6-dimethoxy-N-methylphthalimide , N-trans-feruloyldopamine, N-trans- feruloyltyramine, N-cis-feruloyltyramine	
Others (12)	lichexanthone and 2,6-dimethoxy-p-quinone , scopoletin (coumarin), 4-hydroxy-heptadec-6-enoic acid ethyl ester, tinosinen (phenylpropanoid diglycoside), tinosposides A and B (lignan glucosides), tinosinesides A and B (dinorditerpene glucosides), tinosinenside (cadinane sesquiterpene glycoside), Malabarolide (furanoid bisnorditerpene), 1-deacetyltinosposide A (dinorclerone diterpenoid glycoside)	
Alkaloids (7)	berberine, jatrorrhizine, palmatine, Iwamide, decarine, palmatrubin, magnoflorine	
Furanoditerpenoids (17)	Tinosinenosides A- F, 4-epi-tinosinenoside D, 2-deacetyltinosinenoside D, 4-epi-2-deacetyltinosinenoside D, 2-deacetoxytinosinenoside D, menispermacide, tinosporicide, 10 α -hydroxycolumbin, tinosposinensides A-C, malabarolide B_1	

3.2. Experimental Section

3.2.1. General experimental procedures

NMR spectra were obtained on Bruker AU III 400 or 500 MHz NMR spectrometers using $CDCl_3$ or CD_3OD solvents. The mass spectra were recorded on an Agilent technology 6200 series mass spectrometer. Rudolph Research Analytical Autopol IV automatic polarimeter was used to obtain the specific rotations. The IR spectra were recorded on an Agilent Technologies Cary 630 FTIR. UV spectrum was obtained on a Thermoscientifc UV-Visible Spectrophotometer Evolution 200 Series. Chromatographic procedures were carried out using aluminum-backed, TLC plates, precoated with silica gel F254 (200 μm, 60 Å, Merck), flash silica gel (40 μm, 60 Å, J. T. Baker), reversed phase RP-C_{18} silica (Polarbond, J.T. Baker) and sephadex LH20 (sigma) for gravity column chromatography. Sep-Pak cartridges (C_{18}, 10 g) were purchased from Supelco. Detection was carried out at UV-254 nm and 1% vanillin sulphuric acid spray followed by heating. Purification was achieved using preparative-TLC plates (20 cm × 20 cm, 500 μm).

3.2.2. Plant Material

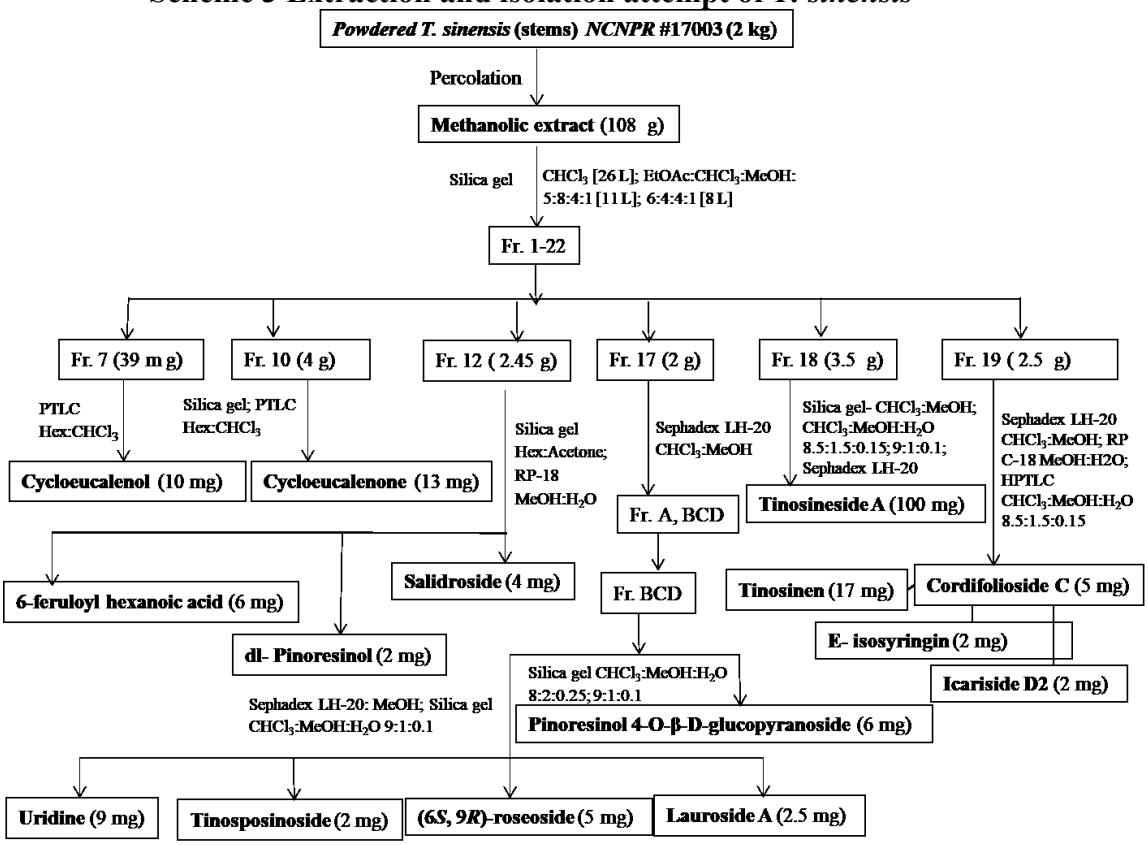
Stems samples of *T. sinensis* were obtained from commercial sources (NCNPR# 17003) and identified by co-TLC with authentic samples of *T. sinensis* (NCNPR# 17322) deposited at the Botanical Repository in the National Center for Natural Products Research, University of Mississippi, University, MS, USA.

3.2.3. Extraction and Isolation of Constituents

Powdered plant material (2 kg) was repeatedly extracted with methanol by percolation until exhaustion of plant material at room temperature to obtain a yield of 108 g **(Scheme 3)**. The methanolic extract was fractionated by column chromatography over silica gel using mixtures of chloroform (26 L), ethyl acetate: chloroform:methanol:water 15:8:4:1 (11 L) and 6:4:4:1 (8 L) respectively as mobile phase in order of increasing polarity over silica gel to afford 22 major fractions. Cycloeucalenone (10 mg) was obtained from fraction 7 (39 mg) by preparative thin layer chromatography using chloroform:hexanes 3:7. Cycloeucalenol (10 mg) was isolated from fraction 10 (4 g) by repeated column chromatography [silica gel (121 cm × 2.5 cm), hexanes:chloroform, 6:4 (1 L), 7:3 (3 L), 8:2, (3 L); silica gel (92 cm), hexanes:chloroform: ethyl acetate 6.5:3:0.5 (5 L); silica gel (82 cm × 3.8 cm) hexanes: ethyl acetate 10:1 (1 L)- 9:1 (1 L); PTLC, hexanes: ethyl acetate 8:2]. Fraction 17 (2 g) was chromatographed repeatedly over [sephadex LH-20 (97 cm × 3.3 cm), chloroform: methanol 1:1 (1 L); Sep-Pak cartridge (10 g) water:methanol 8:2-5:5; Sep- Pak cartridge (10 g), water:methanol 9:1-8:2] to obtain four fractions Fr. A-D. Fraction B, C and D were mixed and divided into two parts BCD_1 and BCD_2. Uridine (9 mg), tinosposinoside (2 mg), (6*S*, 9*R*)-roseoside (5 mg) and lauroside A (2.5 mg) were obtained from BCD_1 following repeated column chromatography over [sephadex LH-20 (92 cm × 1.2 cm) methanol 400 mL; silica gel (60 cm × 1 cm) chloroform:methanol: water 9:1:0.1]. (-)-Pinoresinol 4-O-β-D-glucopyranoside (6 mg) was obtained from BCD_2 by CC over [silica gel (92 cm × 2.5 cm chloroform:methanol:water 8:2:0.25 (1.2 L); silica gel (36 cm × 0.1 cm) chloroform:methanol:water 9:1:0.1 (150 mL)]. Repeated column chromatography of fraction 12 (2.45 g) [silica gel, hexanes:acetone 7:3 (5 L), reversed phase C_{18} water:methanol 4:6 (200 mL)

and 5:5 (400 mL)] yielded 6-feruloyl hexanoic acid (6 mg), dl- pinoresinol (2 mg) and salidroside (4 mg). Tinosineside A (100 mg) was isolated from fraction 18 (3.5 g) by repeated column chromatography [silica gel (92 cm × 3.8 cm) chloroform: methanol 100:0- 8:2 (2.5 L × 5); silica gel (77 cm × 2.5 cm) chloroform:methanol:water 9:1:0.1 (1 L) and 8.5:1.5:0.15 (1 L); sephadex LH-20 (5 cm × 1 cm)]. Tinosinen (17 mg), cordifolioside C (5 mg), icariside D2 (2 mg) and E- isosyringin (2 mg) were isolated from fraction 19 (2.5 gm) by column chromatography [sephadex LH-20 (88 cm × 3 cm) methanol (1 L); reversed phase C18 (61 cm × 2.5 cm) with water:methanol 6:4 (1 L); sephadex LH-20 (76 cm × 1cm) chloroform:methanol 1:1 (150 mL); silica gel (91 cm × 2.5 cm) chloroform: methanol: water 8:2:0.25 (600 mL); Preparative TLC in chloroform:methanol:water 8.5:1.5:0.15].

Scheme 3 Extraction and isolation attempt of *T. sinensis*



3.3. Results and Discussion

The methanolic extract of the stems of *T. sinensis* was subjected to different chromatographic techniques and fifteen compounds were isolated. Two cycloartanes, cycloeucalenol and cycloeucalenone, two megastigmanes, (6*S*,9*R*)-roseoside and lauroside, a ferulic acid ester, two lignans, (-)-pinoresinol monomethyl ether O-β -D-glucopyranoside and dl-pinoresinol were isolated among other known compounds. **(Fig. 3.1)**. Two new compounds were isolated and were named tinosposinoside and cordifoliside C. The structures of the compounds were established and elucidated with the help of 1D and 2D NMR spectra as well as HRESIMS. The chemical constituents isolated from *Tinospora sinensis* are detailed as follows. The identity of the known compounds was confirmed by HRESIMS and was in agreement with the published literature.

3.3.1. *Tinosposinoside* (**1**)

White powder; HR-ESI-MS *m/z*, 815.4206[M + Na] $^{+}$ (calcd for $C_{42}H_{64}O_{14}$+Na, 815.4194); V_{max} 3380,2924,1653,1377,1077,1034 ; $[\alpha]^{25}{}_{D}$: - 60 (c=0.1, MeOH) ; UV/Vis λmax (MeOH) nm (log ϵ): 210(4.2), 255(4.1); ^{1}H NMR (400 MHz, Methanol-d_4) δ 5.76 (br.s, 1H), 4.49 (d, *J* =7.8 Hz, IH), 3.81 (dd , *J*=5.3,11.99 Hz, IH), 3.67 (dd, *J* = 2.29, 11.99Hz, 1H) , 3.38 (t, *J*=8.7 Hz, 1H), 3.30 (overlapped, 1H) , 3.28 (overlapped, 1H) , 3.15 (dd , *J*=7.8, 8.7Hz, 1H), 3.13 (s, 1H), 2.74 (d, *J* = 6.7, 1H), 2.15 (dd, *J* = 12.3, 6.0 Hz, 1H), 2.09 (d, *J* = 6.7 Hz, 1H), 2.06 (br.s, 3H), 1.97 (dd, *J* = 12.7, 8.5 Hz, 1H), 1.83(overlapped, 2H), 1.66 (dd, *J* = 12.7, 8.0 Hz, 1H), 1.23 (s, 3H), 1.16 (s, 3H), 0.99 (s, 3H).^{13}C NMR (101 MHz, Methanol-d_4) δ 207.61(C-1), 174.59(C-3), 121.66(C-2), 98.12(C-1′), 81.63(C-11), 78.32(C-3′), 77.51(C-5′), 75.10(C-2′), 71.66(C-4′),

62.72(C-6′), 58.53(C-6), 58.35(C-4), 55.85(C-5), 55.69(C-10), 49.44(C-9), 37.65(C-7), 24.28(C-12), 23.85(C-14), 22.46(C-13), 21.61(C-8), 20.33(C-15)

3.3.2. *Cordifolioside C* (2)

Colorless solid; HR-ESI-MS *m/z* 547.1694 $[M-H+HCO_2H]^-$ (calcd for $C_{22}H_{30}O_{13}$ -H+HCO_2H 547.1663); V_{max} 3308,1636; $[\alpha]^{25}_D$: - 29 (c=0.1, MeOH) ; UV/Vis λmax (MeOH) nm (log ϵ): 234(3.38), 311(3.37);^{1}H NMR (400 MHz, Methanol-d_4) δ 9.66 (d, *J* = 7.7 Hz, 1H), 7.64 (d, *J* = 15.8 Hz, 1H), 7.05(s, 2H), 6.79 (dd, *J* =16, 7.7 Hz, 1H), 5.33 (d, *J* = 2.8 Hz, 1H), 5.09 (d, *J* =7.7, 1H), 4.16 (d, *J* = 9.6 Hz, 1H), 4.04 (d, *J* = 2.8 Hz, 1H), 3.92 (s, 6H), 3.82 (d, *J* =9.6 Hz, 1H), 3.79 (dd, *J* =9.7, 12 Hz, 1H), 3.71 (dd, *J* = 12, 3.5 Hz, IH), 3.63 (overlapped, 2H), 3.54 (t, *J* = 8.8Hz, 1H), 3.48 (t, *J* = 8.8 Hz, 1H), 3.27 (ddd, *J*= 9.2, 5.1, 2.4 Hz, 1H). ^{13}C NMR (101 MHz, Methanol-d_4) δ 196.09(C-1), 154.62(C-3,3′,5′), 138.61(C-4′), 131.99(C-1′), 129.20(C-2), 111.34(C-1‴), 107.75(C-2′,6′), 104.39(C-1″), 85.27(C-3″), 80.5(C-3‴), 78.16 (C-5″), 77.87 (C-2‴)75.36(C-2″), 74.98(C-4‴), 69.81(C-4″), 65.14(C-5‴), 62.51(C-6″), 57.13(-OCH_3).

3.3.3. *Tinosineside A* (3)

White crystals; HRESIMS *m/z* 579.2018 $[M+Na]^+$ (calcd. for [$C_{26}H_{36}O_{13}$+Na], 579.2054); ^{1}H and ^{13}C NMR data matched with the literature (Yonemitsu M., et al. 1995)

3.3.4 *Tinosinen* (4)

White crystals; HRESIMS *m/z* 427.1732 $[M+Na]^+$ (calcd. for [$C_{22}H_{30}O_{13}$+Na], 427.1741); ^{1}H and ^{13}C NMR data matched with the literature ((Yonemitsu, Michiko et al., 1993)

3.3.5. *Cycloeucalenol* (5)

White crystals; HRESIMS *m/z* 449.3509 $[M+Na]^+$ (calcd. for [$C_{30}H_{50}O$+Na] 449.3759); ^{1}H and ^{13}C NMR data matched with the literature (Khuong-huu et al. 1975; Kongkathip et al. 2002)

3.3.6. *Cycloeucalenone* (**6**)

White crystals; HRESIMS *m/z* 447.3442[M+Na]$^+$ (calcd. for [$C_{30}H_{48}O$+Na], 447.3603); ^{1}H and ^{13}C NMR data matched with the literature (Khuong-huu et al., 1975; Kongkathip et al., 2002)

3.3.7. *(-)-pinoresinol 4-O-β-D-glucopyranoside* (**7**)

White crystals; HRESIMS *m/z* 543.1840 [M+Na]$^+$ (calcd. for [$C_{26}H_{32}O_{11}$+ Na], 543.1842); ^{1}H and ^{13}C NMR data matched with the literature (W. Li et al. 2004)

3.3.8. *dl-Pinoresinol* (**8**)

White crystals; HRESIMS *m/z* 357.1363[M-H]$^-$ (calcd. for [$C_{20}H_{22}O_6$-H], 357.1338); ^{1}H and ^{13}C NMR data matched with the literature (Greca et al., 1998).

3.3.9. *Salidroside* (**9**)

White crystals; HRESIMS *m/z* 323.1108 [M+Na]$^+$ (calcd. for [$C_{14}H_{20}O_7$+Na], 323.1107); ^{1}H and ^{13}C NMR data matched with the literature (T. A. O. Li, He, and Luo 2017)

3.3.10. *Icariside D2* (**10**)

White crystals; HRESIMS *m/z* 345.1202 [M-H+HCO_2H]$^-$ (calcd. for [$C_{14}H_{20}O_7$-H+HCO_2H], 345.1186); ^{1}H and ^{13}C NMR data matched with the literature (Miyase et al. 1989)

3.3.11. *E-isosyringin* (**11**)

White crystals; HRESIMS *m/z* 395.1323 [M+Na]$^+$ (calcd. for [$C_{26}H_{32}O_{11}$+H], 395.1318); ^{1}H and ^{13}C NMR data matched with the literature (Greca et al. 1998)

3.3.12. *Uridine* (**12**)

White crystals; HRESIMS *m/z* 243.0627 [M-H]$^-$ (calcd. for [$C_9H_{12}N_2O_6$-H], 243.0695); ^{1}H and ^{13}C NMR data matched with the literature (Khan S, Iqbal M, Khan D, Badshah S 2018)

3.3.13. (*6S, 9R*)-*Roseoside* (**13**)

White crystals; HRESIMS *m/z* 431.1933 $[M-H+HCO_2H]^-$ (calcd. for $[C_{19}H_{30}O_8-H+HCO_2H]$, 431.1917); ^1H and ^{13}C NMR data matched with the literature (Masayuki Yoshikawa et al. 1997)

3.3.14. *Lauroside A* (**14**)

White crystals; HRESIMS *m/z* 433.2103 $[M-H+HCO_2H]^-$ (calcd. for $[C_{19}H_{32}O_8-H+HCO_2H]$, 433.2074); ^1H and ^{13}C NMR data matched with the literature (De Marino et al. 2004)

3.3.15. *6-Feruloyloxyhexanoic acid* (**15**)

White crystals; HRESIMS *m/z* 331.1124 $[M+Na]^+$ (calcd. for $[C_{16}H_{20}O_6+Na]$, 331.1158); ^1H and ^{13}C NMR data matched with the literature (Song et al. 2017)

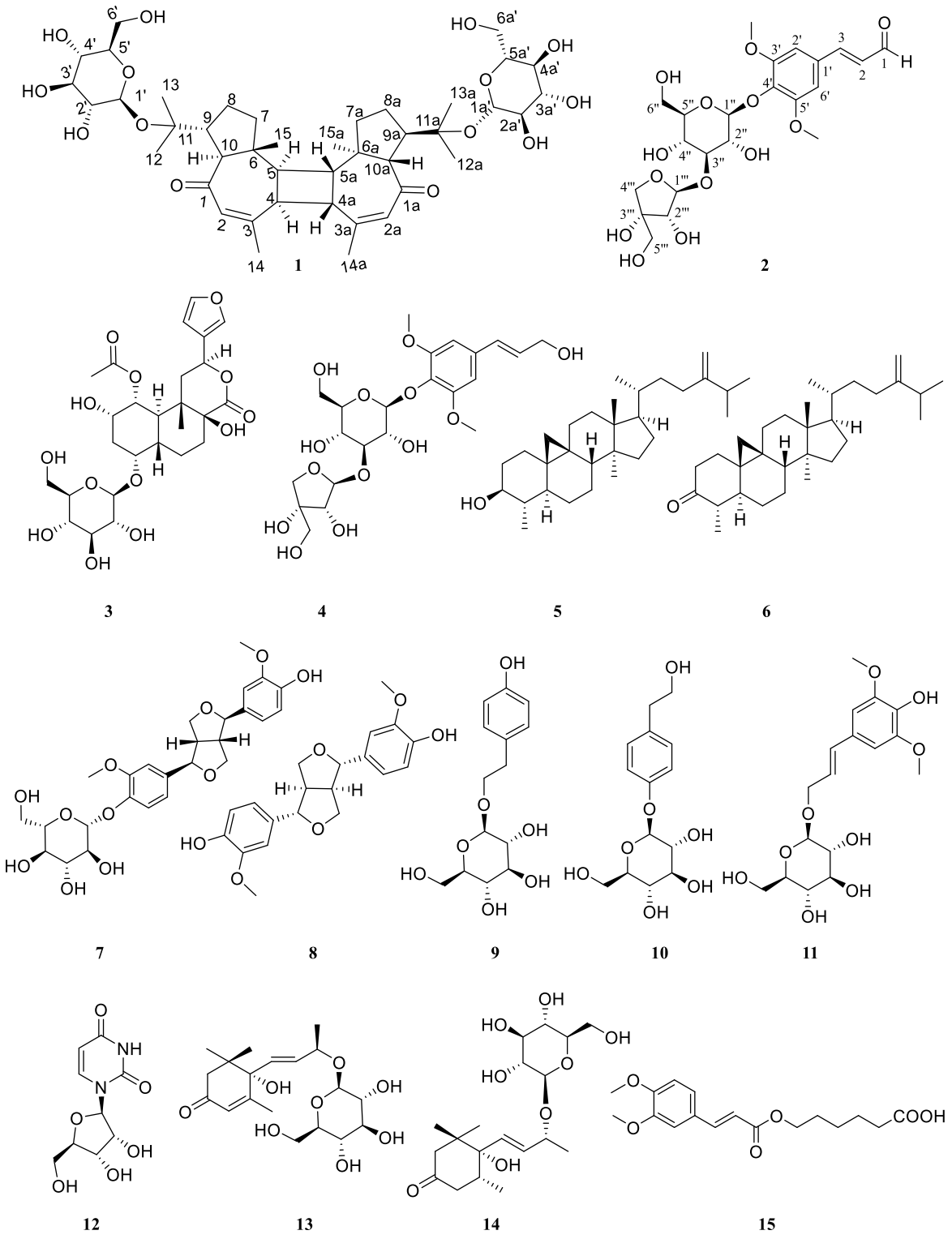


Figure 3.1. Structures of compounds isolated from *T. sinensis*

3.3.1 Structural elucidation of new compounds

Tinosposinoside (**1**) was obtained as white powder and showed $[M+Na]^+$ ion at *m/z*. 815.4206, (calcd for $C_{42}H_{64}O_{14}$+Na, 815.4194) corresponding to the formula $C_{42}H_{64}O_{14}$. The IR spectrum showed absorption at 3380, 2924, 1653, 137 and 1034 cm^{-1} indicating the presence of hydroxy and an α, β-unsaturated ketone. The UV spectrum showed absorption at 210 and 255 nm. The ^{1}HNMR spectrum gave rise to four signals at δ 1.23, 2.06, 1.16 and 0.99 corresponding to the four methyls respectively. The two methine groups of the cyclobutane ring resonated at δ 2.74 (d, *J* = 6.7, 1H), and 2.09 (d, *J* = 6.7 Hz, 1H) **(Fig. 3.2)**. Due to symmetric nature of the dimeric molecule, the NMR data of only one unit is discussed

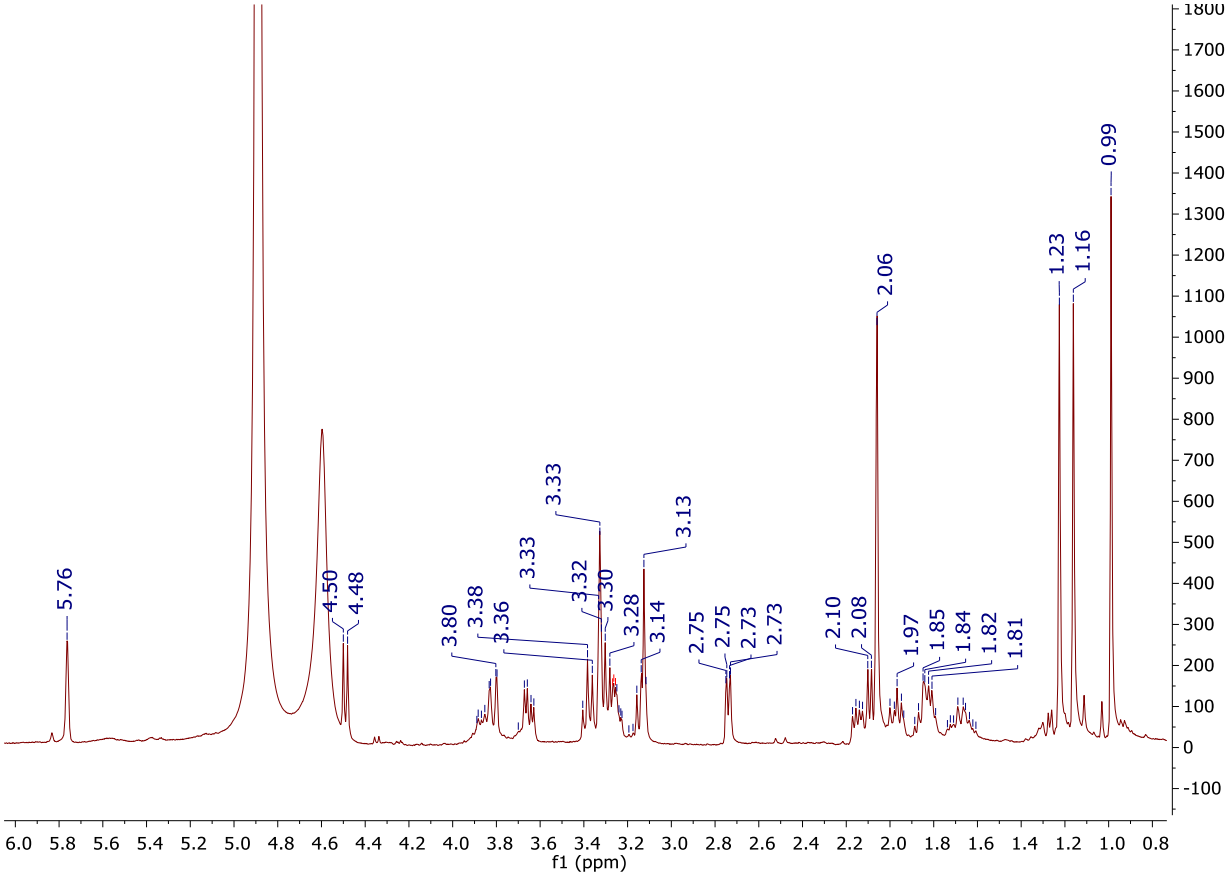


Figure 3.2. ^{1}H NMR spectrum of compound 1.

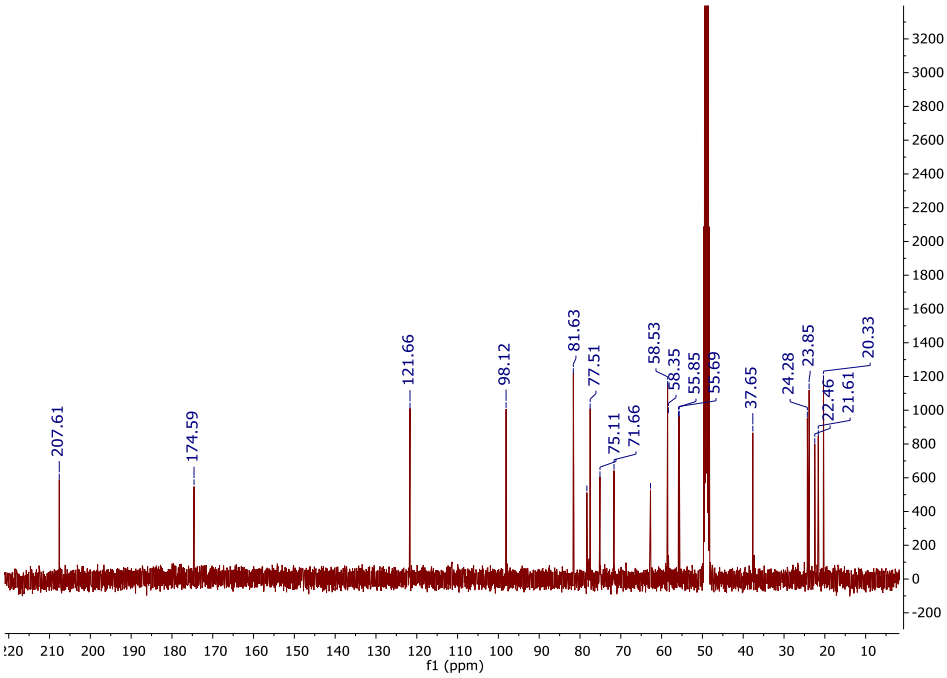


Figure 3.3. ^{13}C NMR spectrum of compound 1.

The decoupled ^{13}C NMR spectrum **(Fig. 3.3)** showed 21 resonances which were differentiated by DEPT 135 NMR spectrum as four nonprotonated carbons (one at δ 58.53, an olefinic carbon at δ174.59, one oxygenated carbon at δ81.63 and a carbonyl function supported by resonance at δ207.61), five methines (two CH at δ49.44, δ55.69, δ55.85 and δ58.35 and one olefin carbon at δ121.66). Two methylenes (δ 21.61 and 37.65) and four methyls (δ 24.28, 23.85, 22.46, and 20.33) were exhibited in the DEPT spectrum.

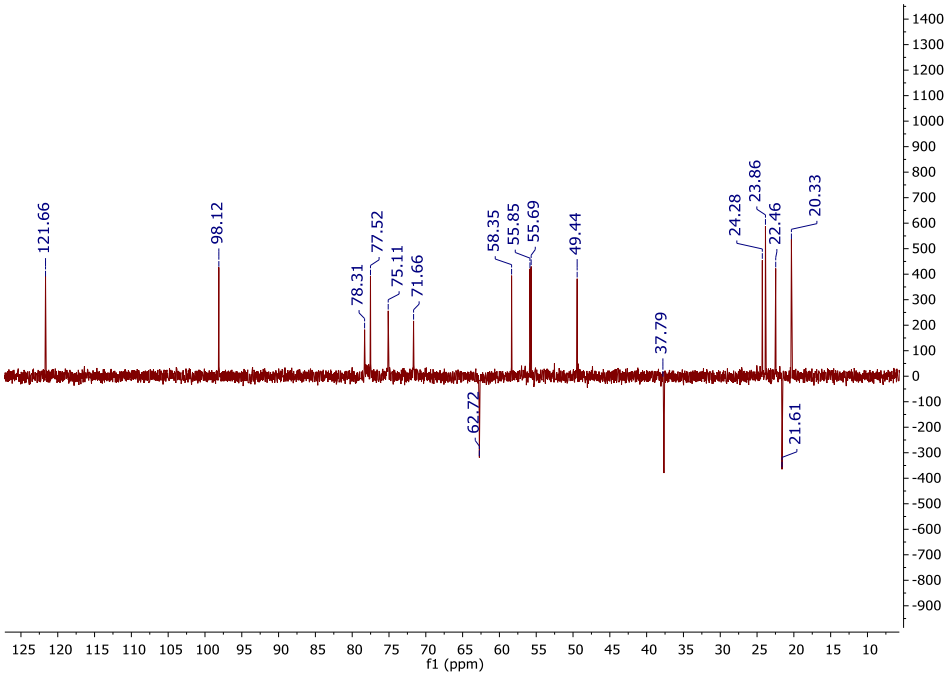


Figure 3.4. DEPT NMR spectrum of compound 1.

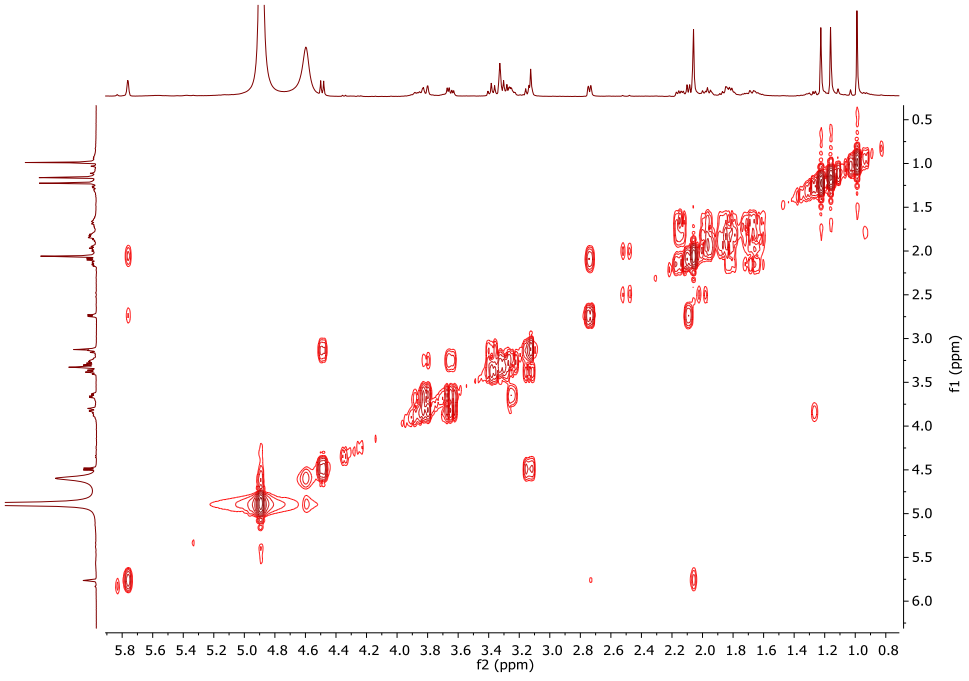


Figure 3.5. COSY spectrum of compound 1.

The HMBC correlations of methyl protons (H_3-15, δ 0.99) with C-5 (δ 55.8), and H-4 (δ 2.09) with C-14 (δ 23.85) and H-2 (δ 5.76) with C-4 (δ 58.35) confirmed the positions of C-4 and C-5 on the seven membered ring **(Fig. 3.6)**. The relative configuration for compound **1** was elucidated on the basis of NOESY correlations **(Fig. 3.7)**. The β-axial orientation of H-4 was deduced by the NOESY correlation between H-4 (δ2.09) and H_3-15 (δ 0.99). H-10 (δ 3.13) exhibited NOESY correlations with H-9 (δ 2.15) and H-4 (δ2.09). H-5 (δ 2.74) showed correlations with H-8 (δ 1.66) and C-13 (δ 22.4).

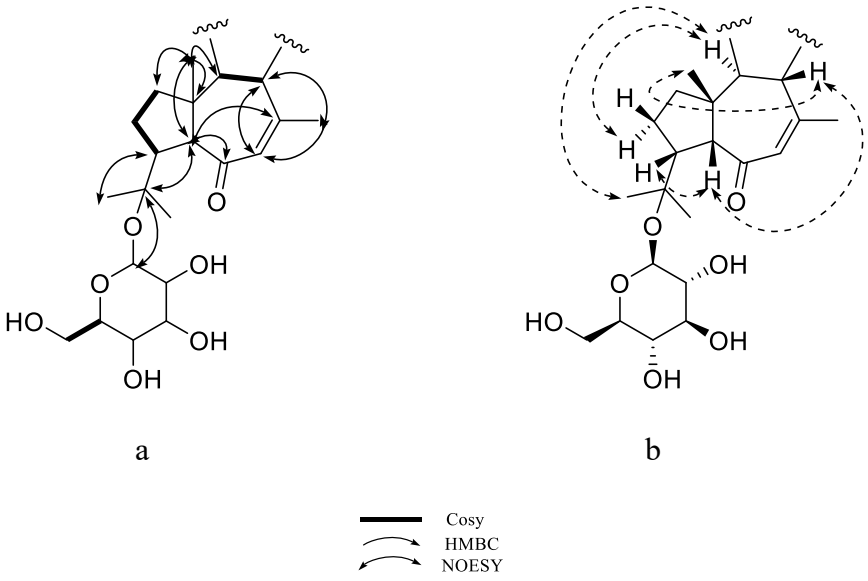


Figure 3.6. Key HMBC (a) correlations and NOESY (b) correlations

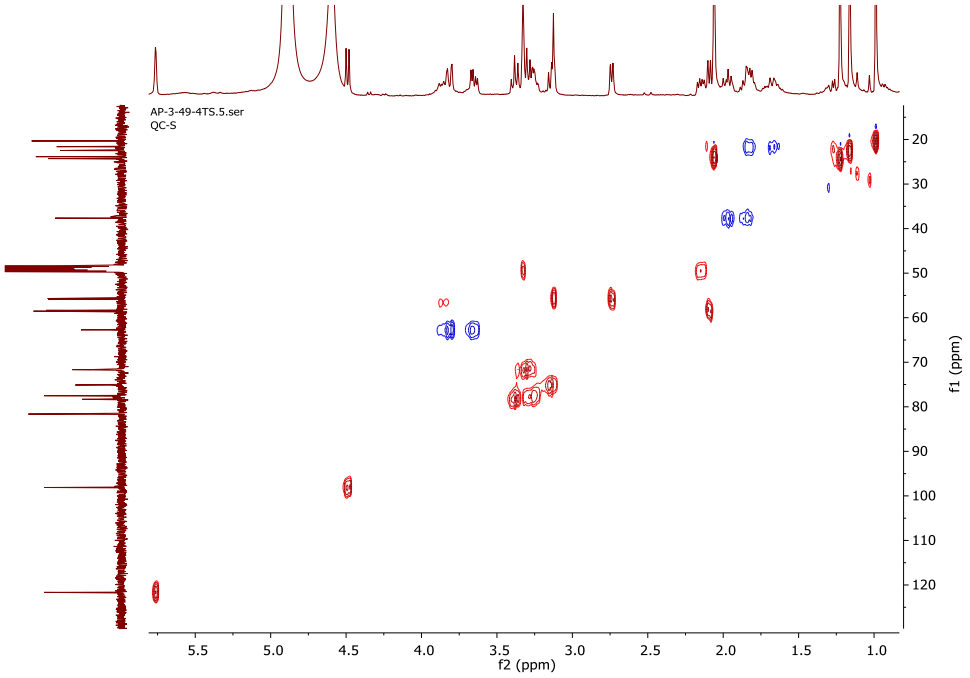


Figure 3.7. HSQC spectrum of compound 1.

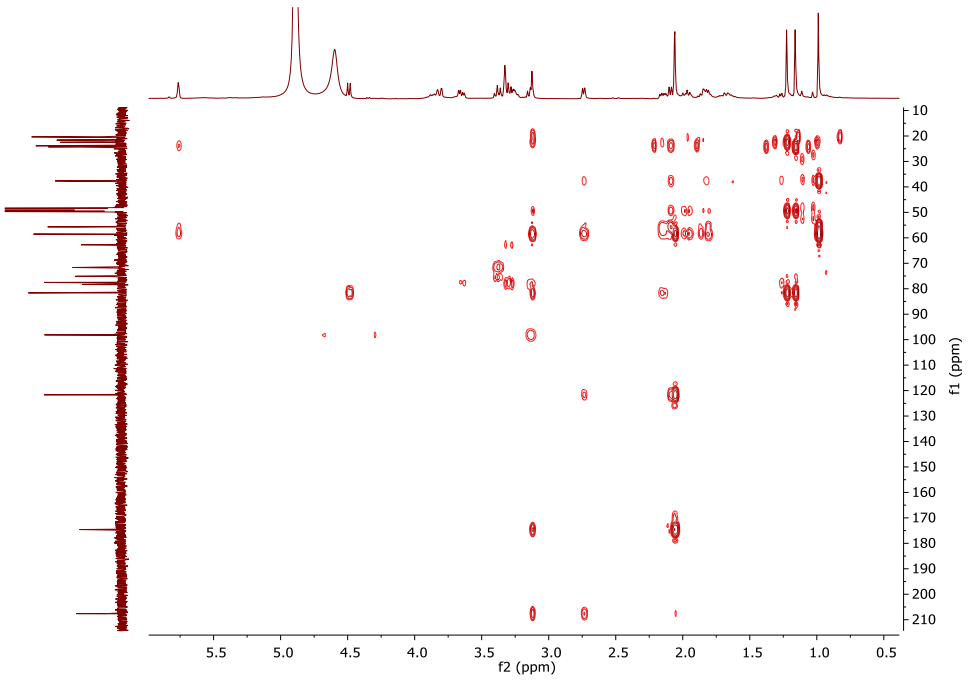


Figure 3.8. HMBC spectrum of compound 1.

Table 3.2. ^{1}H NMR (400 MHz, methanol-d_4) and ^{13}C NMR (101 MHz, methanol-d_4) data of 1.

Position	δ_C, Mult.	δ_H Mult. (*J* in Hz)
1	207.61, C	-
2	121.66, CH	5.76 br s
3	174.59 C	-
4	58.35, CH	2.09 d (6.7)
5	55.85, CH	2.74 d (6.7)
6	58.53, C	-
7	37.65, CH_2	1.83 overlapped, 1.97 dd (12.7, 8.0)
8	21.61, CH_2	1.83 overlapped, 1.66 dd (12.7, 8.5)
9	49.44, CH	2.15 dd (12.3, 6.0)
10	55.69, CH	3.13 overlapped
11	81.63, C	-
12	24.28, CH_3	1.23 s
13	22.46, CH_3	1.16 s
14	23.85, CH_3	2.06, br s
15	20.33, CH_3	0.99, br s
1′	98.12, CH	4.49, d (7.8)
2′	75.10, CH	3.15, dd (7.8, 8.7)
3′	78.32, CH	3.38, t (8.7)
4′	71.66, CH	3.30, overlapped
5′	77.51, CH	3.28, overlapped
6′	62.72, CH_2	3.67 dd (2.29, 11.99), 3.81 dd (5.3, 11.99)

Cordifoliside (**2**) was isolated as white crystals and HRESIMS indicated the occurrence of an ion peak at *m/z* 547.1694 $[M-H+HCO_2H]^-$ corresponding to the molecular formula $C_{22}H_{30}O_{13}$. The ^{1}HNMR and ^{13}CNMR exhibited characteristic resonances for an aldehyde function [δ_H/δ_C δ 9.66 (d, *J* = 7.7 Hz, 1H)/ 196.06 (C-1)], two trans olefinic protons [δ_H/δ_C 7.64 (d, *J* = 15.8 Hz, 1H)/ 154.62 (C-3), 6.79 (dd, *J* =16, 7.7, 1H/129.20 (C-2)] , two aromatic methines [δ_H/δ_C 7.05(s, 2H)/107.75 (2′, 6')] , two methoxy [δ_H/δ_C 3.92 (s, 6H)/ 57.13-OMe], a glucopyranose [δ_H/δ_C 5.09 (d, *J* =7.7, 1H)/104.39(C-1″), 3.63 (overlapped, 2H)/ 75.36(C-2″), 3.54 t (J=8.8 Hz, IH)/85.27(C-3″),3.48 t (J=8.8 Hz, , IH)/69.81(C-4″),3.27 ddd (*J*= 9.2, 5.1, 2.4 Hz, IH) /78.16(C-5″), 3.79 dd (J = 9.7, 12 Hz, IH), 3.71 dd (12, 3.5, IH)/ 62.51(C-6″)], and an apiose [δ_H/δ_C 5.33 (d, *J* = 2.8 Hz, 1H)/ 111.34(C-5‴), 4.04 (d, *J* = 2.8 Hz, 1H)/ 77.87 C-4‴], 4.16 (d, *J* = 9.6 Hz, 1H), 3.82 (d, *J* =9.6 1H)/74.98 (C-4‴)], 3.63 (overlapped, 2H)/65.14 (C-5‴)].

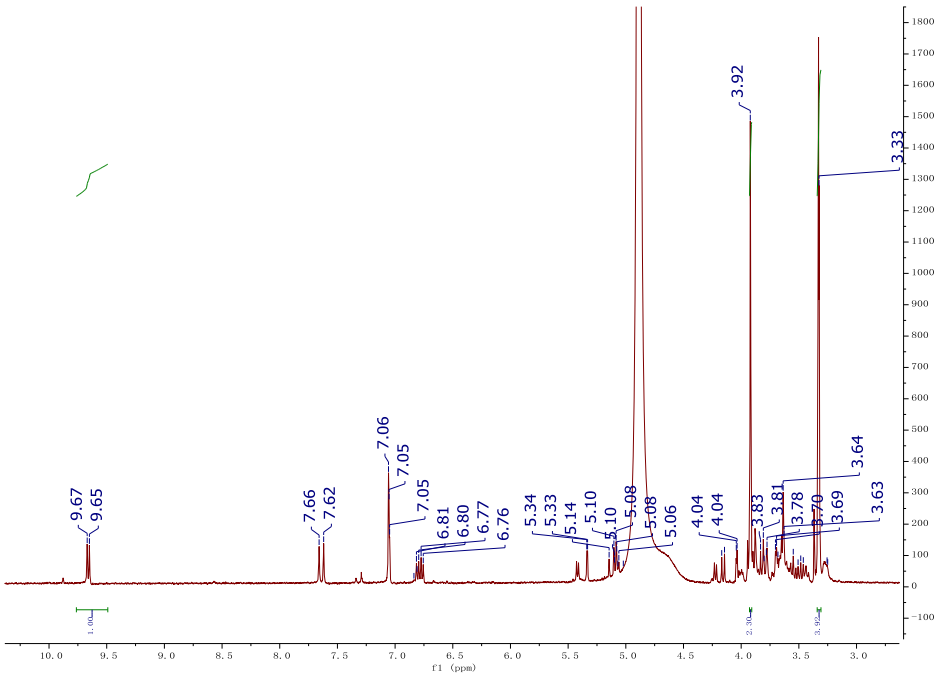


Figure 3.9 ^{1}H NMR spectrum of compound 2.

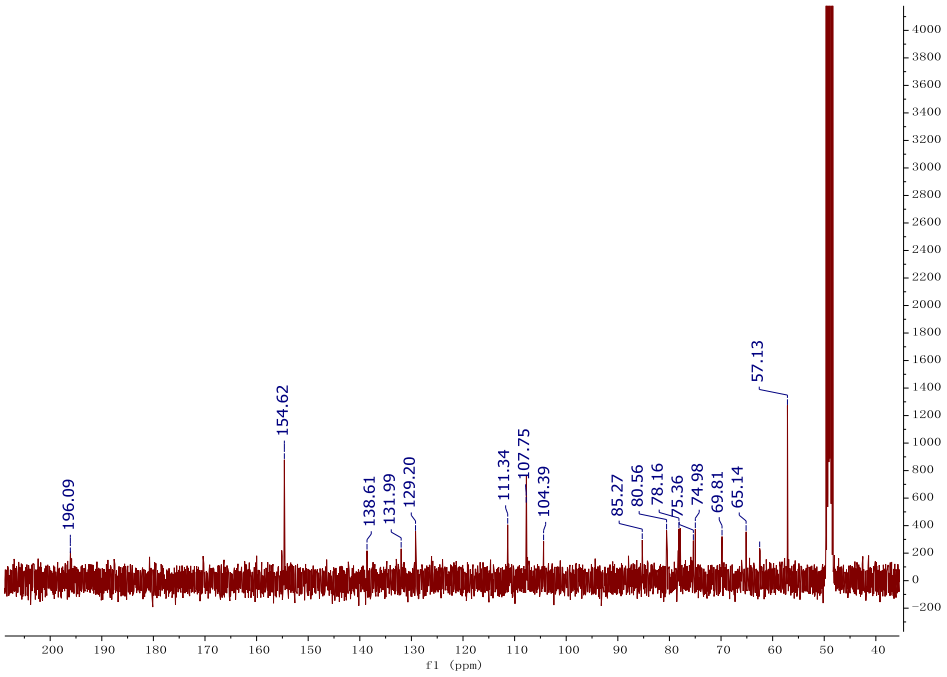


Figure 3.10^{13}C NMR spectrum of compound 2.

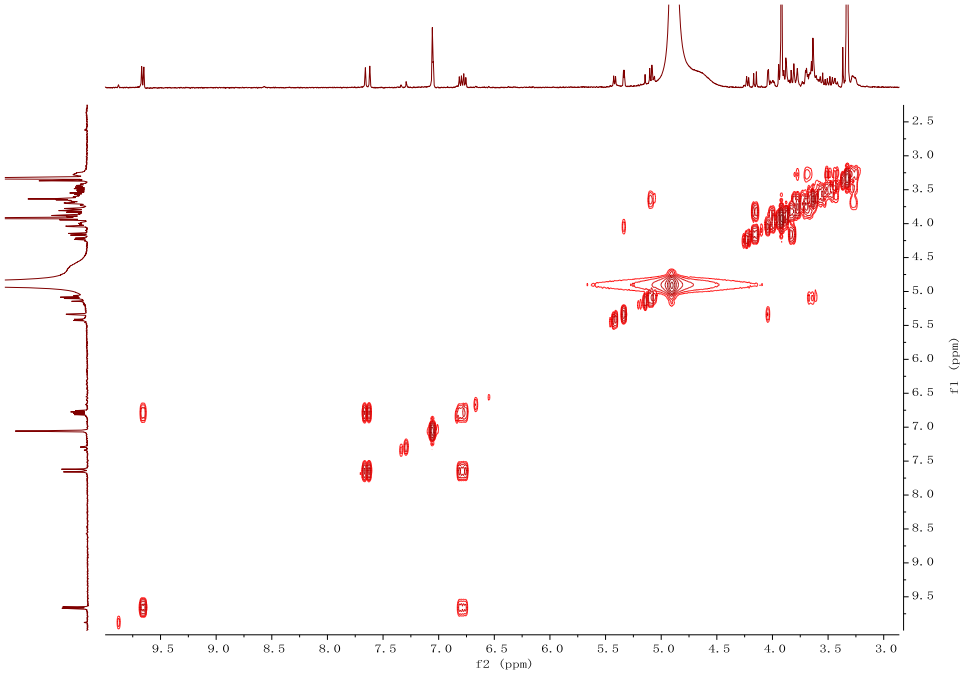


Figure 3.11. COSY spectrum of compound 2.

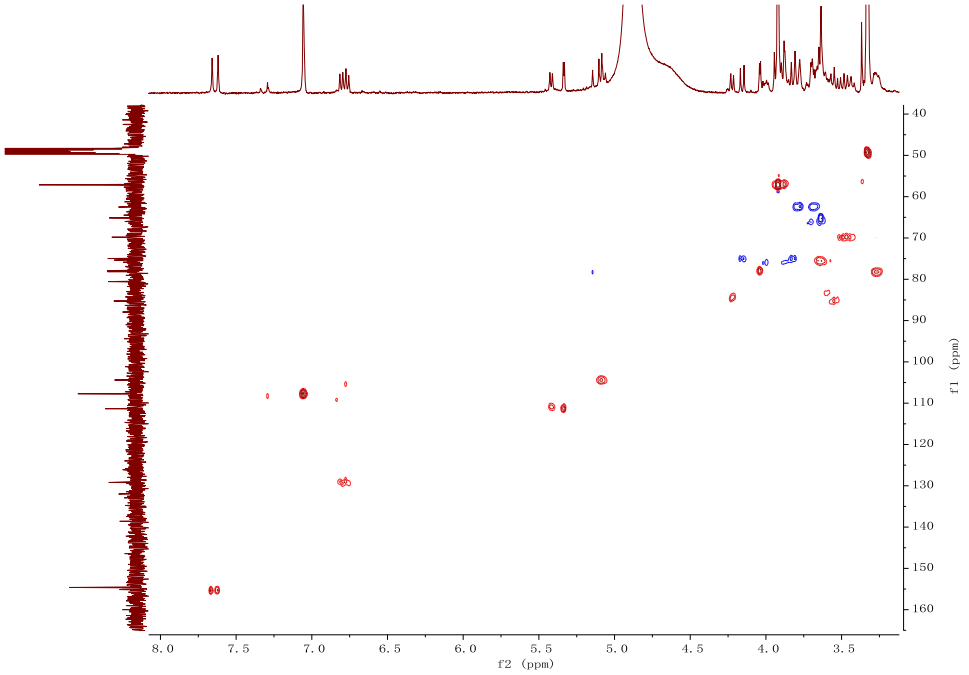


Figure 3.12. HSQC spectrum of compound 2.

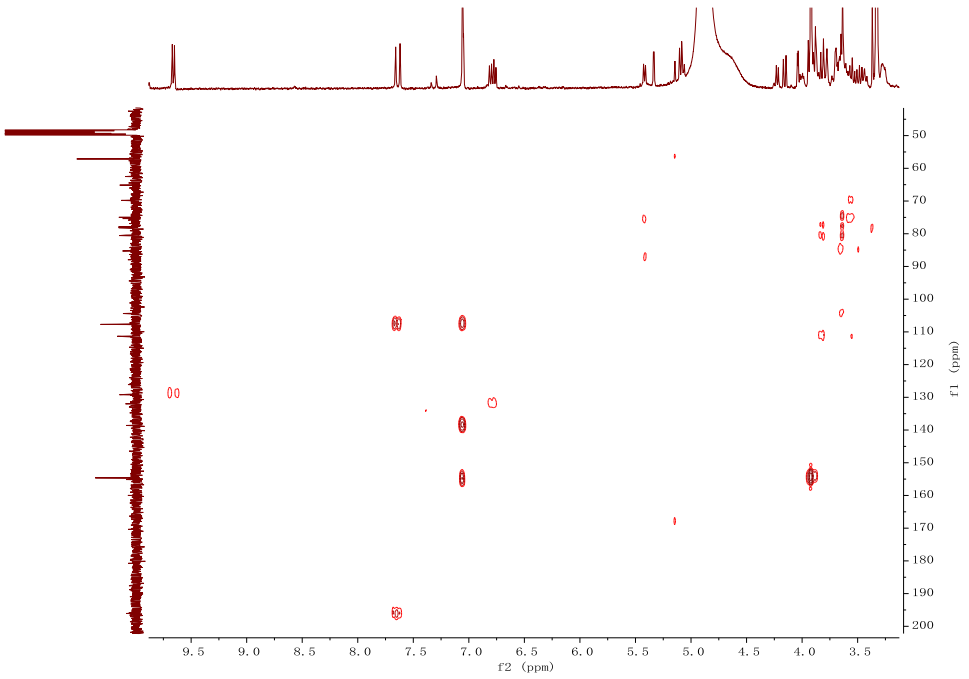


Figure 3.13 HMBC spectrum of compound 2.

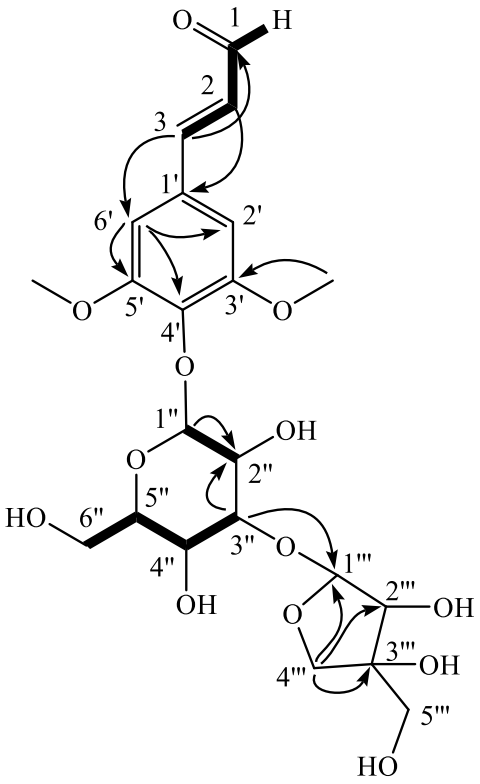


Figure 3.14. Key HMBC correlations for compound 2.

The HMBC correlation between H-3″ (δ 3.55) and an anomeric carbon (C-1‴) of the apiose moiety at δ 111. 3 confirmed the inter-glycosidic linkage. The HMBC correlations of H-3 (δ 7.64) with C-1 (δ 196.0) and C-2′, C-6' (δ107.7), and of H-2 (δ6.79) with C-1′ (δ 131.9) confirmed the position of the olefinic and aldehyde functions. The HMBC correlations shown by the aromatic protons H-2′, H-6' (δ7.05) with C-3′, C- 5′ (δ 154.6), C-4′ (δ 138.6) confirmed the assignment of the aromatic ring. The location of the methoxy groups was confirmed by the HMBC correlation of their proton (δ 3.92) with C-3′, C- 5′ (δ 154.6).

Table 3.3. ^{1}H NMR (400 MHz, Methanol-*d*$_4$) and^{13}C NMR (101 MHz, Methanol-*d*$_4$) data of 2.

Position	δ_C, Mult.	δ_H Mult. (*J* in Hz)
1	196.09, C	9.66 d (7.7)
2	129.20, CH	6.79 dd (16, 7.7)
3	154.62, CH	7.64 d (15.8)
1′	131.99, C	-
2′, 6'	107.75, CH	7.05 s
3′, 5'	154.62, C	-
4′	138.61, C	-
OMe	57.13, C	3.92 s
OMe	57.13, C	3.92 s
1″	104.39, CH	5.09 d (7.7)
2″	75.36, CH	3.63 overlapped
3″	85.27, CH	3.54 t (8.8)
4″	69.81, CH	3.48 t (8.8)
5″	78.16, CH	3.27 ddd (9.2, 5.1, 2.4)
6″	62.51, CH_2	3.79 dd (9.7, 12), 3.71 dd (12, 3.5)
1‴	111.34, CH	5.33 d (2.8)
2‴	77.87, CH	4.04 d (2.8)
3‴	80.5, C	-
4‴	74.98, CH_2	4.16 d, (9.6), 3.82 d (9.6)
5‴	65.14, CH_2	3.63 overlapped

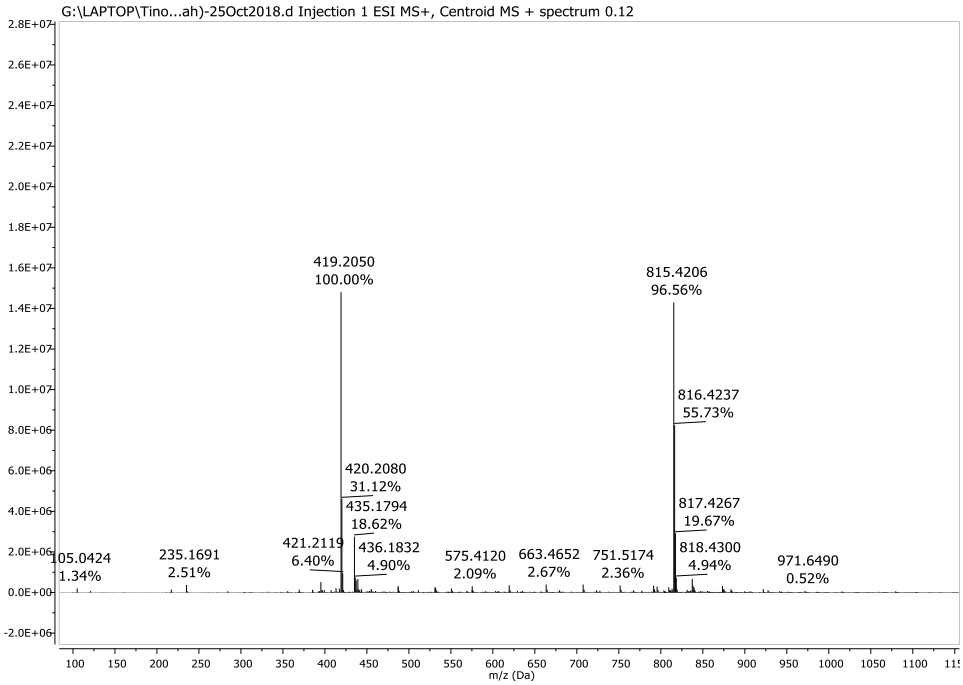


Figure 3.15. ESI mass spectrum of compound 1

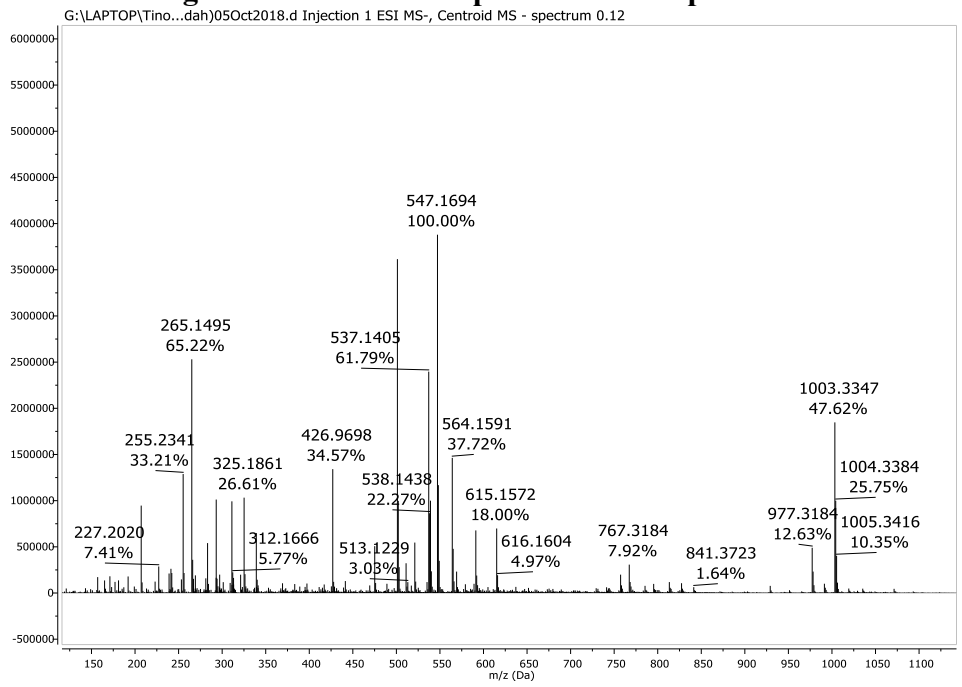


Figure 3.16. ESI mass spectrum of compound 2.

3.4. Conclusions

Fifteen compounds were isolated from *T. sinensis*, of which two were new compounds whereas five compounds qualified for first report from *T. sinensis*. Tinosposinoside and cordifoliside C are new compounds whereas cycloeucalenone, lauroside A, (6*S*, 9*R*)-roseoside, E-isosyringin and 6-feruloyl hexanoic acid are reported for the first time from this plant. Tinosineside A was isolated as the major compound and being unique to *T. sinensis;* it will be instrumental as a chemical marker for *T. sinensis* for the analytical studies.

Chapter 4

EVALUATION OF THE EFFECT OF *TINOSPORA CRISPA*, ITS MAJOR FURANODITERPENOIDS AND *TINOSPORA SINENSIS* ON LIVER

4.1. Introduction

4.1.1. Herbal drug supplements and Hepatotoxicity

The terms hepatotoxicity and drug induced liver injury (DILI) are used interchangeably. The liver plays a major role in the metabolism of drugs, which makes it vulnerable to toxic insults from xenobiotics. DILI is a term increasingly being recognized as potential complication of many drugs. It is defined as a liver injury caused by a xenobiotic, which may either be a drug, herbal and/or a dietary supplement when all other possible causes for DILI have been ruled out (Björnsson 2015). DILI can be natural (due to dose dependent hepatotoxicity) or idiosyncratic (due to genetic or other unknown factors). Dysregulation of specific cytokines resulting from other drugs (Aithal et al. 2004), and inhibition of hepatobiliary transporters (Pauli-Magnus and Meier 2006) are few examples of the mechanisms of DILI. As herbal dietary supplements (HDS) continue to grow in popularity in the United States, HDS related liver injury has increased over the past decade according to Drug Induced Liver Injury Network (DILIN). The retail sales of HDS continue to increase consecutively over a decade with retail sales for HDS reaching an estimated total of $ 8.085 in 2017, surpassing $8 billion mark (Smith Tyler et al., 2018).

According to the National Health and Nutrition Examination Survey (NHANES) 2003-2006, 50% of all Americans and 70% of adults (70 years and above) use dietary supplements (Bailey et al. 2011). According to the DILIN, HDS related liver injury is common and on the rise in the United States. The most common cause of DILI has been reported due to weight loss supplements. The direct cause of liver injury is difficult to establish owing to the fact that HDS can be taken concurrently with other conventional medicines or dietary supplements (Zheng and Navarro 2015). The quality of the HDS remains a concern as a potential determinant of toxicity. Factors such as the possibility of alteration in chemical profile of a botanical due to a variety of conditions including improper storage, processing or adulteration may lead to contamination and thus unfavorable untoward effects such as acute liver failure. HDS are believed to be safer than the OTC or prescription drug, in general. Considered to be regulated by the FDA, dietary supplements users are more likely to self-medicate. On the contrary, HDS are regulated under the Dietary Supplement Health and Education Act (DSHEA) 1994, under which they are not held to the same standards as prescription drugs. They can be marketed without strong evidence of clinical safety studies (Navarro et al. 2014).

4.1.2. Role of Inflammation in Drug Induced Liver Injury

Inflammation plays an important role in potentiating liver toxicity by several toxins. Even a modest episode of inflammation can enhance liver susceptibility and trigger hepatotoxic insult. Regardless of the source (injury or infection) and intensity (modest or severe) of inflammation, concurrent administration of drugs may trigger a hepatotoxic response and thus DILI.

Lipopolysaccharide, LPS, is an endotoxin that produces subtle inflammation as a result of leaky gut syndrome; during which LPS is released by the gut micro flora and inflamed traverse

intestinal walls into the venous portal circulation where it stimulates the release of cytokines altering cellular homeostasis (Roth, R. A., 1997). Exogenous LPS, when administered, may lead to accumulation of platelets and neutrophils in the liver in addition to the activation of endothelial cells. (Eipel et al.,2004). Pro-inflammatory and lipid mediators like cytokines including TNF-α, IL-1, and prostaglandin, reactive oxygen species and toxic proteases are released as a result of the activation of transcription factors (Beutler 2001). It has been reported that LPS at sub toxic doses (6 mg/kg body weight i.p), does not lead to an increase in ALT levels but upon concurrent administration with a hepatotoxicant, results in an amplified toxic response. Examples of such hepatotoxicants include galactosamine (Galanos, Freudenberg, and Reutter 1979), carbon tetrachloride (Formal et al., 1963), ethanol (Bhagwandeen et al., 1987), T-2 toxin (Tai and Pestka 1988), allyl alcohol (Sneed et al., 1997), monocrotaline (Abdel-Bakky et al., 2010), acetaminophen (Gardner et al. 1998), and ranitidine (Luyendyk et al. 2009).

4.1.3. *Tinospora* species: previous toxicological studies and knowledge gap

Hepatotoxicity is the major reported adverse effect of *Tinospora crispa* (Sangsuwan et al., 2004). Chronic administration of 1.28 gm/kg of the ethanolic extract of the stems of *T. crispa* for six months showed elevated ALT, ALP and creatinine levels in rats. No signs of acute hepatotoxicity or mortality were observed at the dose 4 gm/kg in mice (Chavalittumrong et al., 1997). Behavioral signs of toxicity were observed at the dose of 1 gm/kg and 5 gm/kg body weight of the aqueous extract of the stems of *T. crispa* in mice. Doses of 500 mg/kg and less were reported to be nontoxic and no mortality was reported in the acute study. A hepatoprotective effect was reported during *plasmodium berghei* infection in mice by the aqueous extract at the dose of 500 mg/kg (Nutham et al., 2015). However, in another study, the

ethanolic extract of the dried stems of *T. crispa* was shown to potentiate the hepatotoxicity induced by thioacetamide in rats at the oral dose of 100 and 200 mg/kg gavage daily for 8 weeks (Kadir et al., 2011). During a randomized double blind placebo controlled trial for antidiabetic efficacy, *T crispa*, at the dose of 1gm thrice daily for 6 months, caused marked elevation of liver enzymes of two subjects which normalized after discontinuation of the powdered drug (Sangsuwan et al., 2004).

To date, five human hepatotoxicity cases have been reported including one death. These resulted from chronic overuse of the botanical product of *T.crispa* for prophylaxis against malaria (Denis et al. 2007) and for treatment of rheumatism (Langrand et al. 2014) and hepatic disorders (Huang et al. 2019). The recent case reported was of a 53 year old man suffering from acute hepatitis with cholestasis and cytolysis after three weeks of consumption of aqueous extract of the stems of *T. crispa* for detoxification of the liver. Symptoms included fever, asthenia, dark urine and high ALT levels. The condition of the patient ameliorated after two weeks without any treatment (Cachet et al. 2018). Borapetosides are the major compounds (furanoditerpenoids) reported from *T. crispa* and have been implicated in the reported human cases. Animal studies have shown that toxic metabolites formed from furanoditerpenoids by cytochrome P450 3A, may lead to hepatocyte death through the induction of apoptosis (Subehan et al., 2006).

T. sinensis has also been implicated in jaundice and toxic hepatitis along with *T. crispa* (Pelclova et al. 2013). However, in Ayurvedic system of medicine, *T. cordifolia* and *T. sinensis* are used in the treatment of liver diseases in a formulation called satwa. Satwa from the stems of

T. sinensis has been reported to show hepatoprotective activity in alcohol induced toxicity (Chavan et al. 2017) and in acetaminophen induced toxicity in rats (Chavan et al. 2013). *T. cordifolia* and *T. sinensis* satwa reduced ALT, AST and TBIL in paracetamol induced hepatotoxicity in rats at dose of 200 mg/kg, i.p. (Nagarkar 2013).

There seems to be a gap of knowledge, regarding the effect of the *T. sinensis*, *T. crispa* and its furanoditerpenoids i.e. the borapetosides, which are being suggested to play a role in affecting the liver. Moreover, toxicity studies for these compounds of *T. crispa* have not been carried out. In order to elucidate their role on liver, experiments were conducted to assess the hepatotoxic potential of the stems of the *Tinospora* species and the borapetosides isolated from *T. crispa* under normal and health compromised conditions. A seven-day study was conducted to assess the hepatoprotective potential of *T. crispa*, its furanoditerpenoids and *T. sinensis.* The investigations were carried out in mouse model for hepatotoxicity using oral MCT (200 mg/kg) administration, with potentiation of liver injury by co-administration of non-toxic dose (6 mg/kg) of LPS.

4.1.4. MCT-LPS induced hepatotoxicity mice model

Rodent models provide a reliable means to understand the underlying mechanisms involved in a drug induced liver injury. The *in vivo* model of LPS-MCT induced hepatotoxicity was utilized to determine the toxic potential of borapetoside B, C and F isolated from *T. crispa* and the crude extracts of *T. crispa* and *T. sinensis* under health compromised conditions. Monocrotaline (MCT), a pyrrolizidine alkaloid, is a well-known representative example of hepatotoxin of natural product origin (Copple, Ganey, and Roth 2003). Monocrotaline induced hepatotoxicity has been reported either through ingestion as a herbal product or through

contamination of food grains (Yan, C. C, and Huxtable 1998). The induction of a modest inflammatory response decreases the toxic threshold for xenobiotics thereby enhancing liver tissue sensitivity leading to an untoward drug reaction with special emphasis on drug induced liver injury. Concurrent administration of LPS with MCT- results in hepatic parenchymal cell injury (Abdel-Bakky et al., 2010) .

4.2. Objective

The objective of the present study is to investigate the hepatotoxic or hepatoprotective potential of *T. crispa*, its major borapetosides and *T. sinensis in vivo* to elucidate their potential effect on the liver. Hepatotoxicity associated with *T. crispa, its* borapetosides B, C and F (combined in same proportion as present in the crude extract of *T. crispa*) and *T. sinensis,* a short term/sub-acute study (21 days) was conducted. Furthermore, an acute study was conducted to elucidate the role of each borapetoside B, C and F on liver in the mouse model. Hepatoprotective potential of *Tinospora* species and combination of borapetoside B, C and F was assessed in a seven-day study using LPS and MCT induced hepatotoxicity model in mice.

4.3. Experimental section

4.3.1 Materials.

Crotaline (MCT) and Lipopolysaccharide, LPS (from *E. coli*) were purchased from St. Louis, MO, USA. Injectable sterile normal saline was purchased from Hospira, Inc, Lake forest, IL, USA. The crude methanolic extract were prepared from the stems of *T. crispa* (NCNPR#17091) and *T. sinensis* (NCNPR#17003), whereas borapetosides B, C and F were isolated from *T. crispa*.

4.3.2. Animals.

Healthy mice of the strain ND-4, male weighing 15 - 20 g were obtained from Envigo (Indianapolis, Indiana, USA). The animals were housed in micro isolator cages with corncob bedding, on 12 h light/dark cycle, at 72 °F temperature and 35-50 % relative humidity. They were fed on Purina 5001 laboratory chow and water ad libitum. The experimental procedure was in accordance with the regulations of Institutional Animal Care and Use Committee, IACUC at the University of Mississippi (Protocol# 16-009). All mice were fasted overnight before starting experiments.

4.3.3. Treatment preparation

The methanolic extracts of the stems of *T. crispa* and *T. sinensis* were freeze dried to remove traces of solvent. Borapetosides B, C and F - the major furanoditerpenoids were isolated from *T. crispa.* Quantitative analysis of the *T. crispa* extract was performed to determine the ratio of borapetosides B, C and F in the methanolic extract. Treatment dose for combination of borapetosides B, C and F (500 mg/kg body weight) was prepared by combining the pure compounds (B, C and F) in the ratio 7.0:8.4:3.0 (same ratio as present in extract). The test samples were either dissolved in water or prepared as suspension with the help of mortar and pestle. A pinch of gum acacia was added to make a uniform suspension.

4.3.4. Quantitative analysis

Quantitative analysis of the methanolic extract of *T. crispa* was performed on Waters Acquity UPLC system. The quantity of borapetoside B, C and F in the extract were determined by UHPLC-UV. Separation was achieved on an Acquity UPLC HSS T3 column (1.8µm, 2.1x100mm) using mobile phase acetonitrile containing 0.05% formic acid (A) and 0.05%

formic acid aqueous solution (B). The flow rate was 0.3 mL/min. The column and sample temperature were maintained at 55ºC and 10ºC respectively. Analysis was undertaken using gradient elution as follows: 0-12 min, 15% A: 85% B to 37% A: 63% B; 12-13 min, held at 37% A:63% B; 13-15 min, 37% A:63% B to 100% A. Each run was followed by a 3 minutes washing procedure with 100% A and re-equilibration using initial condition for 3.5 minutes. The injection volume was 2 µL. The detection wavelength was 210 nm.

4.3.5. Experimental design.

4.3.5.1. Hepatotoxic study.

The hepatotoxic potential of crude extracts of *T. crispa* and *T. sinensis* and combination of three major furanoditerpenoids isolated from *T crispa* (borapetosides B, C and F) were investigated in healthy mice and mice that were sensitized with a sub-toxic dose of LPS (6mg/kg i.p) to create a health compromised condition. Two experiments were carried out as described under:

i) Experiment 1 (21-day study)

Mice were randomly divided into six test groups (n=5). Group **I** and **II** were given daily treatment of BCF (500 mg/kg body weight) orally. Groups **III** and **IV** were given *T. crispa* (1 g/kg body weight) and groups **V** and **VI** were given *T. sinensis* (1g/kg body weight), Groups **I**, **III** and **V** were also administered a single i.p injection of LPS (6 mg/kg) on the 7th day of the experiment to induce health compromised conditions. Daily mortality of mice was observed and all animals were sacrificed after 21 days of treatment regimen.

ii) Experiment 2 (acute study)

An acute study was carried out for the major borapetosides (B, C and F) under healthy and health compromised conditions (induced by LPS 6 mg/kg i.p). The animals were randomly divided into five groups with three mice in each group. Group **VII** and **VIII** were administered borapetosides B (500 mg/kg body weight), groups **IX** and **X** were given borapetoside C (500 mg/kg body weight) and group **XI** was given borapetosides F (500 mg/kg body weight) orally. Groups **VII**, **IX** and **XI** were also given LPS (6 mg/kg i.p.) 45 minutes after the administration of the drug.

iii) Controls

Four groups served as controls in the aforementioned investigations. One group was administered monocrotaline (MCT), a known hepatotoxicant, at sub-toxic dose of 200 mg/kg orally followed (after 45 mins) by vehicle, saline (i.p). The second group was administered LPS at the dose of 6 mg/kg i.p. after 45 mins of vehicle administration (oral). The third group received MCT (200 mg/kg) orally followed by LPS i.p (6 mg/kg) after 45 min interval and was assigned positive control. The fourth group was given only vehicle and was assigned normal.

4.3.5.2. Hepatoprotective study

i) Experiment 3 (seven-day study)

The animals were divided randomly into five groups (n=3) to assess the hepatoprotective efficacy of *T. sinensis, T. crispa* and BCF. Three test groups comprised of groups **XII**, **XIII** and **XIV** were given orally *T. sinensis* (500 mg/kg body weight) *T. crispa* (500 mg/kg body weight) and BCF (combination, 250 mg/kg body weight) respectively for 7 days. On the 7th

day, each group was given MCT (200 mg/kg) via the oral route followed by i.p injection of LPS (6 mg/kg) after 45 minutes of MCT administration in an attempt to induce hepatotoxicity. Blood was withdrawn on the 8th day followed by euthanasia. Two groups served as controls, one group received MCT (200 mg/kg) orally followed by LPS (6 mg/kg) after 45 minutes whereas the second group was administered vehicle (saline i.p) alone.

4.3.6. Statistical Analysis

The data was analyzed by One way Anova test followed by Tukey-Kramer multiple comparisons using Graph pad prism software (La Jolla, CA). $P < 0.05$ was considered statistically significant.

4.3.7. Blood sample collection

Blood samples were drawn by cardiac puncture under anesthesia on the 22nd day in the short term study and after 24 hours in the acute study. The blood was collected on the 8th day from the mice employed for the seven-day hepatoprotective study. The blood was collected in heparinized and also in EDTA coated micro tubes to be immediately analyzed for clinical chemistry and hematology parameters, respectively, by VetScan dry chemistry analyzer with comprehensive Diagnostic Profiles (Abaxis Union City, CA) and HM2 hematology Analyzer.

4.3.8. Determination of Hepatotoxicity

Clinical chemistry and hematology profile was determined to assess hepatotoxic damage. The clinical chemistry parameters analyzed were alanine phosphate, albumin, alanine transferase, amylase, bilirubin, creatinin, total bilirubin, blood urea nitrogen, globulin, total protein, calcium, potassium, sodium and phosphorus. Red blood cells, white blood cells, hemoglobin, lymphocytes, monocytes, granulocytes, GRA%, MO%, LY%, HCT, HGB, MCV,

MCHC, RWDc and MCHC were among the hematological parameters analyzed from the blood samples.

4.3.9. Histological Studies

For histopathological analysis, liver specimens were collected and fixed in 10% formalin and sliced 5 μm thick, embedded in paraffin. The liver sections were stained with hematoxylin and eosin (H and E).

4.4. Results

4.4.1. Hepatotoxicity Study

4.4.1.1 Experiment 1 (21-day study).

Test groups, **I-II**, **III-IV**, **V-VI** were treated with combination BCF and the crude extracts of *T. crispa* and *T. sinensis* respectively showed results comparable with the normal group in both healthy and health compromised mice.

i) ***Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on ALT levels*:**

The positive control group administered LPS and MCT showed significant elevation in ALT levels. The crude extracts of either *T crispa* **(Table 4.1)**, combination BCF **(Table 4.2)** representing the furanoditerpenoids, and or *T. sinensis* **(Table 4.3)** did not show significant elevation of ALT levels compared with normal group.

ii) ***Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on other blood chemistry parameters***

The clinical chemistry parameters of all test groups showed results that were not demonstrative of a hepatotoxic or nephrotoxic effect under the LPS induced health compromised conditions in mice **(Table 4.1, 4.2, 4.3)**.

iii) Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on hematological parameters

The results on hematological parameters showed normal blood profile in mice treated with *T crispa* **(Table 4.4)**, combination BCF **(Table 4.5)** and *T. sinensis* **(Table 4.6)** under both normal and LPS induced health compromised conditions.

iv) ***Effect on body weight:***

The weight of the animals in each group was averaged and plotted. An increase in the average weight of animals in each test group was observed. **Figure 4.1a-c** shows the comparative weights of mice in group **I** and **II**, **III** and **IV**, **V** and **VI**.

v) ***Effect combination BCF and the crude extracts of T. crispa and T. sinensis on histological architecture****:*

The histopathological analysis of the liver from mice treated with MCT + LPS showed severe necrosis around central vein. Disintegration of the cellular structure with focal lesions were also observed in MCT + LPS group **(Fig.4.2d)**. However, the mice treated with MCT only, LPS only or *T. crispa*, *T. sinensis* or BCF (in combination) showed ballooning of cytoplasm which is due to accumulation of glycogen and is not considered abnormal histological architecture. This was observed under normal as well as under health compromised conditions **(Fig.4.2a-c, e-j)**.

4.4.1.2. Experiment 2 (acute Study):

i) Effect of borapetosides B, C and F on ALT levels and other clinical chemistry parameters:

Assessment of each borapetoside B, C and F in health compromised mice showed negative results, indicating that the furanoditerpenoids either alone or in combination at a high dose of 500 mg/kg body weight did not produce any hepatotoxicity as ALT levels and other chemical parameters were normal compared to the LPS group in ND-4 mice. Borapetoside B and C were assessed in normal mice as well and results comparable with normal group, administered vehicle alone, were observed **(Table 4.7)**.

ii) Effect of Borapetoside B, C and F on hematological parameters.

The hematological parameters consisting of RBC count, WBC count, HCT, HGB, MCV, MCHC, RWDc and MCHC, hemoglobin, lymphocytes, monocytes, granulocytes and their relative percentages for either borapetoside B, C and F administered with or without LPS showed results comparable with normal group given vehicle alone **(Table 4.8).**

iii) Effect on histological architecture

The liver section of animals treated with borapetoside F under LPS induced health compromised conditions in the acute study showed normal vasculature of liver **(Fig.4.2k)** comparable with normal group.

4.4.2. Hepatoprotective study:

4.4.2.1 Experiment 3 (Seven-day study).

i) Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on ALT levels and other blood chemistry parameters:

The results, for the methanolic extract of *T. crispa, T. sinensis* and BCF (combination of borapetosides B, C and F) isolated from *T. crispa*, for a potential hepatoprotective action against LPS mediated potentiation of monocrotaline hepatotoxic potential are shown in **Table 4.9**. A single mouse mortality was observed in each of the three test groups. The clinical chemistry results for test groups **XII**, **XIII** and **XIV** treated with *T. sinensis*, *T. crispa* and BCF showed increased ALT levels, which clearly depicts the absence of a potential hepatoprotective efficacy of either of the test drugs.

ii) Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on hematological parameters*:*

All of the hematological parameters for the test groups, **XII**, **XIII** and **XIV** treated in the seven-day study were comparable with those of the normal group and lied in the normal range **(Table 4.10)**.

iii) Effect of combination BCF and the crude extracts of T. crispa and T. sinensis on liver histology:

Mice in group **XII**, **XIII** and **XIV** were treated with *T. sinensis*, *T. crispa* and BCF to observe a hepatoprotective effect under MCT+LPS induced hepatotoxic conditions. Histopathological results of the liver specimens from each group **(Fig.**

4.3c-e) showed areas of necrosis (cell death), and neutrophilic infiltration. *T. crispa* showed marked accumulation of Kupffer cells and RBC infiltration in sinusoids. The results clearly showed that neither of the test samples provides protection against MCT/LPS induced liver toxicity.

Table 4.1. Clinical chemistry for *T. crispa* in normal and health compromised conditions in short term study (21days)

Clinical Chemistry	*T. crispa* + LPS	*T. crispa* only	MCT + LPS	MCT	LPS	Normal
ALT U/L	24.33 ± 0.88	31.00 ± 4.0	768 ±88.59**	54.00 ± 5.0	25.33 ± 1.3	42.00 ± 2.3
ALB g/dL	3.4 ± 0.32	3.9 ± 0.15	2.3 ± 0.27	3.5 ± 0.10	2.7 ± 0.39	2.5 ± 0.08
TBIL mg/dL	0.27 ± 0.02	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.26 ± 0.033
BUN mg/dL	18 ± 2.8	21 ± 1.1	103.6 ±15.01*	20.50 ± 1.5	14.33 ± 0.88	32.33 ± 2.3
Creatinin mg/dL	0.20 ± 0.0	0.20 ± 0.0	0.52 ± 0.22	0.20 ± 0.0	0.20 ± 0.0	0.26 ± 0.033
ALP U/L	89.75 ± 5.5	76.33 ± 1.6	46.20 ± 6.34***	139 ± 11	73 ± 19	126 ± 2.1
Total protein g/dL	5.5 ± 0.44	5.6 ± 0.05	5.4 ± 0.13	5.1 ± 0.35	5.7 ± 0.06	5.0 ± 0.05
Amylase U/L	625 ± 79.64	540.7 ± 35.90	642.2 ± 65.37*	578 ± 77	734 ± 114	933 ± 10
Globulin g/dL	1.8 ± 0.15	1.9 ± 0.06	1.6 ± 0.30	3.0 ± 0.35	2.4 ± 0.12	1.8 ± 0.15
Phosphorus mg/L	6.9 ± 0.67	5.9 ± 1.1	12 ± 1.9	9.6 ± 0.05	7.4 ± 0.76	9.3 ± 0.30
Sodium mmol/L	139.0 ± 7.6	148.3 ± 0.88	143 ± 2.6	149 ± 1.5	148 ± 2.6	152 ± 0.0
Calcium g/dL	10.38 ± 0.83	11.10 ± 0.23	11.40 ± 0.16	11.80 ± 0.30	10.97 ± 0.27	16.87 ± 5.5
Potassium mmol/L	5.7 ± 0.45	6.0 ± 0.08	7.6 ± 0.41	6.9 ± 0.35	7.3 ± 0.21	6.3 ± 0.17

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group.

Table 4.2. Clinical chemistry for BCF in normal and health compromised conditions in short term study (21 days)

Clinical Chemistry	Comb BCF + LPS	BCF only	MCT + LPS	MCT	LPS	Normal
ALT U/L	28.80 ± 4.2	39.20 ± 15.77	768 ±88.59**	54.00 ± 5.0	25.33 ± 1.3	42.00 ± 2.3
ALB g/dL	3.8 ± 0.39	3.7 ± 0.31	2.3 ± 0.27	3.5 ± 0.10	2.7 ± 0.39	2.5 ± 0.08
TBIL mg/dL	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.26 ± 0.033
BUN mg/dL	21.60 ± 2.2	42.60 ± 19	103.6 ±15.01*	20.50 ± 1.5	14.33 ± 0.88	32.33 ± 2.3
Creatinin mg/dL	0.24 ± 0.02	0.24 ± 0.02	0.52 ± 0.22	0.20 ± 0.0	0.20 ± 0.0	0.26 ± 0.033
ALP U/L	93.60 ± 14	90.80 ± 12	46.20 ± 6.34***	139 ± 11	73 ± 19	126 ± 2.1
Total Protein g/dL	5.9 ± 0.16	5.7 ± 0.19	5.4 ± 0.13	5.1 ± 0.35	5.7 ± 0.06	5.0 ± 0.05
Amylase U/L	1196 ± 87	487 ± 49**	642.2 ± 65.37*	578 ± 77	734 ± 114	933 ± 10
Globulin g/dL	1.9 ± 0.14	3.0 ± 0.30	1.6 ± 0.30	3.0 ± 0.35	2.4 ± 0.12	1.8 ± 0.15
Phosphorus mg/L	6.0 ± 0.76	7.6 ± 0.98	12 ± 1.9	9.6 ± 0.05	7.4 ± 0.76	9.3 ± 0.30
Sodium mmol/L	147 ± 3.2	150 ± 1.3	143 ± 2.6	149 ± 1.5	148 ± 2.6	152 ± 0.0
Calcium mg/dL	11.30 ± 0.10	11.18 ± 0.19	11.40 ± 0.16	11.8 ± 0.30	10.97 ± 0.27	16.87 ± 5.5
Potassium mmol/L	7.2 ± 0.29	6.8 ± 0.22	7.6 ± 0.41	6.9 ± 0.35	7.3 ± 0.21	6.3 ± 0.17

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.3. Clinical chemistry for *T. sinensis* in normal and health compromised conditions in short term study (21 days)

Clinical Chemistry	*T. sinensis* + LPS	*T. sinensis* only	MCT + LPS	MCT	LPS	Normal
ALT U/L	22.00 ± 1.7	29.20 ± 0.96	768 ±88.59**	54.00 ± 5.0	25.33 ± 1.3	42.00 ± 2.3
ALB g/dL	3.6 ± 0.11	3.8 ± 0.13	2.3 ± 0.27	3.5 ± 0.10	2.7 ± 0.39	2.5 ± 0.08
TBIL mg/dL	0.32 ± 0.02	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.30 ± 0.0	0.26 ± 0.033
BUN mg/dL	17.60 ± 0.97	21.80 ± 0.73	103.6 ±15.01*	20.50 ± 1.5	14.33 ± 0.88	32.33 ± 2.3
Creatinin mg/dL	0.22 ± 0.02	0.20 ± 0.0	0.52 ± 0.22	0.20 ± 0.0	0.20 ± 0.0	0.26 ± 0.033
ALP U/L	102 ± 4.5	98.60 ± 6.8	46.20 ± 6.34***	139 ± 11	73 ± 19	126 ± 2.1
Total protein g/dL	5.5 ± 0.06	5.8 ± 0.21	5.4 ± 0.13	5.1 ± 0.35	5.7 ± 0.06	5.0 ± 0.05
Amylase U/L	621 ± 55	699 ± 77	642.2 ± 65.37*	578 ± 77	734 ± 114	933 ± 10
Globulin g/dL	1.6 ± 0.08	2.1 ± 0.30	1.6 ± 0.30	3.0 ± 0.35	2.4 ± 0.12	1.8 ± 0.15
Phosphorus mg/L	7.2 ± 0.33	5.0 ± 0.41	12 ± 1.9	9.6 ± 0.05	7.4 ± 0.76	9.3 ± 0.30
Sodium mmol/L	150 ± 1.2	149 ± 1.0	143 ± 2.6	149 ± 1.5	148 ± 2.6	152 ± 0.0
Calcium mg/dL	10.76 ± 0.97	11.16 ± 0.19	11.40 ± 0.16	11.80 ± 0.30	10.97 ± 0.27	16.87 ± 5.5
Potassium mmol/L	6.7 ± 0.11	6.9 ± 0.20	7.6 ± 0.41	6.9 ± 0.35	7.3 ± 0.21	6.3 ± 0.17

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.4. Hematology for *T. crispa* in normal and health compromised conditions in short term study

Hematology parameters	*T. crispa* + LPS	*T. crispa* only	MCT + LPS	MCT	LPS	Normal
WBC(10^9 /l)	10.68 ± 2.4	14.27 ± 3.9 *	4.7 ± 1.8	16.45 ± 7.6 *	5.12 ± 2.017	2.22 ± 0.65
LYM(10^9 /l)	7.7 ± 2.5	14.17± 0.05 **	2.1 ± 1.5	13.50 ± 6.27**	3.70 ± 2.04	1.58 ± 0.71
MON(10^9 /l)	0.55 ± 0.13	0.91 ± 0.23	0.33 ± 0.12	1.42 ± 1.06**	0.24 ± 0.042	0.067 ± 0.06
GRA(10^9 /l)	2.4 ± 0.21	2.5 ± 0.47	2.3 ± 0.25	1.53 ± 0.27	1.170 ± 0.08	1.32 ± 1.15
LY(%)	65.53 ± 10	80.70 ± 3.17	28.97 ± 9.0	81.95 ± 0.25	63.97 ± 10.63	56.6 ± 13.94
MO(%)	5.1 ± 0.43	5.1 ± 1.1	7.4 ± 1.1	7.1 ± 3.1	5.5 ± 0.98	3.77 ± 5.43
GRA(%)	29.40 ± 10	14.17 ± 2.2	63.67 ± 8.6	10.90 ± 3.4	30.47 ± 9.6	39.65 ± 16.86
RBC(10^{12} /l)	9.3 ± 0.42	10.14 ± 0.38	10.31 ± 0.21	10.01 ± 0.10	9.9 ± 0.30	10.96 ± 2.28
HGB(g/dl)	13.48 ± 0.63	14.53 ± 0.37	14.48 ± 0.24	14.80 ± 0.0	14.77 ± 0.13	14.22 ± 1.11
HCT(%)	39.93 ± 1.9**	43.34 ± 1.4	44.32 ± 0.73	45.22 ± 0.12	46.01 ± 0.50	49.6 ± 7.36
MCV(fl)	43.00 ± 1.0	42.00 ± 0.57	43.00 ± 1.0	45.0 ± 0.0	46.67 ± 1.2	45.5 ± 2.38
MCH(pg)	14.53 ± 0.22	14.10 ± 0.28	14.07 ± 0.32	14.75 ± 0.15	14.97 ± 0.33	13.3 ± 2.16
MCHC(g/dl)	33.8 ± 0.55***	33.53± 0.29**	32.72 ± 0.24**	32.65 ± 0.05	32.13 ± 0.38	29.05 ± 3.32
RDWc(%)	18.95 ± 0.61	17.63 ± 0.58	17.63 ± 0.18	17.90 ± 0.30	17.90 ± 0.05	18.27 ± 1.58

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.5. Hematology for BCF in normal and health compromised conditions in short term study

Hematology parameters	Comb BCF + LPS	BCF only	MCT + LPS	MCT	LPS	Normal
WBC(10^9 /l)	5.6 ± 0.42	8.370 ± 2.04	4.7 ± 1.8	16.45 ± 7.6 *	5.12 ± 2.017	2.22 ± 0.65
LYM(10^9 /l)	3.6 ± 0.27	6.87 ± 1.95	2.1 ± 1.5	13.50 ± 6.27**	3.70 ± 2.04	1.58 ± 0.71
MON(10^9 /l)	0.34 ± 0.14	0.19 ± 0.06	0.33 ± 0.12	1.42 ± 1.06**	0.24 ± 0.042	0.067 ± 0.06
GRA(10^9 /l)	1.6 ± 0.2	1.3 ± 0.36	2.3 ± 0.25	1.53 ± 0.27	1.170 ± 0.08	1.32 ± 1.15
LY(%)	65.54 ± 5.0	69.66 ± 13	28.97 ± 9.0	81.95 ± 0.25	63.97 ± 10.63	56.6 ± 13.94
MO(%)	5.8 ± 2.2	3.3 ± 1.3	7.4 ± 1.1	7.1 ± 3.1	5.5 ± 0.98	3.77 ± 5.43
GRA(%)	28.68 ± 4.4	27.00 ± 12	63.67 ± 8.6	10.90 ± 3.4	30.47 ± 9.6	39.65 ± 16.86
RBC(10^{12} /l)	9.6 ± 0.33	10.66 ± 0.11	10.31 ± 0.21	10.01 ± 0.10	9.9 ± 0.30	10.96 ± 2.28
HGB(g/dl)	14.12 ± 0.16	14.62 ± 0.16	14.48 ± 0.24	14.80 ± 0.0	14.77 ± 0.13	14.22 ± 1.11
HCT(%)	42.38 ± 0.99	43.64 ± 0.62	44.32 ± 0.73	45.22 ± 0.12	46.01 ± 0.50	49.6 ± 7.36
MCV(fl)	42.40 ± 0.67	40.80 ± 0.58*	43.00 ± 1.0	45.0 ± 0.0	46.67 ± 1.2	45.5 ± 2.38
MCH(pg)	14.18 ± 0.10	13.68 ± 0.22	14.07 ± 0.32	14.75 ± 0.15	14.97 ± 0.33	13.3 ± 2.16
MCHC(g/dl)	33.34 ± 0.56***	33.44 ± 0.36***	32.72 ± 0.24**	32.65 ± 0.05	32.13 ± 0.38	29.05 ± 3.32
RDWc(%)	17.58 ± 0.11	17.04 ± 0.13	17.63 ± 0.18	17.90 ± 0.30	17.90 ± 0.05	18.27 ± 1.58

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.6. Hematology for *T. sinensis* in normal and health compromised conditions in short term study

Hematology parameters	*T. sinensis* + LPS	*T. sinensis* only	MCT + LPS	MCT	LPS	Normal
WBC(10^9 /l)	7.2 ± 0.47	12 ± 1.4*	4.7 ± 1.8	16.45 ± 7.6 *	5.12 ± 2.017	2.22 ± 0.65
LYM(10^9 /l)	6.2 ± 0.41	10.32 ± 1.2 *	2.1 ± 1.5	13.50 ± 6.27**	3.70 ± 2.04	1.58 ± 0.71
MON(10^9 /l)	0.26 ± 0.06	0.37 ± 0.08	0.33 ± 0.12	1.42 ± 1.06**	0.24 ± 0.042	0.067 ± 0.06
GRA(10^9 /l)	0.72 ± 0.15	1.5 ± 0.27	2.3 ± 0.25	1.53 ± 0.27	1.170 ± 0.08	1.32 ± 1.15
LY(%)	86.38 ± 1.7	83.96 ± 2.2	28.97 ± 9.0	81.95 ± 0.25	63.97 ± 10.63	56.6 ± 13.94
MO(%)	3.5 ± 0.93	3.2 ± 0.88	7.4 ± 1.1	7.1 ± 3.1	5.5 ± 0.98	3.77 ± 5.43
GRA(%)	10.12 ± 2.3	12.78 ± 1.5	63.67 ± 8.6	10.90 ± 3.4	30.47 ± 9.6	39.65 ± 16.86
RBC(10^{12} /l)	9.1 ± 0.28	10.0 ± 0.23	10.31 ± 0.21	10.01 ± 0.10	9.9 ± 0.30	10.96 ± 2.28
HGB(g/dl)	13.16 ± 0.58	14.48 ± 0.41	14.48 ± 0.24	14.80 ± 0.0	14.77 ± 0.13	14.22 ± 1.11
HCT(%)	40.32 ± 1.4**	43.18 ± 1.0	44.32 ± 0.73	45.22 ± 0.12	46.01 ± 0.50	49.6 ± 7.36
MCV(fl)	44.00 ± 0.31	42.60 ± 0.24	43.00 ± 1.0	45.0 ± 0.0	46.67 ± 1.2	45.5 ± 2.38
MCH(pg)	14.38 ± 0.10	14.28 ± 0.08	14.07 ± 0.32	14.75 ± 0.15	14.97 ± 0.33	13.3 ± 2.16
MCHC(g/dl)	32.64 ± 0.31**	33.54 ± 0.22***	32.72 ± 0.24**	32.65 ± 0.05	32.13 ± 0.38	29.05 ± 3.32
RDWc(%)	17.54 ± 0.13	16.66 ± 0.11	17.63 ± 0.18	17.90 ± 0.30	17.90 ± 0.05	18.27 ± 1.58

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

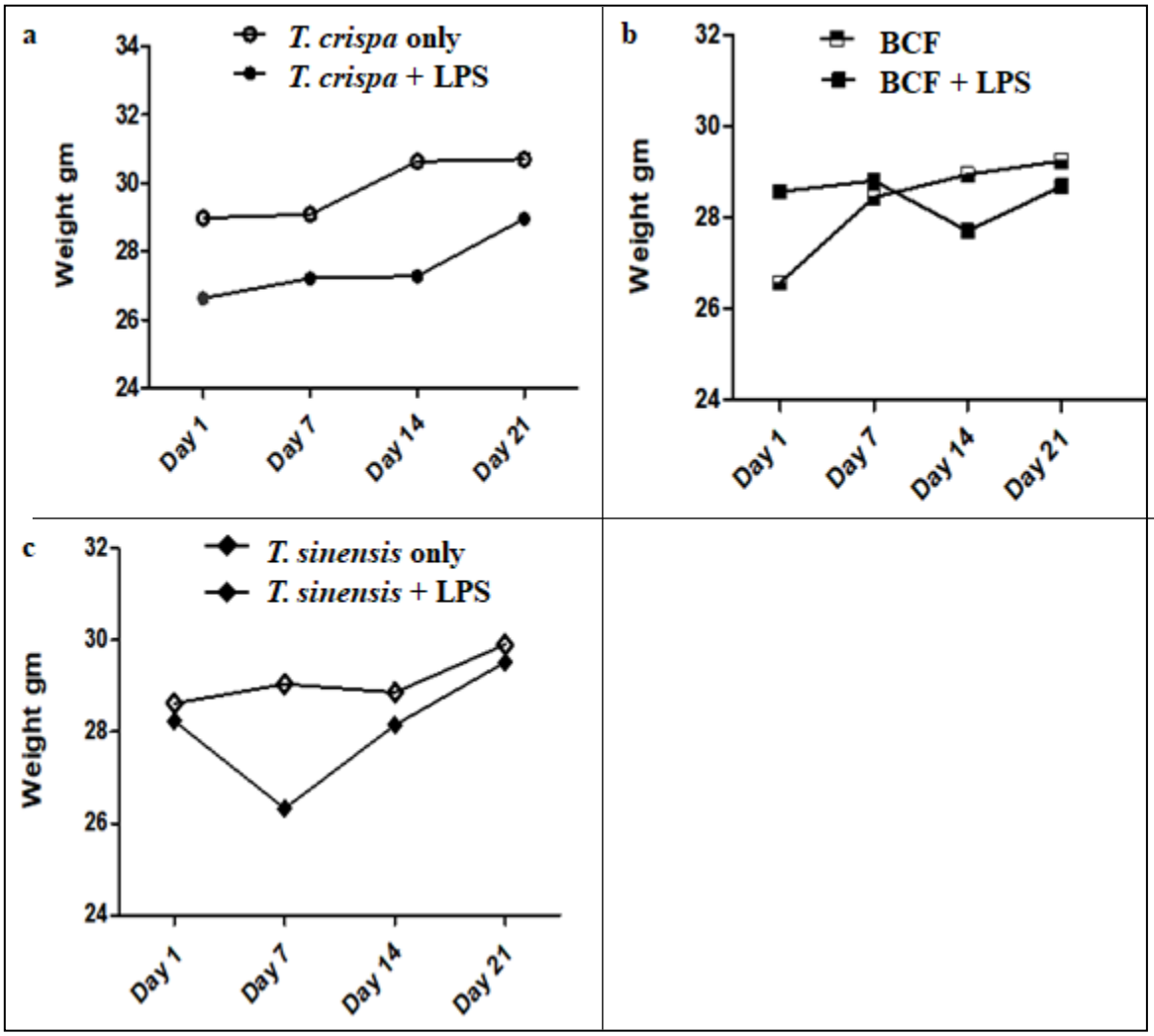


Figure 4.1. Comparative weights of mice in group in short term study (21 days)

Table 4.7. Results of Clinical chemistry parameters for Acute study (24hrs)

	Normal animals		Health compromised conditions			Controls	
Clinical Chemistry	**Comp B**	**Comp C**	**Comp B + LPS**	**Comp C+LPS**	**Comp F + LPS**	**MCT + LPS**	**Normal**
ALT U/L	31.33 ± 0.66	29.00 ± 1.52	24.33 ± 2.3	24 ± 3.0	29.67 ± 2.3	768 ±88.59***	42 ± 2.3
Albumin g/dL	3.5 ± 0.18	3.5 ± 0.10	2.4 ± 0.10	2.25 ± 0.05	2.2 ± 0.03	2.3 ± 0.27	2.5 ± 0.08
Total bilirubin g/dL	0.30 ± 0.0	0.30 ± 0.0	0.26 ± 0.03	0.25 ± 0.05	0.30 ± 0.0	0.30 ± 0.0	0.26 ± 0.03
BUN mg/dL	17.00 ± 2.08	17.33 ± 1.45	18.33 ± 0.88	17.50 ± 1.5	19.67 ± 0.33	103.6 ±15.01***	32.33 ± 2.3
Creatinin mg/dL	0.20 ± 0.0	0.20 ± 0.0	0.20 ± 0.0	0.20 ± 0.0	0.20 ± 0.0	0.52 ± 0.22	0.26 ± 0.03
Alkaline phosphatase U/L	174.0 ± 26.86	179.3 ± 5.04	73.67 ± 0.88	63.50 ± 11.50	78.67 ± 4.4	46.20 ± 6.34**	126.7 ± 2.1
Total protein g/dL	5.2 ± 0.08	5.2 ± 0.05	5.6 ± 0.17	5.6 ± 0.10	5.1 ± 0.17	5.4 ± 0.13	5.0 ± 0.05
Amylase U/L	787.3 ± 151.3	817.7 ± 34.7	618.7 ± 37.08	728 ± 23.00	683.3 ± 282.3	642.2 ± 65.37*	933 ± 10.79
Globulin g/dL	1.6 ± 0.14	1.70 ± 0.11	3.3 ± 0.20	3.3 ± 0.05	2.9 ± 0.20	1.6 ± 0.30	2.4 ± 0.12
Phosphorus mg/L	9.6 ± 0.58	9.3 ± 0.17	7.63 ± 0.26	8.8 ± 0.50	7.9 ± 0.10	12 ± 1.9	9.3 ± 0.30
Sodium mmol/L	149 ± 1.20	150 ± 0.57	146 ± 4.04	143 ± 3.0	146 ± 0.57	143 ± 2.6	152 ± 0.0
Calcium mg/dL	11.73 ± 0.13	11.73 ± 0.21	11.27 ± 0.16	11.25 ± 0.25	11.10 ± 0.25	11.40 ± 0.16	11.17 ± 0.17
Potassium mol/L	6.60 ± 0.37	6.6 ± 0.17	7.7 ± 0.17	7.3 ± 0.35	6.8 ± 0.18	7.6 ± 0.41	6.3 ± 0.17

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.8. Results of Hematology parameters for Acute study

	Normal animals		Health compromised conditions			Controls	
	Comp B	**Comp C**	**Comp B + LPS**	**Comp C+ LPS**	**Comp F + LPS**	**MCT + LPS**	**Normal**
WBC(10^9 /l)	12 ± 0.45*	9.7 ± 1.7	3.9 ± 1.0	6.2 ± 0.30	3.6 ± 0.46	4.7 ± 1.8	2.22 ± 0.65
LYM(10^9 /l)	11.09 ± 0.48	8.5 ± 1.8	2.1 ± 0.60	3.9 ± 0.27	1.8 ± 0.25	2.1 ± 1.5	1.58 ± 0.71
MON(10^9 /l)	0.29 ± 0.09	0.20 ± 0.06	0.27 ± 0.05	0.59 ± 0.04	0.23 ± 0.03	0.33 ± 0.12	0.067 ± 0.06
GRA(10^9 /l)	1.0 ± 0.11	0.97 ± 0.05	1.5 ± 0.52	1.7 ± 0.07	1.61 ± 0.23	2.3 ± 0.25	1.32 ± 1.15
LY(%)	89.27 ± 1.6	86.90 ± 2.9	53.7 ± 4.0	62.65 ± 1.3	49.67 ± 2.5	28.97 ± 9.0	56.6 ± 13.94
MO(%)	2.3 ± 0.79	2.3 ± 0.82	7.7 ± 1.5	9.6 ± 1.2	6.4 ± 1.1	7.4 ± 1.1	3.77 ± 5.43
GRA(%)	8.4 ± 0.85	10.77 ± 2.2	38.53 ± 4.9	27.75 ± 0.15	43.90 ± 1.6	63.67 ± 8.6	39.65 ± 16.86
RBC(10^{12} /l)	9.6 ± 0.29	8.30 ± 0.80	9.7 ± 0.20	10.01 ± 0.21	9.2 ± 0.65	10.31 ± 0.21	10.96 ± 2.28
HGB(g/dl)	14 ± 0.41	11.87 ± 1.3	14.77 ± 0.31	14.95 ± 0.45	13.63 ± 1.0	14.48 ± 0.24	14.22 ± 1.11
HCT(%)	48.96 ± 1.7	38.12 ± 4.5	47.16 ± 1.1	47.13 ± 1.2	43.63 ± 2.8	44.32 ± 0.73	49.6 ± 7.36
MCV(fl)	51.33 ± 0.88*	45.67 ± 1.3	48.67 ± 0.88	47.00 ± 0.0	46.67 ± 0.33	43.00 ± 1.0	45.5 ± 2.38
MCH(pg)	14.60 ± 0.05	14.23 ± 0.27	15.17 ± 0.17	14.95 ± 0.15	14.67 ± 0.13	14.07 ± 0.32	13.3 ± 2.16
MCHC(g/dl)	28.63 ± 0.54	31.17 ± 0.31	31.30 ± 0.11	31.75 ± 0.15	31.23 ± 0.37	32.72 ± 0.24 *	29.05 ± 3.32
RDWc(%)	19.60 ± 0.25	17.77 ± 0.23	18.67 ± 0.20	18.30 ± 0.30	18.23 ± 0.12	17.63 ± 0.18	18.27 ± 1.58

Each value represents mean ± S.E., n = 5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

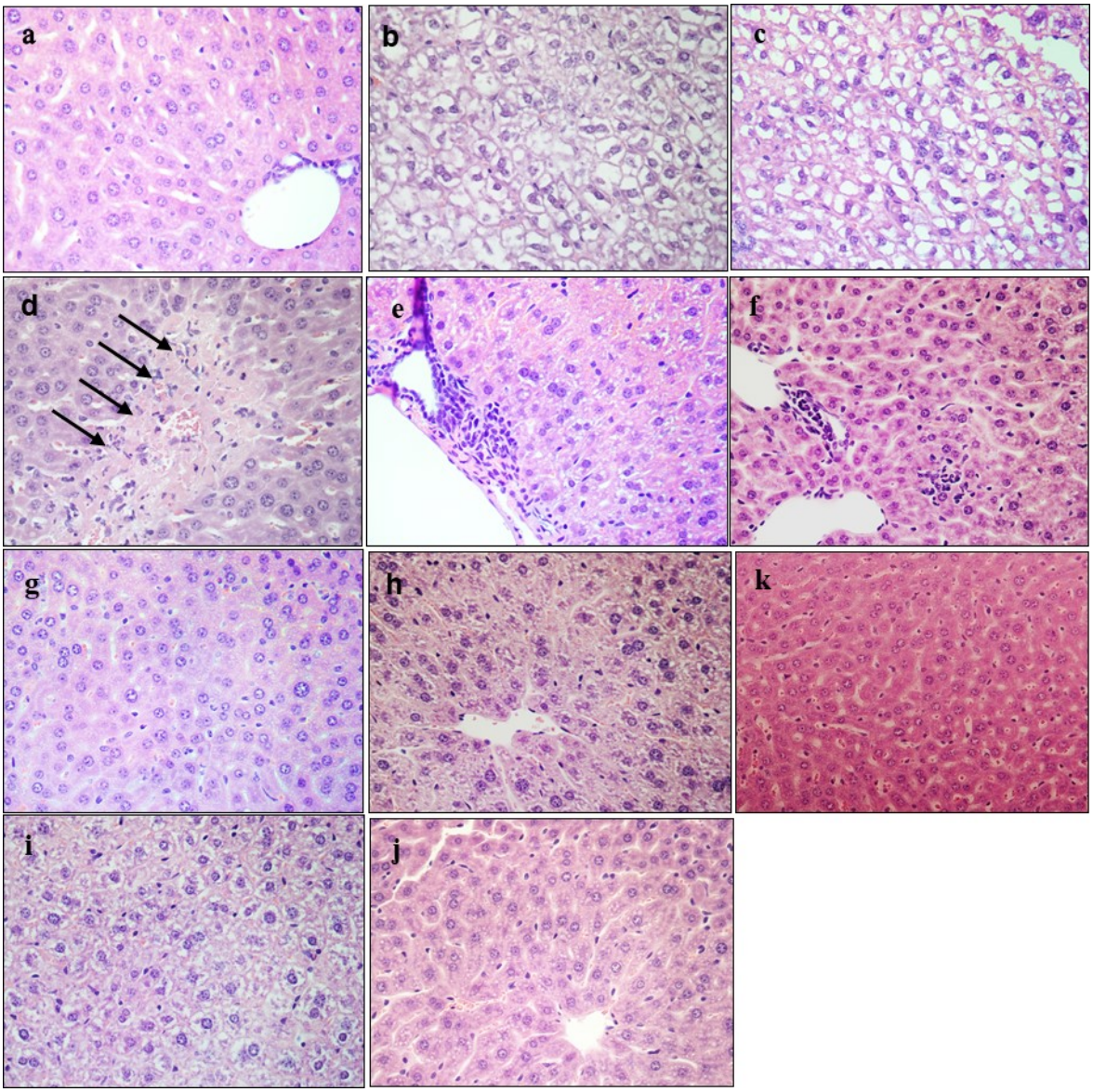


Figure 4.2. Photomicrographs show histopathological architecture of liver tissues from mice groups treated with *T. sinensis*, *T. crispa* and BCF under normal and health compromised conditions as compared with controls.

(**a**) Normal control; (**b**) MCT (200 mg/kg oral); (**c**) LPS (6 mg/kg i.p) (**d**) MCT + LPS (MCT: 200 mg/kg oral and LPS: 6 mg/kg i.p) (**e**) *T. crispa* only (1 gm/kg/day oral for 21 days) (**f**) *T. crispa* (1 g/kg/day oral for 21 days) + LPS (6 mg/kg. i.p); (**g**): BCF only (500 mg/kg/day oral for 21 days) (**h**) BCF (500 mg/kg/day oral for 21 days) + LPS (6 mg/kg. i.p); **(i**) *T. sinensis* only (1 gm/kg/day oral for 21 days) **(j**) *T. sinensis* (1 g/kg/day oral for 21 days) + LPS (6 mg/kg. i.p); (**k**) Borapetoside F (500 mg/kg, single dose, oral) + LPS (6 mg/kg. i.p).

Results Hepatoprotective study:

Experiment 3 (Seven-day study).

Table 4.9. Results of Clinical chemistry parameters for 7-day study

Clinical Chemistry	*T. crispa* + LPS+ MCT	*T. sinensis* + LPS+ MCT	Comb B C F + LPS+ MCT	MCT + LPS	Normal
ALT U/L	180.0 ± 21.0	790 ± 107.2**	2000 ± 57.74***	768 ±88.59**	42.00 ± 2.3
Albumin g/dL	3.0 ± 0.0	2.6 ± 0.17	2.53 ± 0.76	2.3 ± 0.27	2.5 ± 0.08
TBIL mg/dL	0.30 ± 0.0	0.30 ± 0.0	0.33 ± 0.033	0.30 ± 0.0	0.26 ± 0.03
BUN mg/dL	75 ± 10	145.5 ± 13.50**	85.67 ± 9.35	103.6 ±15.01*	32.33 ± 2.3
Creatinin mg/dL	0.45 ± 0.15	0.46 ± 0.13	0.36 ± 0.12	0.52 ± 0.22	0.26 ± 0.03
ALP U/L	58.50 ± 4.5***	50.00 ± 5.1***	89.33 ± 8.19*	46.20 ± 6.34***	126 ± 2.1
Total protein g/dL	5.4 ± 0.30	5.6 ± 0.12	4.3 ± 1.15	5.4 ± 0.13	5.0 ± 0.05
Amylase U/L	399 ± 134.0	1766 ± 88.82***	1683 ± 176.8**	642.2 ± 65.37*	933 ± 10
Globulin g/dL	2.40 ± 0.30	3.0 ± 0.25	2.26 ± 0.23	1.6 ± 0.30	1.8 ± 0.15
Phosphorus mg/L	13.15 ± 6.2	13.63 ± 3.8	13.70 ± 5.7	12 ± 1.9	9.3 ± 0.30
Sodium mmol/L	154 ± 16.0	146.7 ± 2.9	153.0 ± 4.0	143 ± 2.6	152 ± 0.0
Calcium mg/dL	10.75 ± 0.65	11.93 ± 0.29	11.05 ± 0.15	11.40 ± 0.16	16.87 ± 5.5
Potassium mmol/L	7.9 ± 0.40	7.1 ± 0.70	6.86 ± 1.6	7.6 ± 0.41	6.3 ± 0.17

Each value represents mean ± S.E., n = 3-5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

Table 4.10. Results of hematology parameters in seven-day study

	T. crispa + LPS+ MCT	*T. sinensis* + LPS+ MCT	Comb BCF + LPS+ MCT	MCT + LPS	Normal
WBC (10^9 /l)	5.79 ± 1.7	4.8 ± 0.57	2.13 ± 1.0	4.7 ± 1.8	2.22 ± 0.65
LYM (10^9 /l)	2.95 ± 1.2	1.24 ± 0.39	0.89 ± 0.29	2.1 ± 1.5	1.58 ± 0.71
MON (10^9 /l)	0.19 ± 0.13	0.28 ± 0.04	0.19 ± 0.07	0.33 ± 0.12	0.067 ± 0.06
GRA (10^9 /l)	2.64 ± 0.45	3.2 ± 0.2	1.1 ± 0.52	2.3 ± 0.25	1.32 ± 1.15
LY (%)	49.25 ± 5.6	24.70 ± 6.4	42.40 ± 3.7	28.97 ± 9.0	56.6 ± 13.94
MO (%)	3.10 ± 1.1	5.8 ± 0.57	9.0 ± 0.50	7.4 ± 1.1	3.77 ± 5.43
GRA (%)	47.65 ± 6.7	69.50 ± 6.1	48.60 ± 4.2	63.67 ± 8.6	39.65 ± 16.86
RBC (10^{12} /l)	10.96 ± 0.02	10.53 ± 0.54	10.88 ± 0.82	10.31 ± 0.21	10.96 ± 2.28
HGB (g/dl)	15.30 ± 0.10	14.93±0.65	15.60 ± 1.2	14.48 ± 0.24	14.22 ± 1.11
HCT (%)	45.55 ± 0.44	44.27±2.3	46.04 ± 3.6	44.32 ± 0.73	49.6 ± 7.36
MCV (fl)	41.50 ± 0.50	42.00±0.0	42.00 ± 0.0	43.00 ± 1.0	45.5 ± 2.38
MCH (pg)	14 ± 0.10	14.17±0.17	14.30 ± 0.10	14.07 ± 0.32	13.3 ± 2.16
MCHC (g/dl)	33.60 ± 0.0	33.73±0.50	33.90 ± 0.0	32.72 ± 0.24 **	29.05 ± 3.32
RDWc (%)	17.70 ± 0.10	17.00±0.10	17.05 ± 0.15	17.63 ± 0.18	18.27 ± 1.58

Each value represents mean ± S.E., n = 3-5, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the corresponding value for Normal group

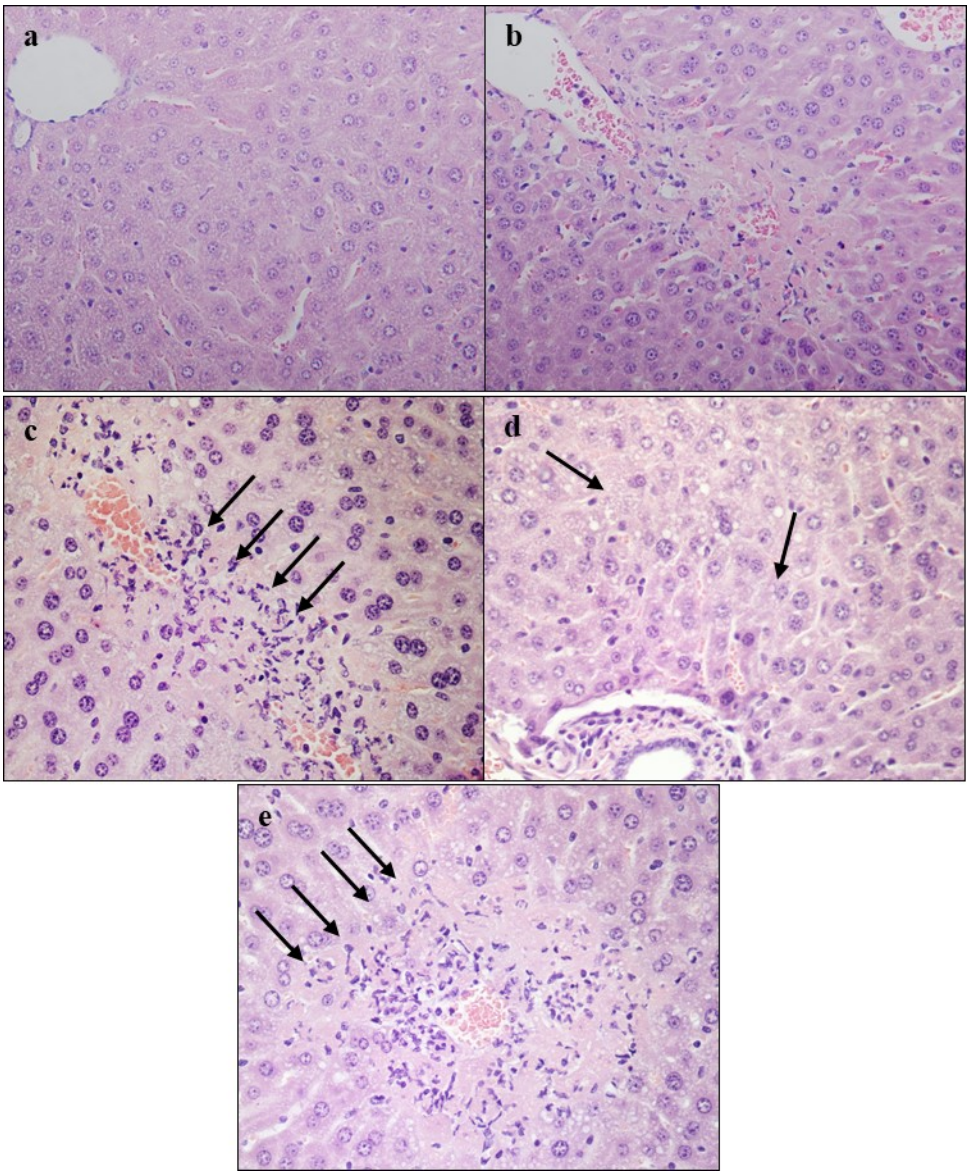


Figure 4.3. Photomicrographs show histopathological architecture of liver tissues from mice groups treated with *T. sinensis, T. crispa* and BCF in LPS +MCT induced hepatotoxicity model compared with controls

(a) Normal control; (b) MCT + LPS (MCT: 200 mg/kg; oral and LPS: 6 mg/kg i.p) (c) *T. sinensis* + MCT + LPS (500 mg/kg/day oral for 7 days; MCT: 200 mg/kg; oral and LPS: 6 mg/kg i.p given on 7th day) (d) *T. crispa* + MCT + LPS (500 mg/kg/day oral for 7 days; MCT: 200 mg/kg, oral and LPS: 6 mg/kg i.p given on 7th day) (e) BCF+ MCT + LPS (250 mg/kg/day oral for 7 days; MCT: 200 mg/kg, oral and LPS: 6 mg/kg i.p given on 7th day).

4.5. Discussion

Herbal medicines have been an integral part of the general health care system since ancient times and 80% of the world population still relies on it as a primary form of health care (Ekor 2014). They are used for the treatment for various diseases such as, diabetes, hypertension, rheumatism, cancer etc. and being natural, are believed to be safe with least or no side effects. Besides being pharmacologically active, herbal drugs have been reported to cause toxicities. Incidence of herbal drug related liver toxicities are reversed after discontinuation of the treatment (Liu et al., 2016). Since the use of dietary supplements/herbal medicines is expeditiously increasing, potential toxicity associated with their consumption such as drug-induced liver injury (DILI) resulting from herbal supplements is rapidly becoming a serious medical issue. LPS is a known inflammagen and its sub-toxic doses in combination with drugs or herbal products have been used to induce experimental hepatotoxicity. LPS is believed to lower the threshold of toxicity and therefore remains an important experimental tool in investigating the hepatotoxic potential of xenobiotics. The present study was attempted to determine the hepatotoxic potential of *T. crispa, T. sinensis* and the combination of borapetosides, B, C and F, isolated from *T. crispa* in normal mice and mice that were sensitized with LPS (health compromised conditions).

Alanine Transaminase (ALT) is a cytoplasmic enzyme mainly present in higher levels in liver followed by kidney, heart and muscles. As a result of liver injury, ALT is released from hepatocytes and its level in blood is elevated. It is, therefore, considered an important liver toxicity marker (Gowda et al. 2009). Total bilirubin (TBL), composed of conjugated and unconjugated bilirubin, is not a sensitive biomarker for identifying risk of DILI. A change in the

level of TBL is observed only after substantial liver damage (Gupta 2019). Clinical measurement of creatinine, blood urea nitrogen, total protein give an indication of kidney function and diagnosis of kidney disease (Salazar 2014; Wu et al. 2012). *T. crispa* has been reported to cause hepatotoxicity, yet the chemical constituents responsible remain unidentified. We investigated three major furanoditerpenes isolated from *T. crispa* and attempted to elucidate their hepatotoxic potential under conditions with decreased threshold for hepatotoxicity. The three borapetosides, B, C and F given in combination BCF (500 mg/kg b. wt./day) did not alter the ALT levels. Similarly, the histopathology did not show remarkable morphological change. The whole extracts of *T. crispa* and *T. sinensis* (1g/kg b.wt./day) also did not affect the ALT levels or histopathological morphology of mice treated under normal or LPS induced health compromised conditions. The overall results for *T. crispa*, *T. sinensis* and BCF liver remained comparable with those of the untreated normal mice. Similar results were observed in acute study performed for each purified compound. Our findings show that the combination of purified compounds BCF representing the major furanoditerpenoids of *T. crispa* did not show any preference for initiating a hepatotoxic episode under LPS induced health compromised state as compared with the whole extract of *T. crispa*. The potential to trigger a hepatotoxic episode by the furanoditerpenoid glycosides or *T. crispa* or *T. sinensis* was not supported by the results observed in the acute or 21-day study as the biomarkers indicating abnormality in liver and kidney function were within the normal range. Literature on hepatotoxicity of *T. crispa* either *in vivo* or from case reports have all been derived from the administration or consumption of extracts of *T. crispa.* These findings are useful in elucidating the effect of borapetosides B, C and F on liver function. Borapetoside F did not elevate ALT levels in acute study at the dose of 500 mg/kg b. wt. The

mere presence of the borapetosides in the plant material or its high content cannot be attributed to a pathological response without actual experimental work. These three purified compounds did not generate a toxic response from the liver and neither did any of the two extracts. Herb-drug interaction has been identified as a cause of liver injury in toxicity cases resulting from the consumption of herbal supplements (Brown, 2017).

The hepatoprotective effect of a drug aims to prevent the necrosis of hepatocytes and to promote their regeneration (Nitin et al., 2012). Different modes of restorative action on liver were shown to affect a differential hepatoprotective activity by the satwa (starchy extract) of *T. sinensis* and *T. cordifolia* (Chavan et al. 2013). Literature depicting the safety of *T. cordifolia* show it to be safe in phase 1 clinical trial in normal volunteers (Dahanukar et al., 1999), an LD_{50} value higher than 1 g/kg in animals when administered orally (Rege et al., 1999) and no observance of acute toxicity study in wistar albino rats at the dose of 3g/kg or abnormality in liver and renal function in rats at the dose of 100 mg/kg administered over 12 weeks (Agarwal et al., 2002). While studies have been conducted regarding the safety aspect of *T. cordifolia*, no assessment of the safety of *T. sinensis* has been carried out. Safety evaluation of *T. sinensis* for its effect on liver becomes crucial since the herbal medicine is often substituted for *T. cordifolia*.

In the 7-day study undertaken to determine the hepatoprotective potential of *T. sinensis,* the hepatotoxicity of monocrotaline manifested under LPS induced health compromised conditions in mice was not alleviated by *T. sinensis*. The ALT levels were elevated as a result of the MCT+LPS induced hepatotoxic insult and the histology of the liver tissue corroborated the liver damage. Our observation is supported by the case reported by Wu et al., 2013. Two males, aged 55-65 years, took *T. sinensis* to treat “liver fire” and edema for over 3 months. Marked

elevation of liver enzymes and bilirubin was observed in both individuals in addition to duodenal ulcer with bleeding, leukocytosis, monocytosis in one and eosinophilia in the other individual. Health was restored through supportive care. The restorative role of *T. sinensis* in liver function therefore, could not be established. Our results negate the hepatoprotective assertions of Chavan et al. 2017 for *T. sinensis* in rats. The established range for the normal ALT levels in healthy rats from a variety of sources lies between 35-80 U/L. A closer analysis of their literature revealed that the ALT levels for normal groups (154.67±2.4) and groups treated with hepatoprotective agent, silymarin (174.77 ± 1.0) and *T. sinensis* (131.58 ± 2.9) were inconsistent. Their findings seem to be perceived in alignment with the popular beliefs in folklore thereby perpetuating the notion of a hepatoprotective efficacy for *T. sinensis* while the results contain a disputable error. The popularity of herbal drugs and the belief that herbal drugs are safe has potentially exposed the consumers of herbal supplements to unwanted adverse effects from botanicals that are not standardized in terms of quality, efficacy and safety. The safety and efficacy of complex extracts containing a pool of principles whose efficacy and/or toxicity may be potentially modulated by unknown factors remains largely uninvestigated. Cases of hepatotoxicity resulting thereof continue to present a health hazard to consumers as well as a challenge for agencies responsible for monitoring public health. Substitution of one *Tinospora* species for the other beseeches serious consideration and vigilance, as each species has phytochemical, pharmacological and toxicological variability that warrants caution and increased awareness with regards to the use of the *Tinospora* species in particular

4.6. Conclusion

The effect of *Tinospora* species on the liver was evaluated by conducting experiments to assess both hepatotoxic and hepatoprotective effect. The borapetosides administered in combination in 21-day study or separately in acute study in normal mice and in LPS induced health compromised mice presented a data that does not support a direct liver injury response; however, that does not rule out other possible mechanisms of liver injury. The methanolic extracts of *T. crispa* and *T. sinensis* did not exhibit hepatotoxicity upon oral administration at the dose of 1gm/kg b.wt./day in the 21-day short term study. The seven-day study, conducted to evaluate the hepatoprotective potential, gave an insight in to the claims of hepatoprotective effect of *T. sinensis*, or a health promoting role of liver by *T. crispa*. The investigation presented the possibility that the two *Tinospora* species lack an evident hepatoprotective effect against xenobiotics with hepatotoxic potential such as monocrotaline resulting in a hepatotoxic effect. A detailed investigation in to the mechanisms would increase awareness regarding herb-herb or herb-drug interaction and also highlight the importance of recognizing each species of *Tinospora* as a different botanical with its own distinct pharmacological and toxicological dynamics.

Chapter 5

EVALUATION OF THE CHEMICAL CONSTITUENTS ISOLATED FROM *TINOSPORA CRISPA* AND *TINOSPORA SINENSIS*

5.1. Introduction

The methanolic extracts of *T. crispa* and *T. sinensis* and purified compounds were evaluated for cytotoxicity, cytochrome P450 inhibition and Pregnane X receptor activation through *in vitro* bioassays. Activation of PXR, a nuclear receptor, mediates CYP3A4 induction as a mechanism to metabolize and eliminate xenobiotics from the body (Sinz 2013). Inhibition of CYP3A4, on the other hand, may lead to the delayed clearance of xenobiotics, leading to toxic response. Cytochrome P450 s (CYPs) and Pregnane X receptor (PXR) are two of the three main modulators of drug-drug interactions that may lead to adverse drug effects as a result of alteration in pharmacokinetics (Leake, 1975; Sionneau, 1995; Subhuti, 2000). *Tinospora* dietary supplements availability and possible use with clinical drugs highlights the need to study and determine the potential of the crude methanolic extracts of *Tinospora* species and the constituents isolated from *T. crispa* and *T. sinensis* to affect CYP3A4 or PXR activity. Alteration in pharmacokinetic profile results upon inhibition of the CYP450 enzymes due to alteration in the metabolic clearance of the concomitantly administered drug leading to adverse drug effects. Above mentioned *in vitro* assays may, thus, aid to predict the risk of herb-drug interactions resulting from the concomitant use of herbal supplements and clinical drugs.

A review for the literature for *T. crispa* shows that inhibition of metabolism mediated by CYP3A4 and CYP2D6 is exhibited by its methanolic extract (Usia et al., 2006a, b; Subehan et al.) However, active phytoconstituents responsible for the cytochrome P450 activity have not been identified. There are no reports in literature concerning the inhibitory activity for the methanolic extract of *T. sinensis* or its purified constituents. The *Tinospora* plants have been reported to contain quaternary alkaloids viz berberine (Bisset and Nwaiwu 1983), palmatine (Dong et al. 2010), magnoflorine (Choudhary et al., 2010; Yusoff, Hamid, & Houghton, 2014) and jatrorrhizine (Maurya et al. 2009), which were included in this study. These alkaloids are widely distributed in nature (Grycová, Dostál, and Marek 2007). No reports are available on the effects of *Tinospora* extracts and their purified chemical constituents on PXR.

5.2. Experimental Section

5.2.1. General Methods

5.2.1.1. Cytotoxicity assay

A preliminary *in vitro* analysis for cytotoxicity was undertaken. The extracts from *T. crispa* and *T. sinensis* and the borapetosides, B, C and F were tested i*n vitro* to assess signs of cytotoxicity in liver and kidney cell lines (HepG2, LLC-$PK_{1)}$ and Vero at six concentrations starting at 100 µg/mL for the extracts and 10 µg/mL for pure compounds. Cytotoxicity towards these cell lines was determined by a cell proliferation assay (Orfali et al. 2018). Cells at a density of 10,000 cells/well were seeded in 96-well plates and incubated for 24 h. Test samples at various concentrations were added followed by the incubation of the cells for 48 h. Cell viability was determined by using tetrazolium dye (WST-8) which, in the presence of 1-methoxy PMS by the activity of cellular dehydrogenases, is converted to formazan, a water soluble product. The

amount of formazan produced is directly proportional to the number of viable cells and is measured by reading the absorbance at 450 nm. Percent viability of sample treated cells was calculated against the vehicle treated cells and the IC_{50} was calculated from the concentration response curves. Doxorubicin was used as positive control.

5.2.1.2. Cytochrome P450 inhibition assay

CYP 3A4 inhibition assay was conducted as reported earlier (Crespietal., 1997; Manda et al., 2013) in a total volume of 200 µL in 96-well microplates. Test samples or positive controls were serially diluted in a solution (100 µL) of cofactors mix, control protein (0.05 mg of protein/mL), and G- 6-PDH to achieve six concentrations (200, 66.67, 22.22, 7.41, 2.47, 0.82 µL for extracts and 100. 33.33, 11.11, 3.70, 1.23, 0.41 µL for compounds). The plates were incubated at 37°C for 10 min. Initiation of the reaction was achieved by the addition of enzyme substrate mixture (100 µL), followed by incubation for 15, 30, or 45 min. 75 µL of ice cold acetonitrile/0.5M Trisbase (80:20) was added to terminate the reaction. Fluorescence was measured on Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) at specified excitation and emission wavelengths. IC_{50} values were obtained from concentration-response curves generated by plotting concentration versus % inhibition. Ketoconazole was used as positive control.

5.2.1.3. Pregnane X receptor activation by luciferase reporter gene assay

The methanolic extracts and the major compounds from *Tinospora* species were tested for PXR activation by method described by Manda et al., 2016. Briefly, transiently transfected HepG2 cells, with pSG5-hPXR (25 µg) and PCR5 plasmid DNA (25 µg), were seeded in 96 well plates at the density of 50,000 cells per well and incubated for 24 hrs. Test extracts, compounds

and positive controls were added at different concentrations (100, 50 and 25 µg/mL) followed by an additional incubation of 24 hours. The media was aspirated and luminescence was measured on Spectramax M5 plate reader (Molecular devices) after the addition of 40 µL luciferase reagent (Promega Corporation). The results for PXR activation were calculated as a fold increase in sample treated cells in comparison to the vehicle treated cells. Rifampicin was used as positive control.

5.2.2. Materials

The methanolic extracts of *T. crispa* and *T. sinensis* were tested *in vitro* for the three activities. Details of the chemical constituents evaluated are given as follows.

a. **Cytotoxic assay**: *In vitro* cytotoxicity was performed for borapetoside B and mixture of borapetoside C and F against liver and kidney cell lines, HepG2, LLC-PK_1 and Vero.
b. **Cytochrome P450 inhibition**: Eleven compounds, borapetosides B, C and F, (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester (Code#AP-4-19-9TC), N-trans feruloyl tyramine, palmatine. tinosineside A, tinosinen, berberine, jatrorrhizine and magnoflorine were tested for CYP3A4 inhibition activity in the present study.
c. **PXR activation**: Test materials included the methanolic extract and the hexane, chloroform, ethyl acetate and n-butanolic fractions of *T. crispa.* Sixteen compounds were evaluated. These included tinosineside A, tinosinen, tinosposinoside and cordifoliside C isolated from *T. sinensis* and borapetosides B, C and F, (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester, baenzigeride A, columbin, cycloeucalenol, N-trans-feruloyl tyramine

and palmatine isolated from *T. crispa.* Jatrorrhizine was purchased from Wuhan ChemFaces Biochemical Co., Ltd (China). Magnoflorine and berberine were made available through the NCNPR natural product repository.

5.3. Results and Discussion

In vitro evaluations of the crude methanolic extracts and the isolated chemical constituents of *T. crispa* and *T. sinensis* were carried out for CYP3A inhibition, PXR activation and cytotoxicity against three cell lines, HepG2, LLC-PK_1 and Vero cell lines. The crude extracts for *T. crispa* and *T. sinensis* and the borapetosides showed no cytotoxicity against all three cell lines **(Table 5.1)**. The negative results are in agreement with the findings reported by Choudhary et al., 2010.

The data for CYP3A4 inhibition and PXR activity are represented as mean ± SD of three independent experiments. The metabolism of xenobiotics is mediated by cytochrome P450 family of heme containing enzymes. Among several isoforms, CYP3A4 mediates the metabolism of 50% of the medicines marketed (Kamel and Harriman 2013). The results for the modulation of CYP3A4 inhibition by the test extracts and compounds are given in **Table 5.2**. The methanolic extracts of *T. crispa* (IC_{50} 5.9 µg/mL) and *T. sinensis* (IC_{50} 8.9 µg/mL) inhibited CYP3A4 likewise. Among the four furanoditerpenes, borapetoside B showed the least activity (IC_{50} 100 µg/mL) whereas borapetoside F showed the highest inhibitory activity with an IC_{50} of 13 µg/mL. N-tans-feruloyl tyramine, an amide showed relatively higher inhibitory activity than borapetoside F. Four quaternary alkaloids, berberine, jatrorrhizine, magnoflorine and palmatine were tested. Two alkaloids, jatrorrhizine and berberine showed high CYP3A4 inhibitory activity whereas magnoflorine and palmatine showed least activity. Rifampicin served as control for

determination of *in vitro* PXR activation at six different test concentrations. The crude extracts were tested at the concentrations of 100, 50, and 25 µg/mL **(Table 5.3)**. No effect on PXR was observed with the methanolic extracts of *Tinospora* species. Among the fractions of *T. crispa* tested, only the ethyl acetate fraction of *T. crispa* showed activation of PXR. The chemical constituents isolated from *T. crispa* and *T. sinensis* were tested for PXR activation and the results are given in **Table 5.4**. Cordifoliside C showed PXR activation potential; tinosineside A, tinosinen, and tinosposinoside did not show strong activity. The furanoditerpenes from *T. crispa* also did not exhibit strong activation of PXR. Columbin showed a threefold (3.58 µg/mL) activation of PXR comparable to rifampicin (3.24 µg/mL). Baenzigeride A (1.96 µg/mL), cycloeucalenol (2.42 µg/mL), N-trans-feruloyl tyramine (2.29 µg/mL) showed two fold PXR activation. Among the alkaloids, only jatrorrhizine showed a 2-fold induction (2.44 µg/mL) at the concentration of 50 µg/mL.

The major furanoditerpenes, borapetosides B and C did not show CYP3A4 inhibition or PXR activation. Borapetoside F caused CYP3A4 inhibition whereas columbin showed higher PXR activation potential. N-trans feruloyl tyramine, an amide and jatrorrhizine, an isoquinoline alkaloid showed both CYP3A4 inhibition and PXR activation potential. Our current finding lead us to hypothesize that induction of hepatotoxicity from *T. crispa* may be contributed by multiple compounds from different classes including furanoditerpenes, alkaloids and cycloartanes.

5.4. Conclusions

Plant extracts are multicomponent systems which confer complexity to a biological response whether efficacious or toxicological. Our results indicate that *T. crispa* and *T. sinensis* contain chemical constituents that could mediate herb- drug interaction through CYP inhibition

and PXR activation. Moreover, no cytotoxicity was observed in HepG2, LLC-PK_1 and Vero cell lines by the *T. crispa* or *T. sinensis* borapetosides B, C and F.

Table 5.1. Results of *In vitro* cytotoxic assay

Sample	IC_{50} µg/mL		
	HEPG2	LLC-PK_1	Vero
Tinospora sinensis extract	NA	NA	NA
Tinospora crispa extract	NA	NA	NA
T crispa chloroform fraction	NA	NA	NA
T crispa butanol fraction	NA	NA	NA
T crispa EtOAc fraction	NA	NA	NA
Borapetosides C+F (Mixture)	NA	NA	NA
Borapetoside B	NA	NA	NA
Doxorubicin	0.36	1.0	>5

Table 5.2. CYP3A4 inhibition by *T crispa* and *T. sinensis* extracts and their compounds

Plant name	Sample Name	CYP 3A4 Inhibition - IC_{50} (µg/mL)
T crispa	Methanolic extract	5.90 ± 1.22
T sinensis	Methanolic extract	8.90 ± 0.73
T. crispa	Borapetoside B	100.0 ± 0.00
T. crispa	Borapetoside C	37.50 ± 12.27
T. crispa	Borapetoside F	13.00 ± 0.82
T. crispa	AP-4-19-9TC	29.00 ± 0.82
T. crispa	N-trans-feruloyl tyramine	2.70 ± 0.08
T.crispa	Palmatine	34.00 ± 0.00
T. sinensis	Tinosineside A	45.00 ± 5.72
T. sinensis	Tinosinen	>100
	Berberine	0.72 ± 0.17
	Jatrorrhizine	9.50 ± 2.29
	Magnoflorine	36.00 ± 3.27
Positive control	Ketoconazole	0.03 ± 0.01

Table 5.3. PXR activation by *Tinospora* methanolic extracts and fractions

Test extracts and fractions	Fold induction in PXR activity at		
	100µg/mL	50µg/mL	25µg/mL
T. sinensis crude methanolic	2.27	1.78	1.58
T. crispa crude methanolic	2.16	2.35	2.26
T. crispa hexane Fr.	1.00	2.56	2.44
T. crispa chloroform Fr.	1.99	2.30	2.02
T. crispa EtoAc Fr.	1.87	1.31	1.07
T. crispa butanolic Fr.	NA	NA	NA
Rifampicin*	3.25	3.24	2.62

*Positive control; Test concentration: 10, 5 and 2.5 µM; NA-No activation

Table 5.4. PXR activation by chemical constituents isolated of *T crispa* and *T. sinensis*

Plant name	Compound	Fold induction in PXR activity at		
		50 µg/mL	25 µg/mL	12.5 µg/mL
T. sinensis	Tinosineside A	1.53 ± 0.11	1.50 ± 0.00	1.42 ± 0.06
T. sinensis	Tinosinen	1.42 ± 0.06	1.67 ± 0.02	1.48 ± 0.06
T. sinensis	Tinosposinoside	1.52 ± 0.15	1.55 ± 0.08	1.30 ± 0.08
T. sinensis	Cordifoliside C	1.95 ± 0.04	1.47 ± 0.03	1.22 ± 0.06
T.crispa	Borapetoside B	NA	NA	NA
T.crispa	Borapetoside C	NA	NA	NA
T.crispa	Borapetoside F	NA	NA	NA
T.crispa	AP-4-19-9TC	1.39 ± 0.11	1.01 ± 0.13	0.93 ± 0.04
T.crispa	Baenzigeride A	1.96 ± 0.16	1.54 ± 0.16	1.18 ± 0.09
T.crispa	Columbin	3.58 ± 0.61	2.89 ± 0.62	2.02 ± 0.02
T.crispa	Cycloeucalenol	2.42 ± 0.30	1.91 ± 0.23	1.42 ± 0.06
T.crispa	N trans-feruloyl tyramine	2.29 ± 0.41	2.16 ± 0.27	1.59 ± 0.07
T. crispa	Palmatine	1.43 ± 0.00	1.30 ± 0.00	1.08 ± 0.00
	Jatrorrhizine	2.44 ± 0.05	1.94 ± 0.04	1.48 ± 0.01
	Magnoflorine	1.41 ± 0.12	1.16 ± 0.15	1.01 ± 0.15
	Berberine	NA	NA	NA

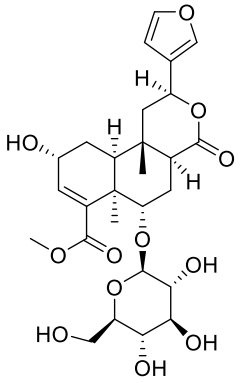
Chapter 6

DEVELOPMENT OF A CHEMICAL FINGERPRINT AS A TOOL TO DISTINGUISH CLOSELY RELATED *TINOSPORA* SPECIES AND QUANTITATION OF MAJOR COMPOUNDS

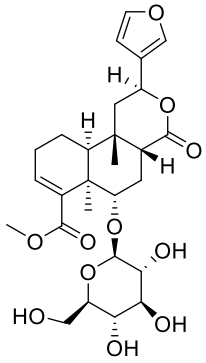
6.1. Introduction

The goal of this project was to develop and validate an analytical method using qualified chemical standards to differentiate *T. crispa* from its closely related species with particular emphasis on *T. sinensis* which is commonly substituted for *T. cordifolia* (Raghunāthan, K., & Mitra 1982). The quality of a botanical is directly related to it being safe for consumption and thus is of critical importance. Qualitative analysis would therefore, enable the development of a validated HPLC-UV-MS method using several marker constituents to achieve decisiveness in not only identifying closely related *Tinospora* species but also differentiating *Tinospora crispa* from *T. sinensis* and other closely related *Tinospora* species. In the present study, ten compounds including a flavonoid, two alkaloids, an amide and six diterpenoids were used as markers to develop a highly sensitive, accurate and rapid HPLC-UV-MS method to enrich quality control for *Tinospora* plants. The markers were identified by analyzing their UV spectra and relevant chromatographic peaks in the plant samples in addition to monitoring by mass detector. The ten compounds used to develop the chemical fingerprint to distinguish *T. crispa* from other closely related species are given in **fig. 6.1**.

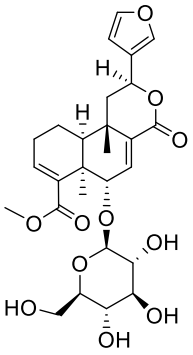
Figure 6.1. Structures of compounds used to develop chemical fingerprint



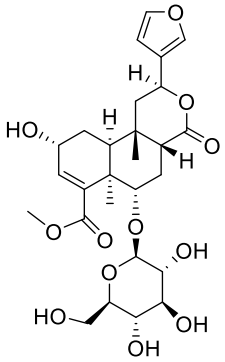
Borapetoside B



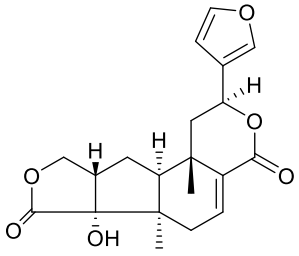
Borapetoside C



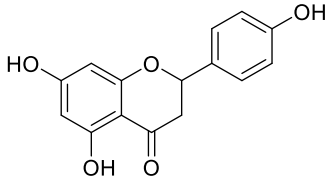
Borapetoside F



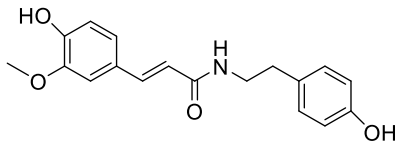
(2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester



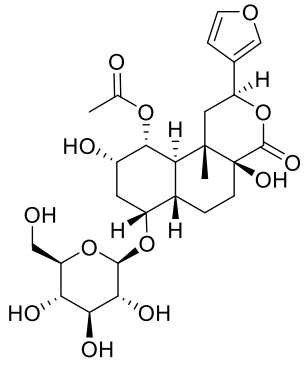
Baenzigeride A



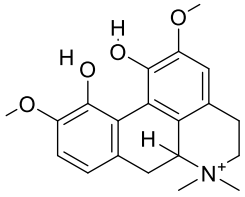
Naringenin



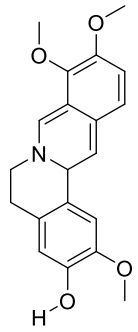
N-trans-feruloyl tyramine



Tinosineside A



Magnoflorine



Jatrorrhizine

6.2. Experimental Section

6.2.1. UHPLC-MS Analysis

The analyses were carried out on a Waters Acuity UPLC system consisting of solvent manager, sampler manager, column heater, PDA and SQD-ESI-MS detectors. The PDA detector was connected to the Waters Empower 2 data station. Separation was achieved by an Acquity UPLC HSS T3 column (1.8 µm, 2.1x100 mm). The column and sample temperature were maintained at 35 ºC and 10 ºC respectively. The mobile phase consisted of acetonitrile (with 0.1% formic acid) (A) and water (with 0.1% formic acid) (B) run at a flow rate of 0.3 mL/min. Analysis was undertaken using gradient elution as follows: 0min, 15% A: 85% B; 10 min to 17% A: 83% B, 17 min to 44% A and 56% B, by 19 min 100% A.

6.2.2. Chemicals

Magnoflorine, tinosineside A, borapetoside B, (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester (code# AP-4-19-9TC), N trans-feruloyl tyramine, naringenin, baenzigeride A, borapetoside F, and borapetoside C, were isolated at the National Center for Natural Products Research (NCNPR), University of Mississippi, University, Mississippi, USA. Jatrorrhizine was purchased from Wuhan ChemFaces Biochemical Co., Ltd (China). HPLC grade acetonitrile and formic acid were purchased from Fischer Scientific (Fair Lawn, NJ, USA). Water for the mobile phase was purified using a Milli-Q system (Millipore).

6.2.3. Plant materials

Seventeen plant samples for *Tinospora* were obtained from various authentic and commercial sources. The accession numbers for all plant samples are listed **(Table 6.1)**. All samples were deposited at the botanical repository of NCNPR, University of Mississippi.

6.2.4. Dietary supplements

Seventeen botanical products of *Tinospora* species were purchased from commercial sources. All products were given accession numbers **(Table 6.2)** and deposited at the botanical repository of NCNPR, University of Mississippi.

6.2.5. Sample preparation

Finely powdered plant material for each plant sample (500 mg) was extracted four times by sonication with 2.5 mL of 80% methanol for 30 min followed by centrifugation for 15 min at 3300 rpm. The supernatant was collected in a volumetric flask and the final volume was adjusted to 10 mL. The extract was filtered through a 0.45 µm PTFE filter. Each sample was injected in triplicate.

Table 6.1. List of *Tinospora* plant samples

	Code#	Botanical name	Source	Plant part	Form
1	17091	*T. crispa*	Thailand	Stem	Powder
2	20869	*T. crispa*	India	Stem	Whole
3	16849	*T. crispa*	India	Stem	Powder
4	17003	*T. sinensis*	China	Stem	c/s
5	3104	*T. sinensis*	India	Stem	Pieces
6	16885	*T. sinensis*	China	Stem	Pieces
7	20863	*T. sinensis*	China	Stem	Pieces
8	20769	*T. cordifolia*	India	Stem	Pieces
9	5799	*T. cordifolia*	India	Stem	Large pieces
10	5212	*T. cordifolia*	India	Stem	Large pieces
11	17303	*T. cordifolia*	India	Stem	c/s
12	8069	*T. cordifolia*	-	Stem	Pieces
13	10107	*T. cordifolia*	-	Stem	Pieces
14	20865	*T. cordifolia*	-	Stem	Pieces
15	20866	*T. cordifolia*	-	Stem	Pieces
16	20867	*T. cordifolia*	-	Stem	Pieces
17	20870	*T. baenzigeri*	Thailand	Stem	Whole
18	20864	*T. cordifolia*	NA	Stem	Powder

Table 6.2. List of *Tinospora* dietary supplements

	# Code	Claimed name	Serving size	Average wt. (mg)	Form	Plant part
1	20754	*T. crispa*	1	511	Capsule	-
2	20756	*T. crispa*	1-2	473	Capsule	-
3	20760	*T. crispa*	1-2	512	Capsule	-
4	20762	*T. crispa*	1	384	Capsule	Stems
5	20798	*T. crispa*	1-2	476	Capsules	-
6	20799	*T. crispa*	2	392	Capsule	-
7	20753	*T. cordifolia*	1	545	Capsule	-
8	29758	*T. cordifolia*	2	577	Capsule	-
9	20759	*T. cordifolia*	1	586	Capsule	-
10	20761	*T. cordifolia*	1	541	Capsule	Stem
11	20763	*T. cordifolia*	1	560	Capsule	Extract
12	20764	*T. cordifolia*	1	392	Capsule	-
13	20864	*T. cordifolia*	1-2.5 g	N/A	Powder	Stems
14	20765	*T. cordifolia*	1	624	Capsule	-
15	20767	*T. cordifolia*	1	558	Capsule	Extract
16	20768	*T. cordifolia*	1	706	Caplets	Stems
17	20766	*T. sinensis*	1g	N/A	Granule	Stems

6.2.6. Preparation of standard solutions

Stock solutions (1 mg/mL) were prepared for each standard in methanol except baenzigeride A which was prepared in isopropanol. The calibration curves were prepared at five different concentrations in triplicate to assess the precision and accuracy of the newly developed method. The range of calibration curves was between 10-500 µg/mL.

6.2.7. Precision and accuracy

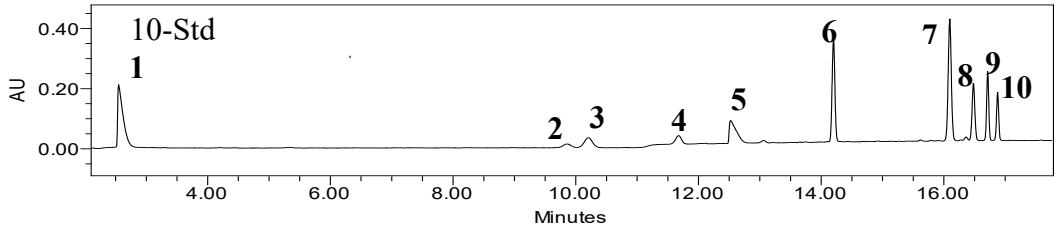
Inter-day and intra-day assay was performed for precision. An analysis of four separate samples of *T. crispa* #17091 was carried out on three consecutive days. The samples were extracted and assayed for precision under optimized conditions. The accuracy of the method was confirmed by a recovery experiment. Duplicate samples were extracted five times to exhaust the samples maximally. The exhausted samples were spiked with a known concentration of standards, then dried and extracted under optimized conditions for analysis.

6.3. Results

Chromatographic conditions were optimized following several trials with mobile phase, temperature and chromatographic columns. Initially, the separation of the borapetosides was tried with UPLC BEH Shield RP18 column and then was successfully achieved using the HSS T3 column, the resolution of additional compounds was satisfactorily optimized by variation in temperature and gradient alone. Optimal separation of the compounds 1-10 was achieved on an Acquity UPLC HSST3 column with a mobile phase composed of acetonitrile and water both acidified with 0.1% formic acid run by gradient elution at a flow rate of 0.3 mL/min and fixed column temperature of 35 ºC. Formic acid was added as a modifier to enhance separation of

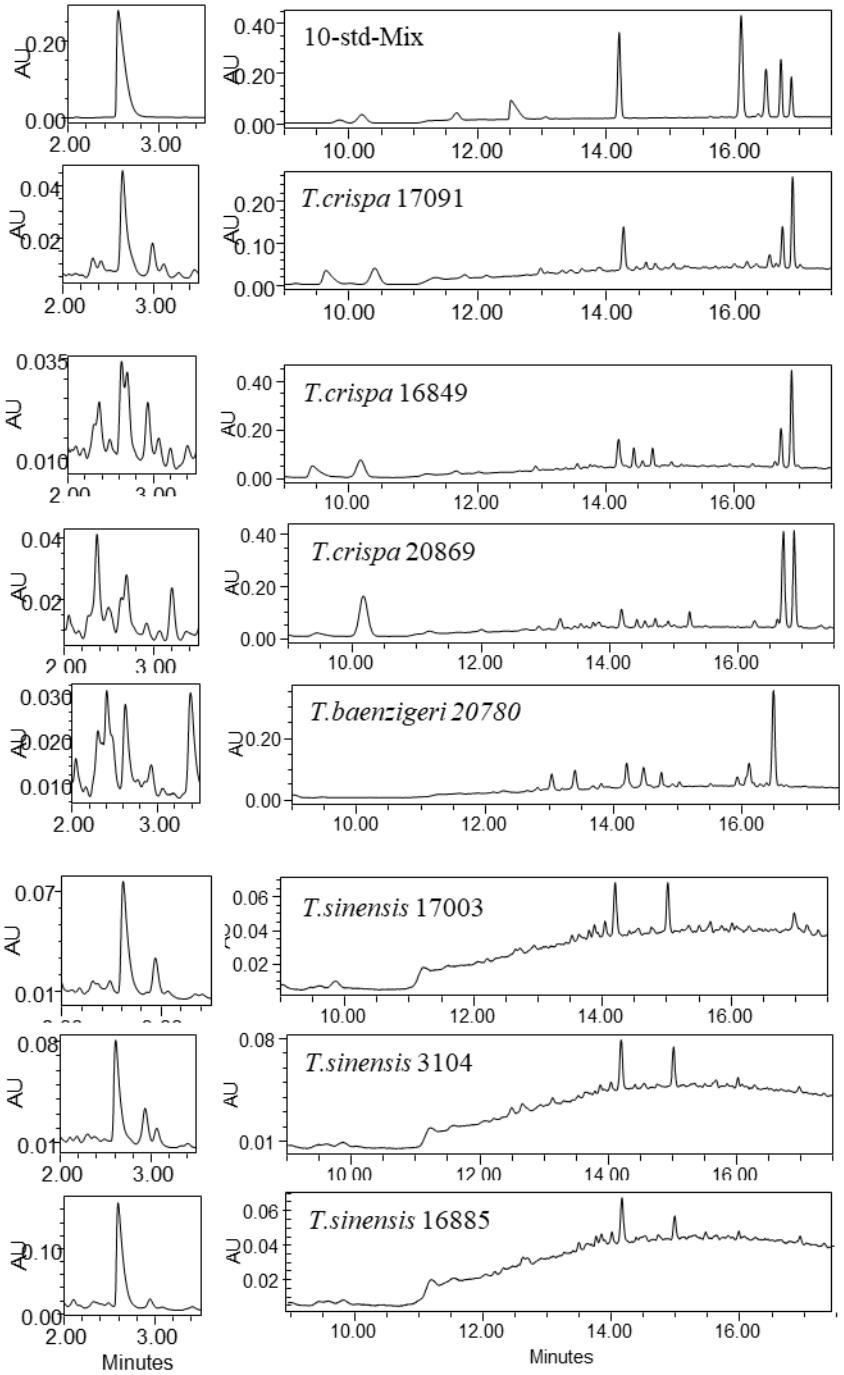
peaks. Acetonitrile is preferred over methanol due to its eluting power and reduction of column back pressure. The standards were injected into UPLC and monitored in PDA and SQD detectors. The developed method used solvent gradient over 22 min to result in the characteristic fingerprint chromatogram depicted in **(Fig. 6.2)**. The chemical fingerprints for *Tinospora* plant samples **(Fig. 6.3)** and dietary supplements analyzed are shown as well **(Fig. 6.4)**.

Figure 6.2. UHPLC-UV Chromatogram of a standards mixture (1–10) at 210 nm



[Magnoflorine (**1**), Tinosineside A (**2**), Borapetoside B (**3**), (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-Epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester (**4**), Jatrorrhizine (**5**), N trans-feruloyl tyramine (**6**), Naringenin (**7**), Baenzigeride A (**8**), Borapetoside F (**9**), and Borapetoside C (**10**)]

Figure 6.3. UHPLC-UV chromatograms of the *Tinospora* samples at 221 nm and 210 nm



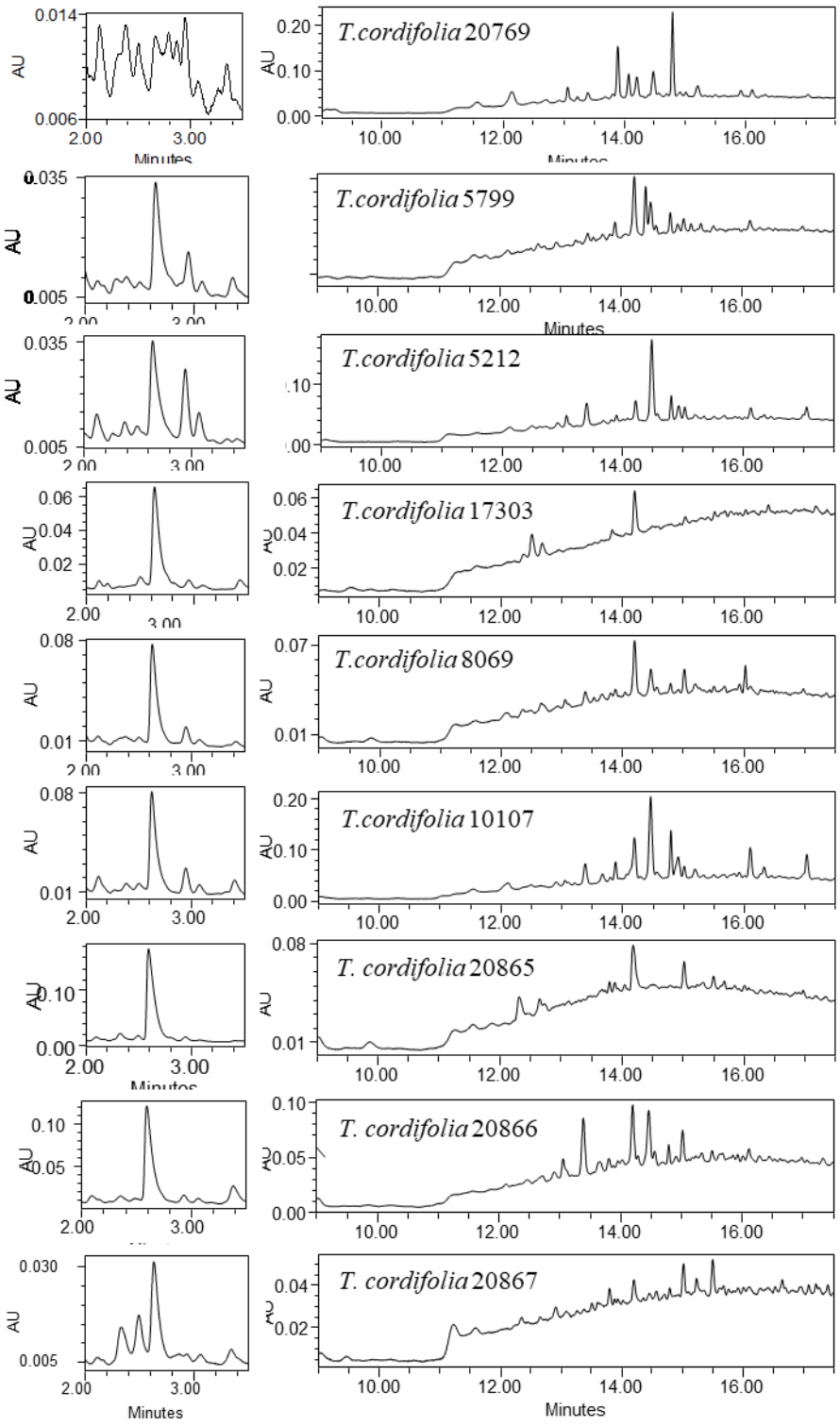
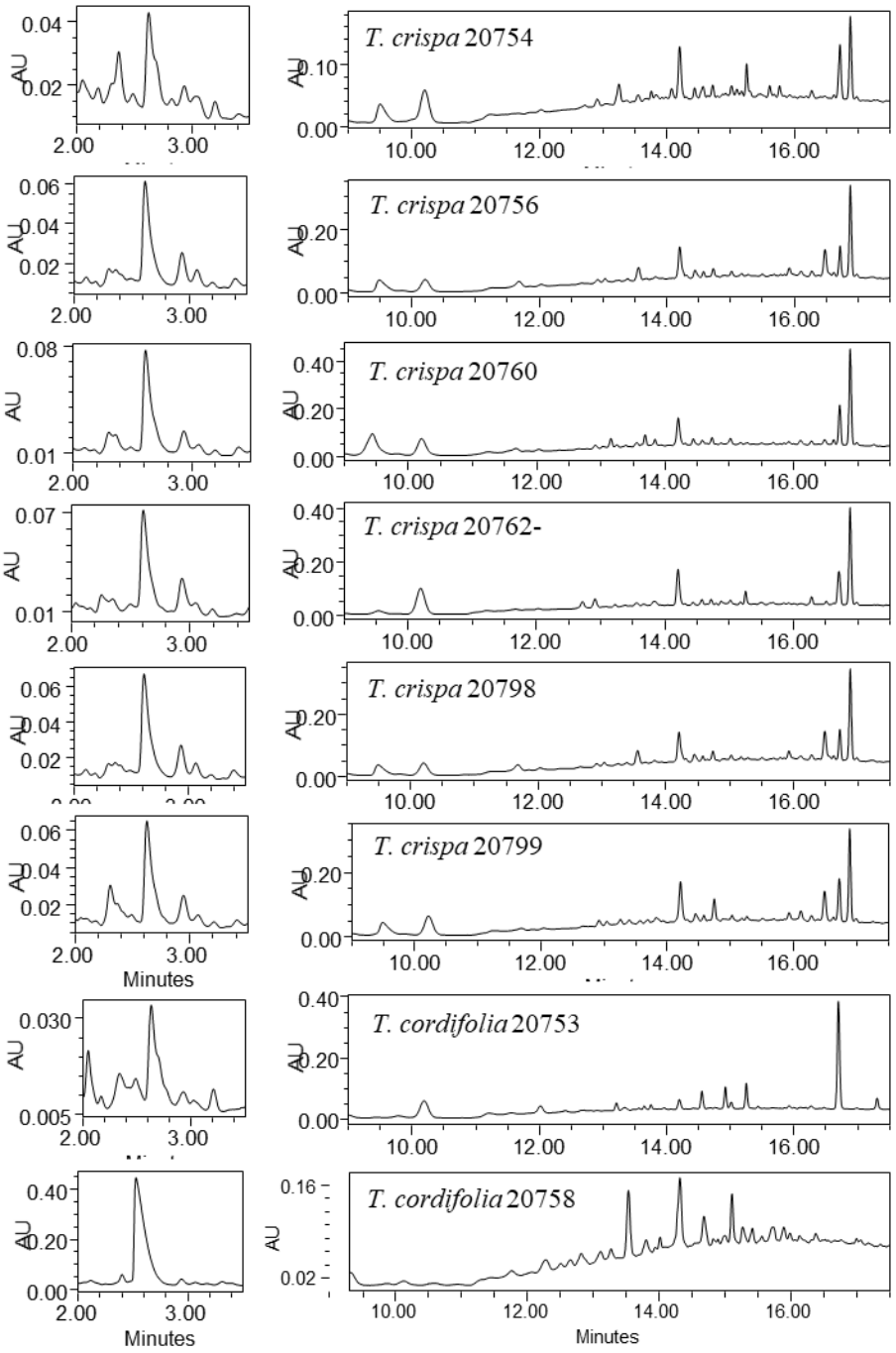
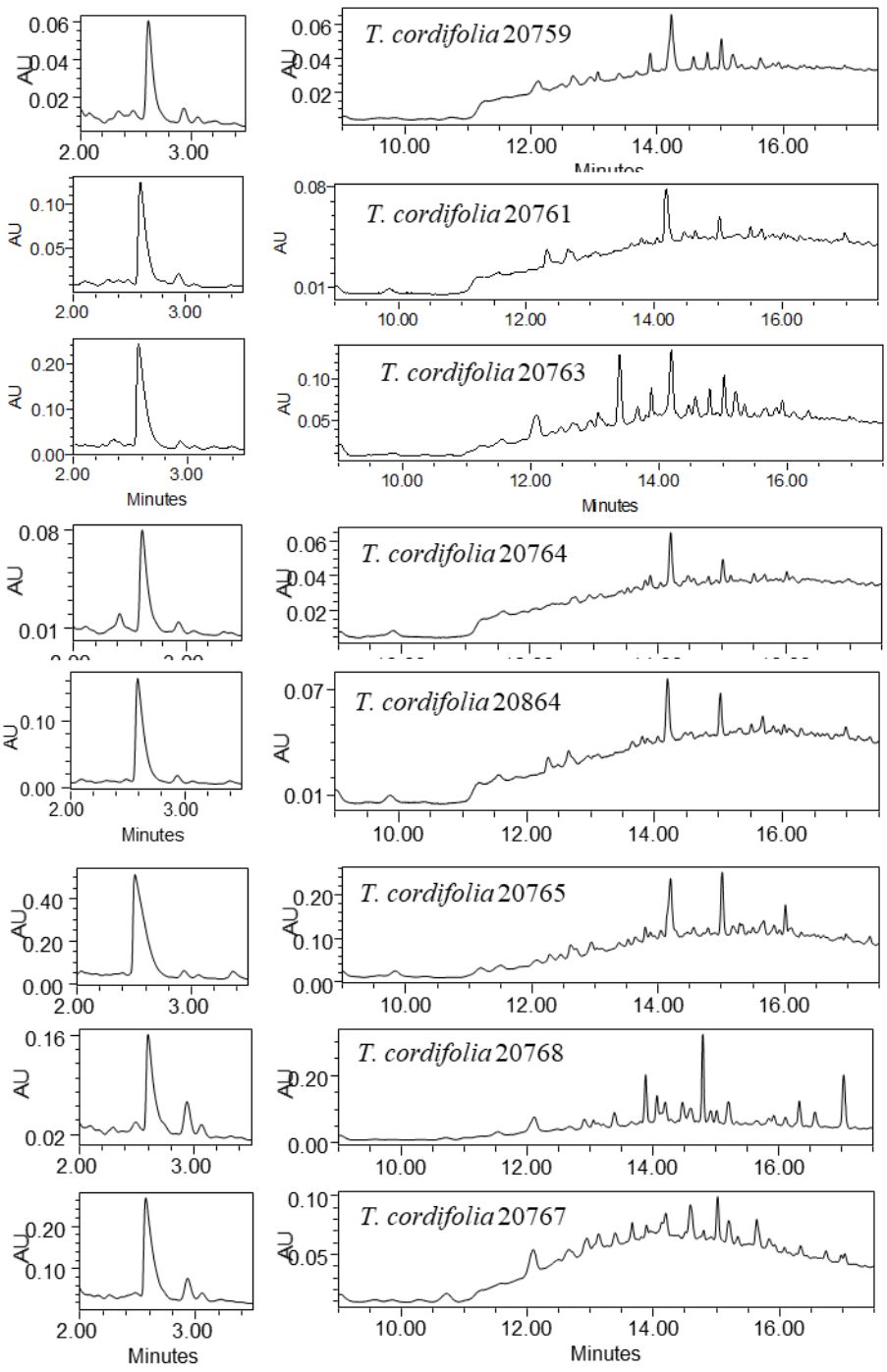

T.cordifolia 20769
T.cordifolia 5799
T.cordifolia 5212
T.cordifolia 17303
T.cordifolia 8069
T.cordifolia 10107
T. cordifolia 20865
T. cordifolia 20866
T. cordifolia 20867
AU
Minutes

Figure 6.4. UHPLC-UV chromatograms of *Tinospora* dietary supplements at 221 and 210 nm

221 nm 210 nm




T. cordifolia 20759
T. cordifolia 20761
T. cordifolia 20763
T. cordifolia 20764
T. cordifolia 20864
T. cordifolia 20765
T. cordifolia 20768
T. cordifolia 20767
AU
Minutes

6.3.1. Linearity, Precision and Accuracy

The five-point calibration curves for each standard showed a linear correlation between concentration and peak area. The statistical and analytical parameters were determined in accordance with the USP to validate the method. The calibration data indicated linearity (r^2 >0.999) of the detector response for all standards from 10-500 µg/ml. The limits of detection were found to be 0.5 µg/mL (magnoflorine), 10 µg/mL (tinosineside A), 10 µg/mL (borapetoside B), 10 µg/mL (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester), 0.5 µg/mL (N-trans-feruloyl tyramine), 2 µg/mL (baenzigeride A), 2 µg/mL (borapetoside F), 2 µg/mL (borapetoside C), 0.5 µg/mL (jatrorrhizine) and 0.3 µg/mL (naringenin).

(Table 6.4).

Extraction efficiency was tested prior to the extraction of the plant samples and the dietary supplements for analysis. Different ratios of methanol were tested by extracting samples of *T. crispa* #17091 four times. The supernatant was removed after each time and analyzed separately. Based on the results, 80% methanol was employed for the optimum extraction based on the abundance values of the components.

Quantitative determination of ten compounds was achieved using the UHPLC-PDA method. The method was tested for sensitivity (LOD, LOQ), linearity, precision, accuracy, specificity, robustness. For precision, inter and intra-day variation of the sample #17091 was determined and was lower than 10. Extraction was performed four times on three different days. The intra-day RSD for replicates was between 0.9 and 6.8% and inter-day RSD was between 1.2 and 9.1%.

A recovery method was performed to confirm the accuracy of the newly developed method. Duplicate samples of *T. crispa* #17091 were extracted five times. The samples exhausted of their contents were dried and then were spiked with known amounts of standards at the concentration of 1 mg/ml. The samples were dried, extracted and analyzed. The recovery rates were in the range from 97%-104% for the compounds in comparison to the theoretical amounts except jatrorrhizine, naringenin and tinosineside A. The results **(Table 6.5)** confirmed the integrity of the extraction procedure and the accuracy of the method.

Quantitative analysis was performed by applying the newly developed UPLC-PDA-MS chemical fingerprint method for the quantitation of the qualified chemical markers in the *Tinospora* related plant samples and botanical products. The % content of compounds 1-10 in *Tinospora* plant and commercial products is given in **Table 6.6** and **6.7** respectively.

Table 6.3. λmax, Retention time, molecular formula, UV nm and m/z [M-H] ⁻ of compounds

	Standards	λmax nm	tR (min)	Mol. formula	UV (nm)	m/z [M – H]⁻
1	Magnoflorine	268 nm	2.6	$C_{20}H_{24}NO_4$	221, 268, 301	340
2	Tinosineside A	210 nm	9.9	$C_{26}H_{36}O_{13}$	208	555
3	Borapetoside B	210 nm	10.3	$C_{27}H_{36}O_{12}$	210	597
4	(2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester	210 nm	11.7	$C_{27}H_{36}O_{12}$	209	597
5	N-trans-feruloyl tyramine	268 nm	14.1	$C_{18}H_{19}NO_4$	219, 317	312
6	Baenzigeride A	210 nm	16.5	$C_{20}H_{22}O_6$	208	357
7	Borapetoside F	210 nm	16.7	$C_{27}H_{34}O_{11}$	209	579
8	Borapetoside C	210 nm	18.8	$C_{27}H_{36}O_{11}$	213	535
9	Jatrorrhizine	268 nm	12.7	$C_{20}H_{20}NO_4$	225, 265, 344	338
10	Naringenin	268 nm	16.1	$C_{15}H_{12}O_5$	288	272

Table 6.4. Regression equation, correlation coefficient (r^2), Limits of detection LOD, limits of quantitation (LOQ) and range of calibration curves

	Standards	Regression equation	r^2	LOD (µg/mL)	LOQ (µg/mL)	Range of calibration curves (µg/mL)
1	**Magnoflorine**	Y = 1.09e+004 X - 1.97e+004	0.99	0.5	2	10-200
2	**Tinosineside A**	Y = 1.16e+003 X - 2.59e+004	0.99	10	30	10-500
3	**Borapetoside B**	Y = 2.82e+003 X - 3.68e+003	0.99	10	30	10-500
4	**AP-4-19-9TC**	Y = 1.64e+003 X + 4.39e+003	0.99	10	30	20-500
5	**N-trans-feruloyl tyramine**	Y = 1.64e+003 X + 4.39e+003	0.99	0.5	2	10-200
6	**Baenzigeride A**	Y = 7.86e+003 X + 1.47e+005	0.99	2	10	10-200
7	**Borapetoside F**	Y = 5.04e+003 X + 1.08e+004	0.99	2	10	10-500
8	**Borapetoside C**	Y = 5.12e+003 X + 2.15e+004	0.99	2	10	10-500
9	**Jatrorrhizine**	Y = 1.01e+004 X + 1.08e+005	0.99	0.5	2	10-200
10	**Naringenin**	Y = 3.70e+003 X + 3.24e+003	0.99	0.3	1	10-200

Table 6.5. Results for Inter-day and Intraday and Recovery assay

Sr. No.	Standards	Intra-Day (n=4)			Inter-day (n=12)	Amount added	Amount Found	Recovery Rate (%)
		Day 1	Day 2	Day 3				
1	**Magnoflorine**	4.69	5.73	6.16	8.65	135.5	132.40	97 %
2	**Borapetoside B**	2.60	2.48	4.51	4.63	266.18	263.51	99 %
3	**AP-4-19-9TC**	2.40	0.54	4.50	0.74	159.08	161.01	101 %
4	**N-trans-feruloyl tyramine**	3.21	1.52	3.06	9.14	220.30	230.07	104 %
5	**Baenzigeride A**	2.92	1.48	4.21	5.17	182.29	177.87	99 %
6	**Borapetoside F**	3.28	1.74	4.43	3.22	174.41	174.73	100 %
7	**Borapetoside C**	3.10	1.63	4.28	2.79	222.06	215.39	97 %
8	**Jatrorrhizine**	4.99	4.55	2.37	1.26	-	-	-
9	**Naringenin**	6.82	6.82	2.42	6.32	12.04	12.44	103 %

Table 6.6. Content of compounds in *Tinospora* plant samples by UPLC-PDA

	#Code	Plant samples	1	2	3	4	5	6	7	8	9	10
1	17091	*T. crispa*	0.017	ND	0.24	DUL	ND	0.079	0.011	0.038	0.105	0.412
2	16849	*T. crispa*	0.016	ND	0.46	0.087	0.022	0.075	0.003	DUL	0.144	0.799
3	20869	*T. crispa*	0.012	ND	1.00	DUL	ND	0.037	ND	DUL	0.383	0.794
4	20870	*T. baenzigeri*	0.013	ND	DUL	ND	ND	0.037	ND	0.35	ND	ND
5	17003	*T. sinensis*	0.044	0.077	ND	ND	ND	0.015	0.003	ND	ND	ND
6	3104	*T. sinensis*	0.043	DUL	ND	ND	0.028	0.017	ND	ND	ND	ND
7	16885	*T. sinensis*	0.102	0.065	ND	ND	0.023	0.015	ND	ND	ND	ND
8	20863	*T. sinensis*	0.041	0.077	ND	ND	ND	0.009	ND	ND	ND	ND
9	20769	*T. cordifolia*	0.005	ND	ND	ND	ND	0.021	0.009	ND	ND	ND
10	5799	*T. cordifolia*	0.015	ND	ND	ND	0.023	0.021	0.005	ND	ND	ND
11	5212	*T. cordifolia*	0.023	ND	ND	ND	ND	0.019	0.011	ND	ND	ND
12	17303	*T. cordifolia*	0.038	ND	ND	ND	0.030	0.035	ND	ND	ND	ND
13	8069	*T. cordifolia*	0.042	ND	ND	ND	0.029	0.026	ND	ND	ND	ND
14	10107	*T. cordifolia*	0.042	ND	ND	ND	ND	0.046	0.031	ND	ND	ND
15	20865	*T. cordifolia*	0.114	ND	ND	ND	0.047	ND	DUL	ND	ND	ND
16	20866	*T. cordifolia*	0.072	ND	ND	ND	0.023	0.034	0.007	ND	ND	ND
17	20867	*T. cordifolia*	0.017	ND	ND	ND	0.026	0.004	0.002	ND	ND	ND

ND: not detected; **DUL**: detected under limits

Magnoflorine (**1**), Tinosineside A (**2**,) Borapetoside B (**3**), (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester (**4**), Jatrorrhizine (**5**), N trans-feruloyl tyramine (**6**), Naringenin (**7**), Baenzigeride A (**8**), Borapetoside F (**9**), and Borapetoside C (**10**)

Table 6.7. Content of compounds in *Tinospora* botanical products by UPLC-PDA

	#Code	Claimed Name	1	2	3	4	5	6	7	8	9	10
1	20754	*T. crispa*	0.021	ND	0.309	DUL	ND	0.049	0.002	ND	0.083	0.179
2	20756	*T. crispa*	0.031	ND	0.246	0.141	0.023	0.061	0.010	0.101	0.080	0.398
3	20760	*T. crispa*	0.043	ND	0.459	0.138	ND	0.069	0.011	0.022	0.148	0.740
4	20762	*T. crispa*	0.037	ND	0.646	DUL	ND	0.080	0.003	DUL	0.142	0.690
5	20798	*T. crispa*	0.034	ND	0.234	0.143	0.023	0.059	0.006	0.092	0.090	0.527
6	20799	*T. crispa*	0.036	ND	0.369	DUL	0.022	0.070	0.018	0.104	0.123	0.482
7	20753	*T. cordifolia*	0.017	ND	0.322	ND	ND	0.015	0.003	ND	ND	ND
8	29758	*T. cordifolia*	0.352	ND	ND	ND	0.038	0.098	0.003	ND	ND	ND
9	20759	*T. cordifolia*	0.032	ND	ND	ND	0.040	0.069	0.002	ND	ND	ND
10	20761	*T. cordifolia*	0.076	ND	ND	ND	0.028	0.056	ND	ND	ND	ND
11	20763	*T. cordifolia*	0.110	ND	ND	ND	0.028	0.048	0.003	ND	ND	ND
12	20764	*T. cordifolia*	0.044	ND	ND	ND	0.025	0.018	0.002	ND	ND	ND
13	20864	*T. cordifolia*	0.106	ND	ND	ND	0.033	0.032	0.002	ND	ND	ND
14	20765	*T. cordifolia*	0.379	0.183	ND	ND	0.048	0.091	ND	ND	ND	ND
15	20767	*T. cordifolia*	0.128	ND	ND	ND	ND	0.008	DUL	ND	ND	ND
16	20768	*T. cordifolia*	0.073	ND	ND	ND	ND	0.026	0.014	ND	ND	ND
17	20766	*T. sinensis*	0.019	0.067	ND	ND	ND	DUL	0.003	ND	ND	ND

ND: not detected; **DUL**: detected under limits

Magnoflorine (**1**), Tinosineside A (**2**), Borapetoside B (**3**), (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester (**4**), Jatrorrhizine (**5**), N trans-feruloyl tyramine (**6**), Naringenin (**7**), Baenzigeride A (**8**), Borapetoside F (**9**), and Borapetoside C (**10**)

6.4. Discussion

Dietary supplements or plant materials sold commercially lack evidence of the authentication. The gap in the provision of quality evidence stems from the lack of analytical resources on part of the source supplier which in most cases is either the manufacturer or the middleman. Although a number of analytical methods have been developed for the *Tinospora* plants (G. V. Srinivasan et al., 2008; Harwoko and Nur Amalia Choironi 2016; Syarifah et al., 2017), yet a validated method to authenticate and distinguish closely related *Tinospora* species is desired for quality control. A qualitative method was developed in this study for chemical fingerprinting with ten reference standards. The method was tested for its usefulness by analyzing commercial plant samples and botanical products of *Tinospora* species. The validated method proved useful in achieving decisive verification of the identity of *T. crispa* and successfully distinguishing it from other *Tinospora* species used in the study. A misidentification/adulteration was successfully detected for two dietary supplements.

The identification of the standards in plant samples and botanical products of *Tinospora* species was based on the retention time, comparison of UV spectra and mass chromatogram. The peaks of interest were free of interference at their respective retention times. A comparative analysis of the results shows that the borapetosides B, C and F are good chemical markers for identifying *T. crispa* and distinguishing it from other *Tinospora* species. In a previous report, the adverse effect of *T. crispa* has been attributed to the presence of high concentration of borapetoside F (Langrand et al. 2014).

The % content of borapetosides B, C and F was in the range 0.23 %-1%, 0.17% - 0.79% and between 0.08- 0.38 respectively. Baenzigeride A can prove to be a useful maker for *T.*

baenzigeri. The content of baenzigeride A in *T. baenzigeri* was found to be higher (0.35 mg/100 mg) than in the *T. crispa* samples where the content was low between 0.022-0.109 %. Moreover, the voucher sample of *T. baenzigeri* did not contain borapetosides C and F whereas borapetoside B was detected under limits. Since, *T. cordifolia* is reported to be commonly substituted by *T. sinensis*; there is a need to authenticate samples claiming to contain *T. cordifolia*. Our newly developed method successfully detected two dietary supplements claimed as *T. cordifolia* to be mislabeled. The dietary supplement claiming *T. cordifolia* # 20765 was found to contain tinosineside A (0.183% content), the chemical marker for *T. sinensis*. Similarly, another dietary supplement of *T. cordifolia* #20753 contained borapetoside B (0.322%), which illustrates the dubious quality of the sample, contrary to the claim. Borapetosides C and F were not detected in this botanical product.

6.5. Conclusion

A UHPLC-UV-MS method was developed to enable the identification of *T. crispa* and distinguishing it from other closely related *Tinospora* species. The validated method was successfully employed for the qualitative and quantitative analysis of thirty-four *Tinospora* plant samples and dietary supplements. Two dietary supplements were found to be mislabeled. The present study for *Tinospora* species highlights the importance of qualitative and quantitative analysis of herbal dietary supplements for the public safety.

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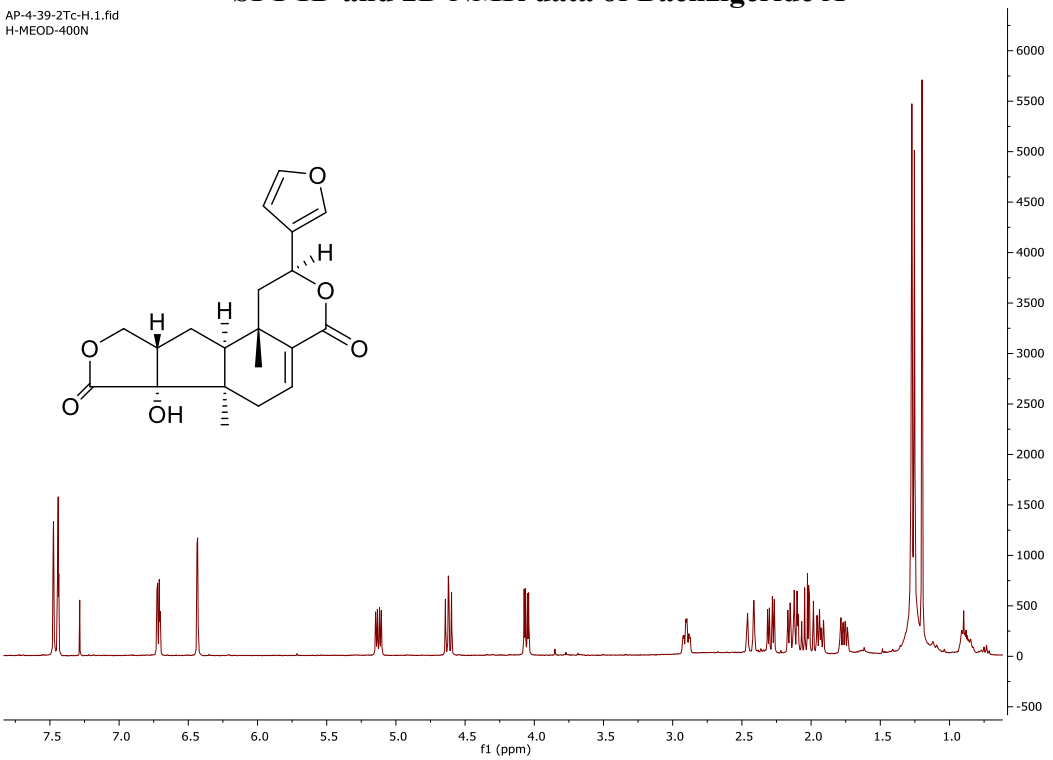
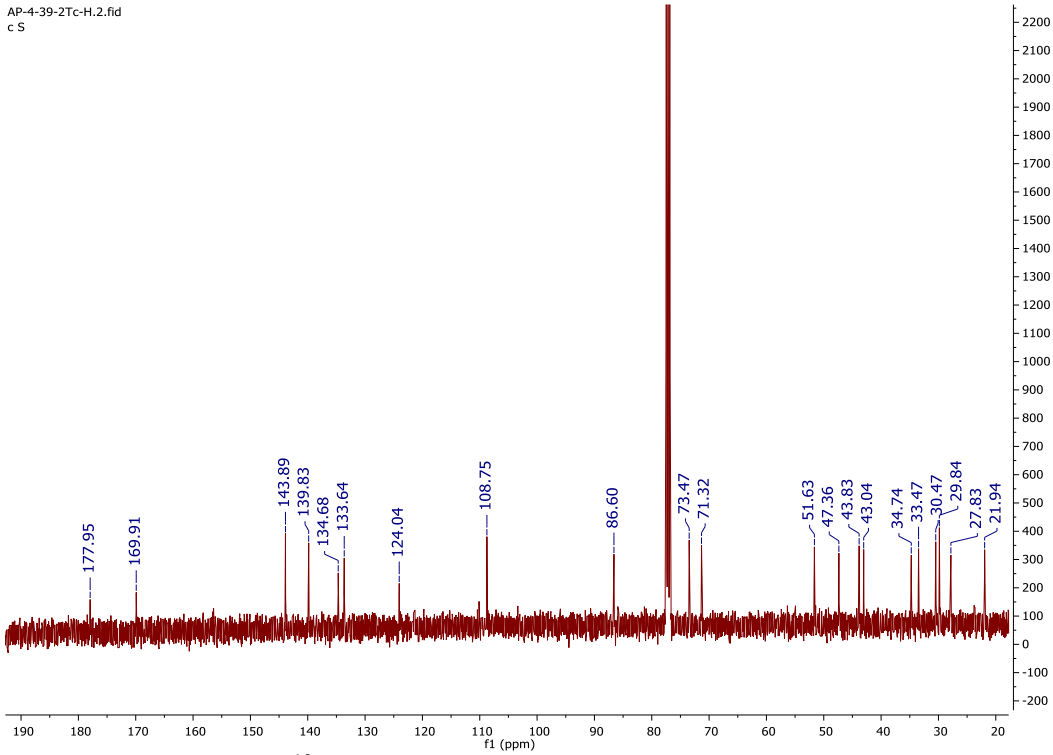
APPENDICES

APPENDIX I-SUPPLEMENTARY INFORMATION-CHAPTER 2

SI 1 1D and 2D NMR data of Baenzigeride A



^{1}H-NMR spectrum of Baenzigeride A



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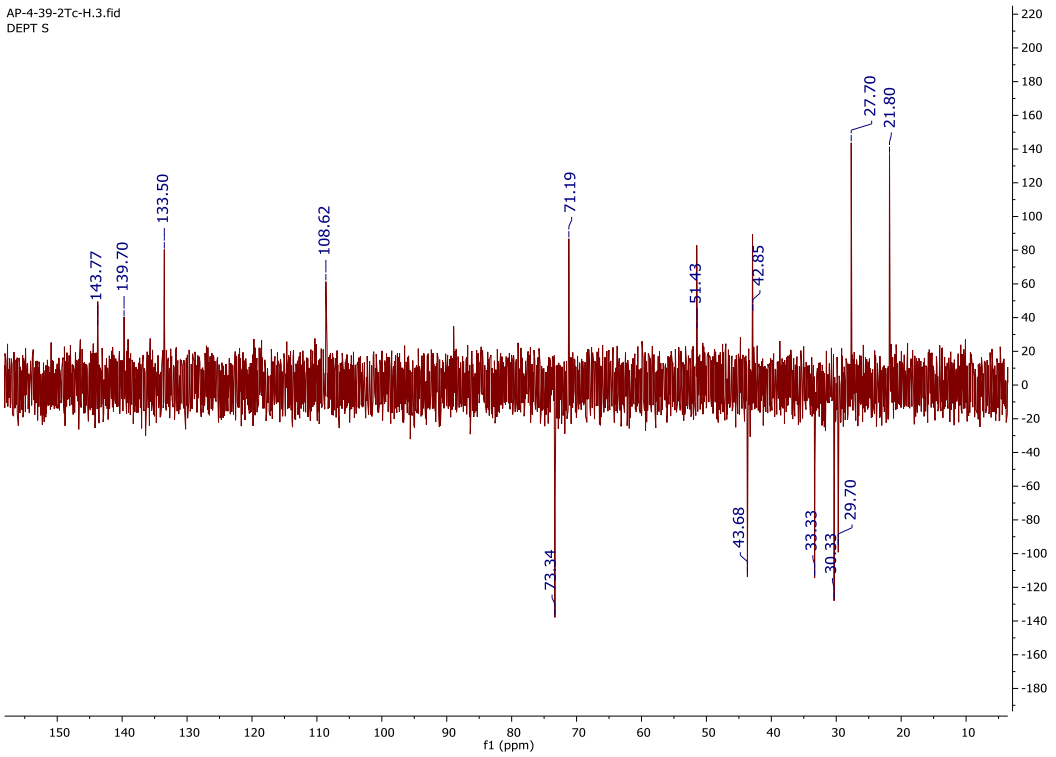
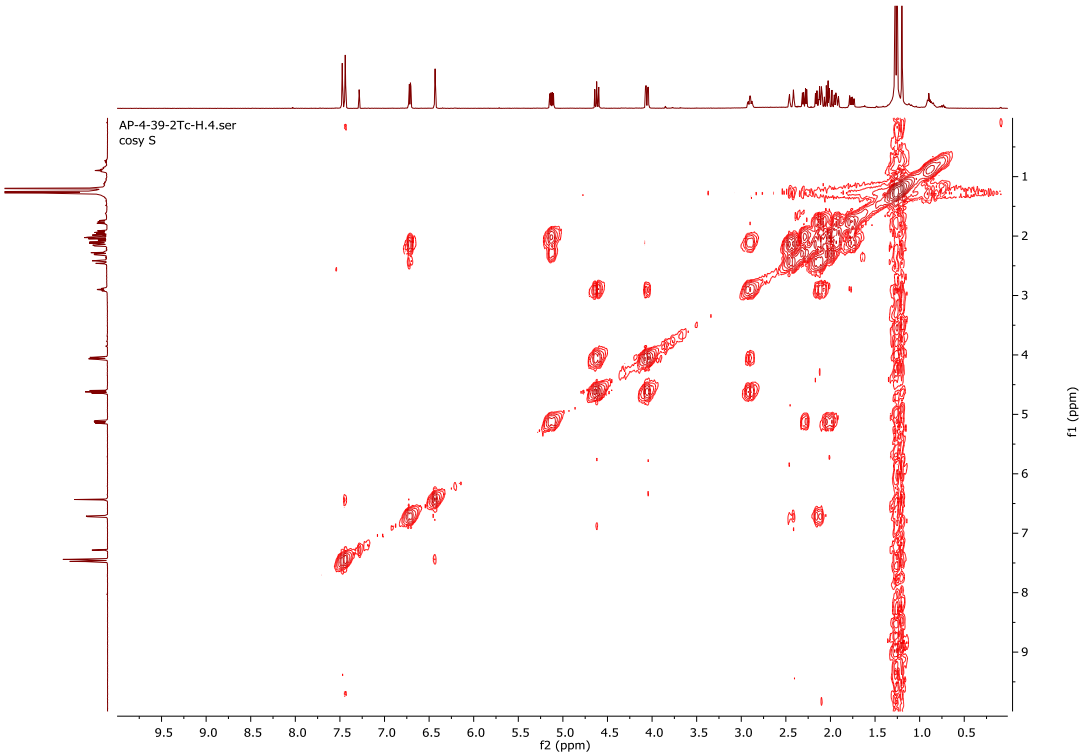
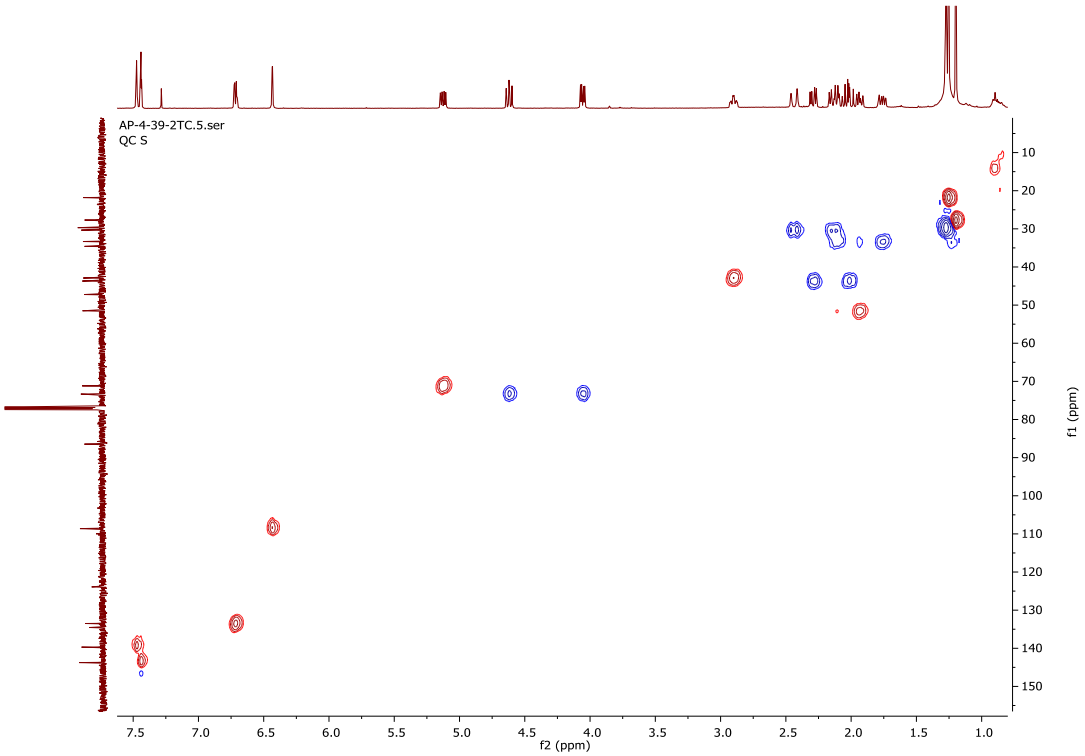


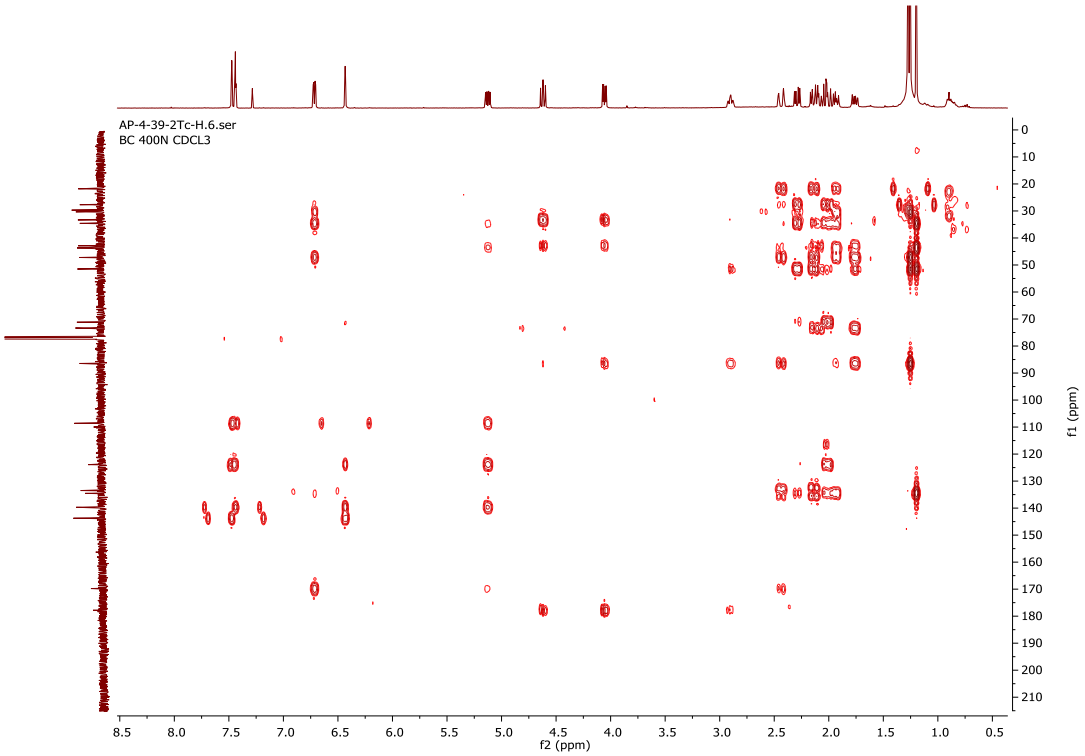
DEPT135 spectrum of Baenzigeride A



COSY spectrum of Baenzigeride A

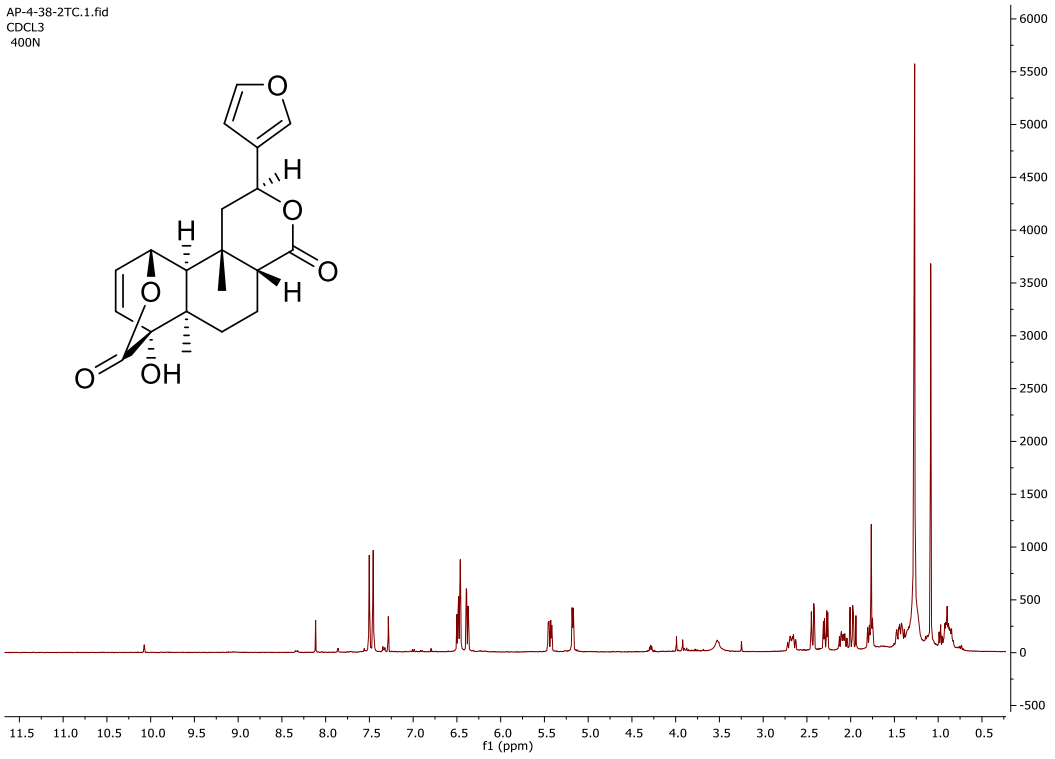


HSQC spectrum of Baenzigeride A



HMBC spectrum of Baenzigeride A

SI 2 1D and 2D NMR data of Columbin



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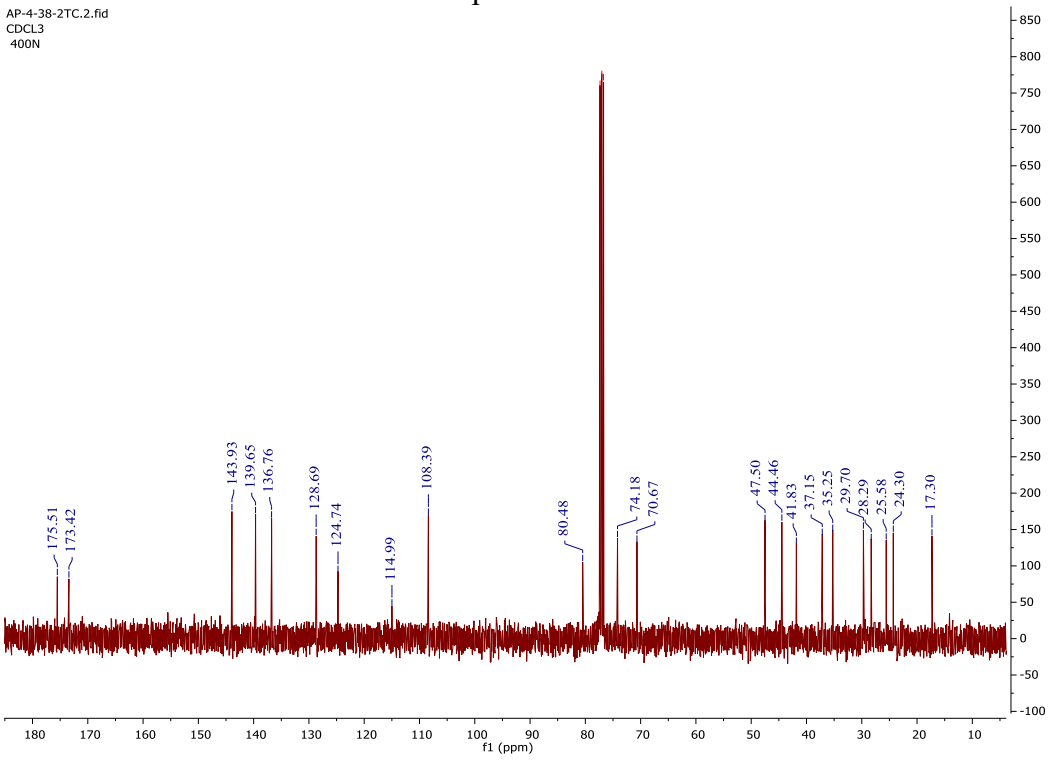
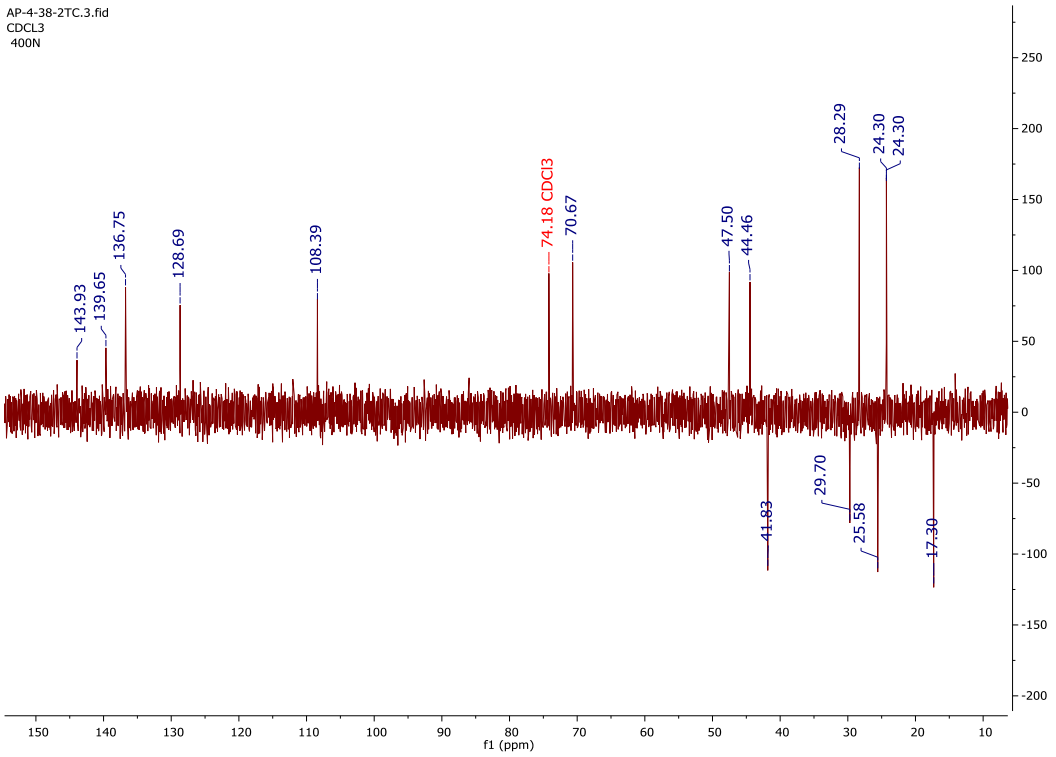


^{13}C-NMR spectrum of Columbin



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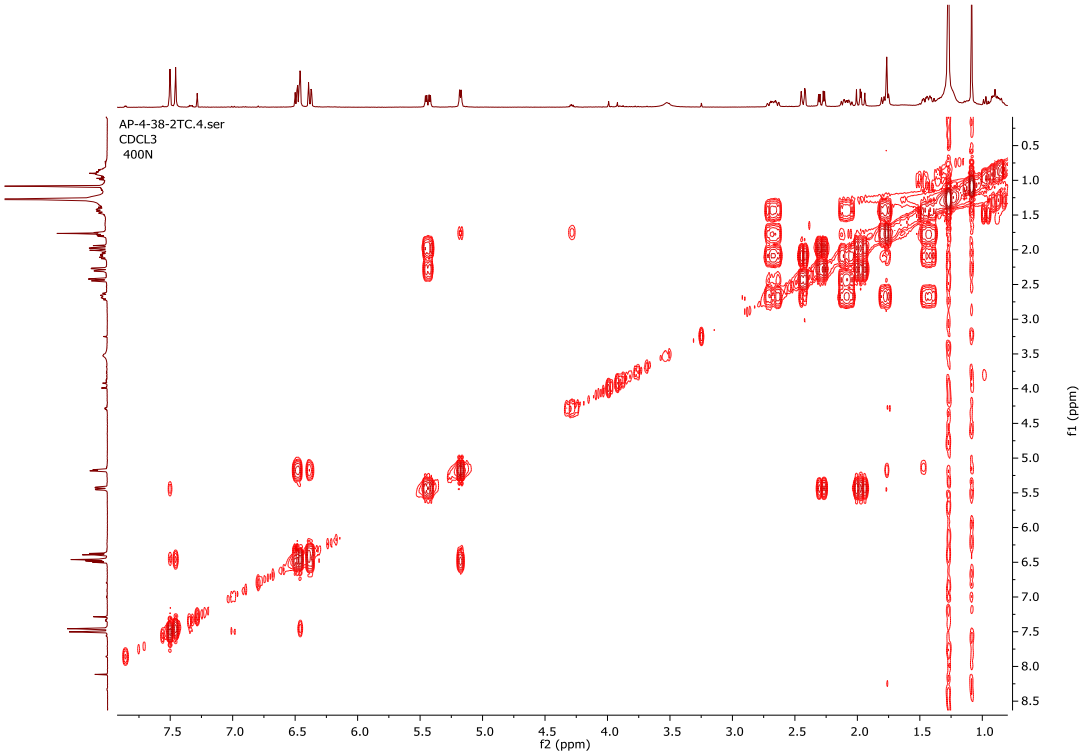

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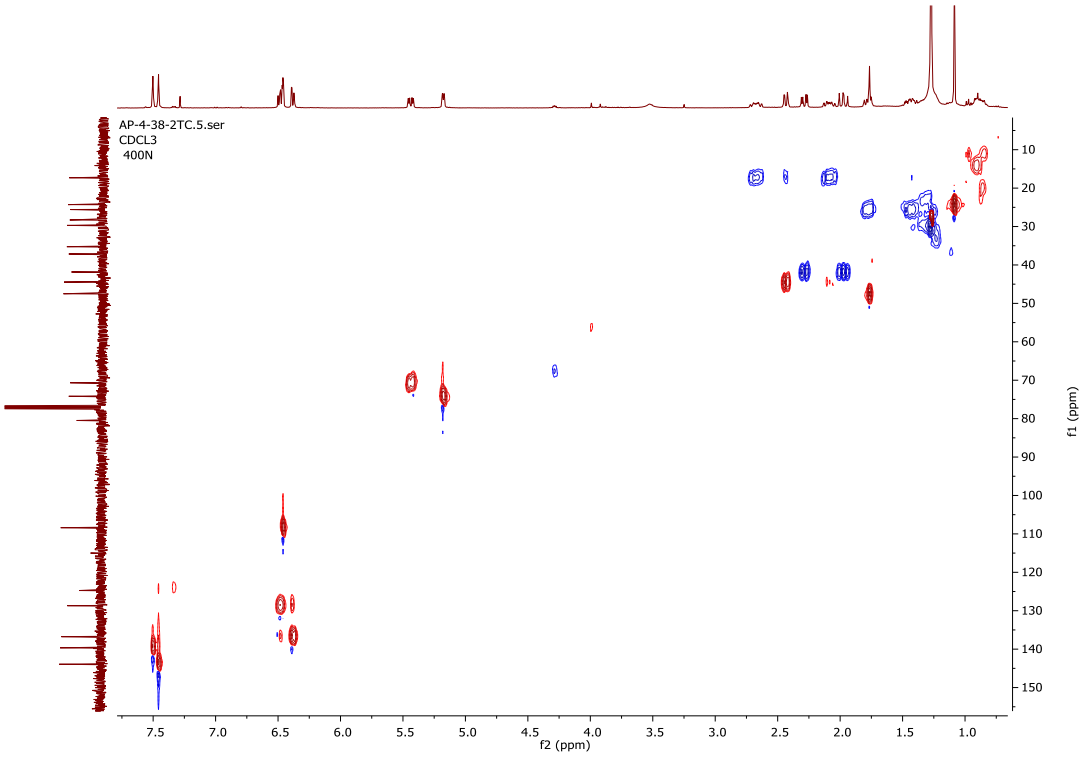
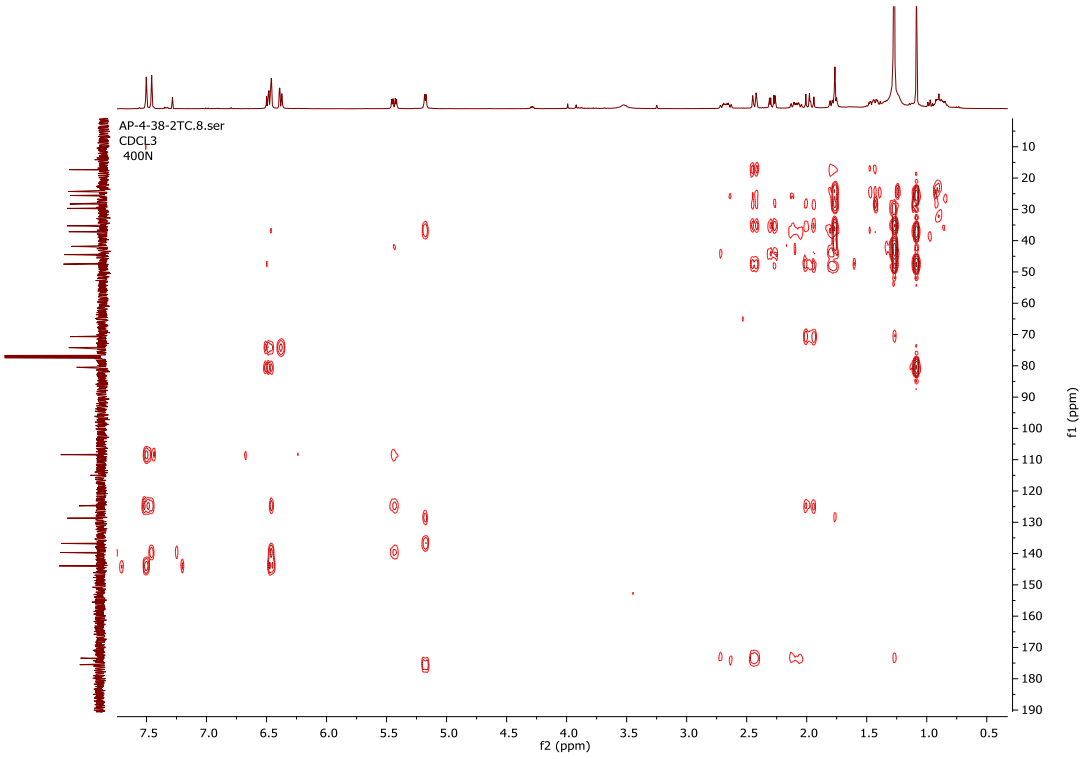
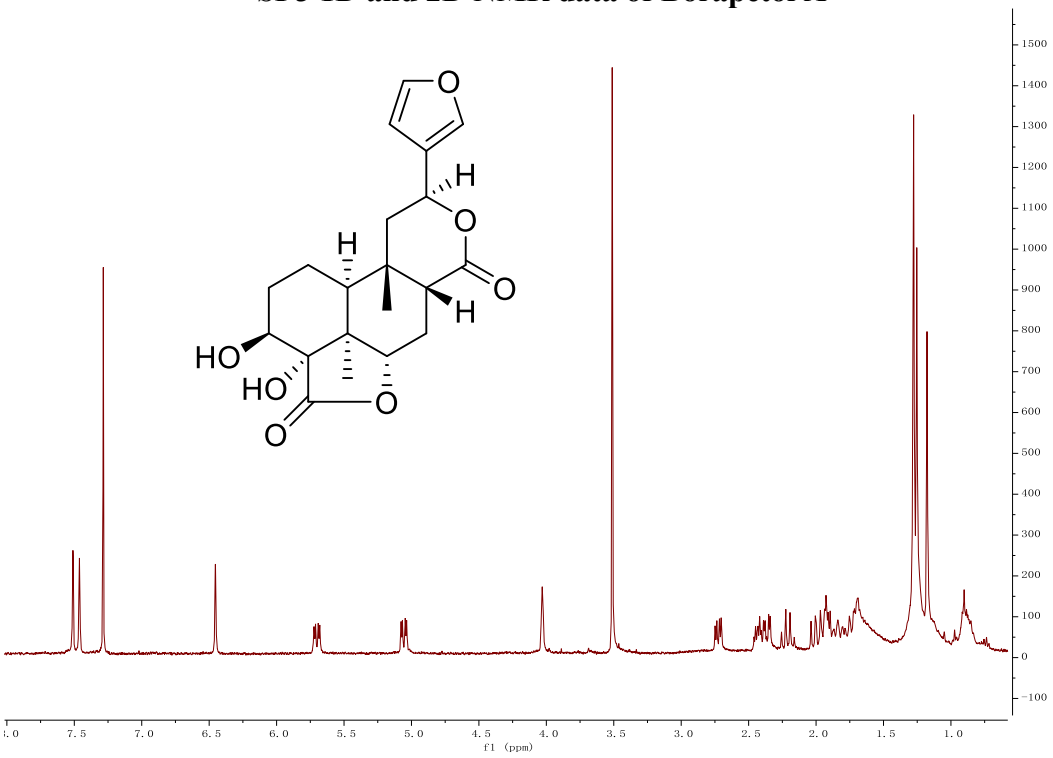


HSQC spectrum of Columbin



HMBC spectrum of Columbin

SI 3 1D and 2D NMR data of Borapetol A



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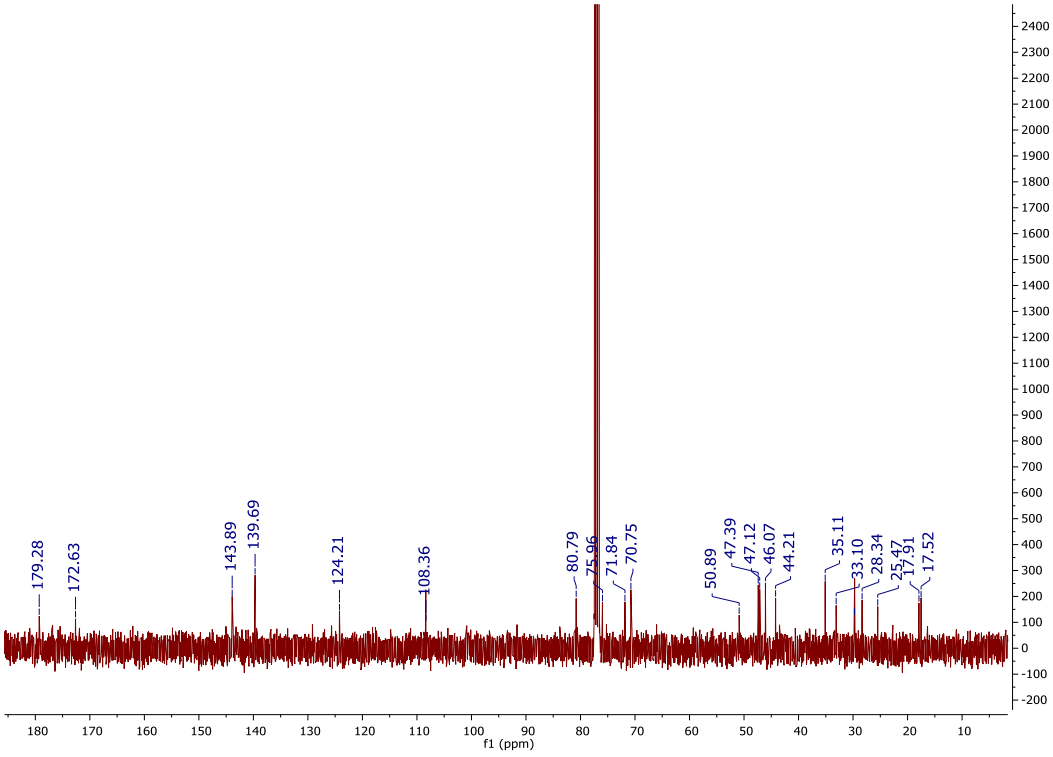

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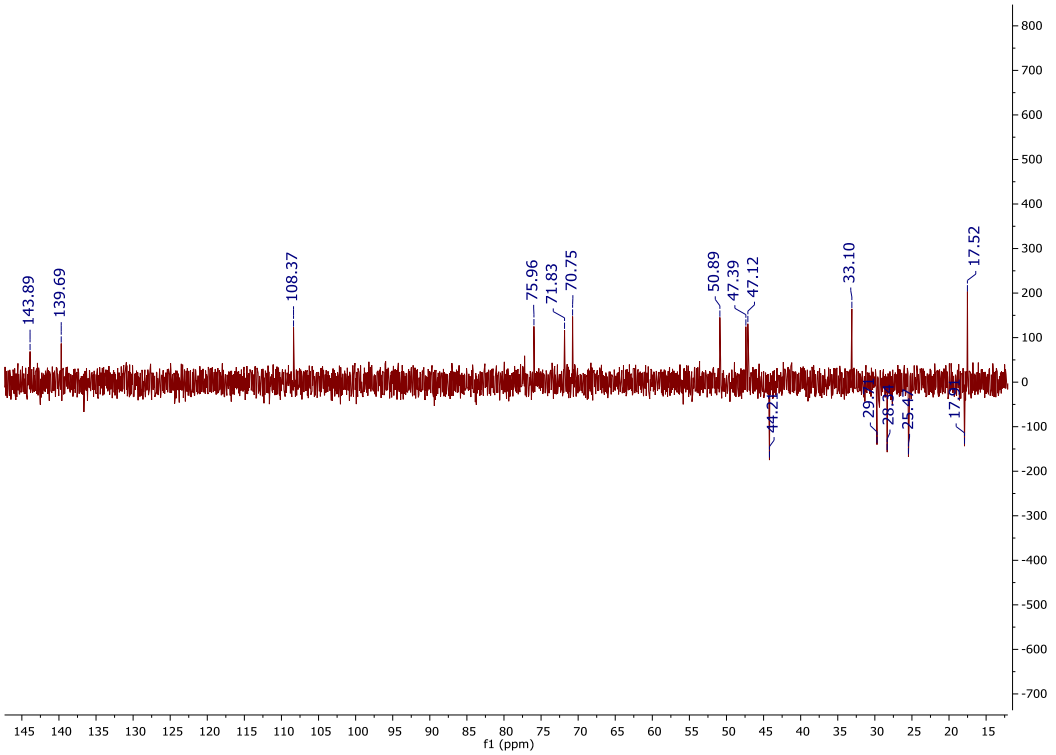

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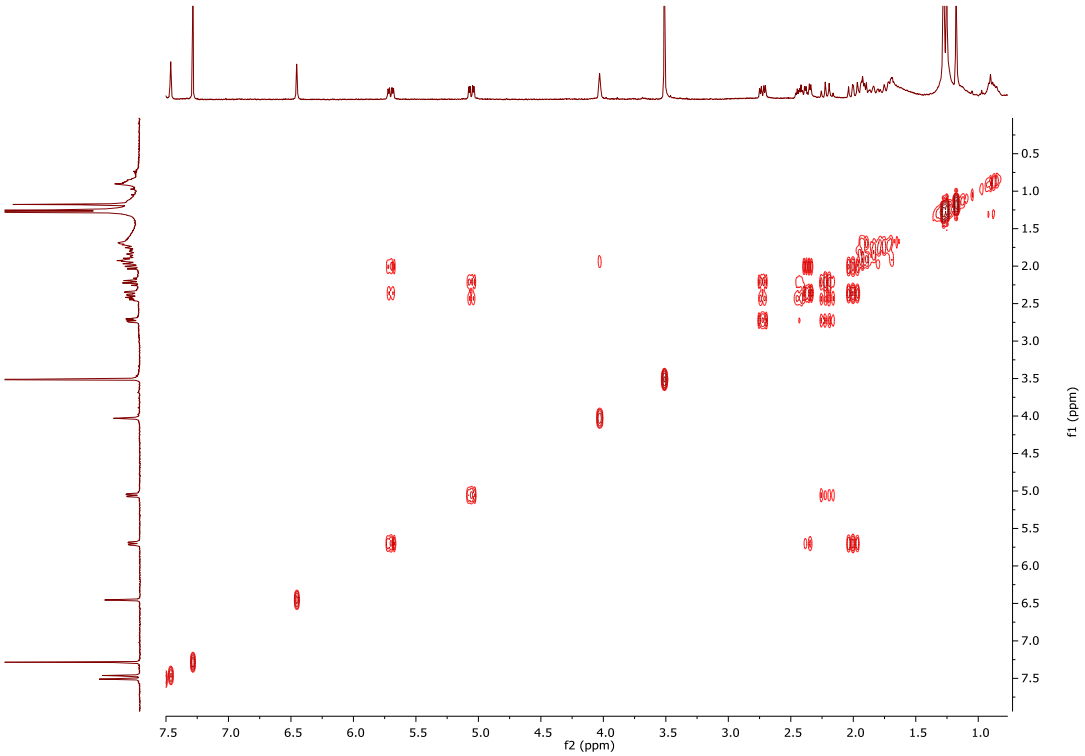

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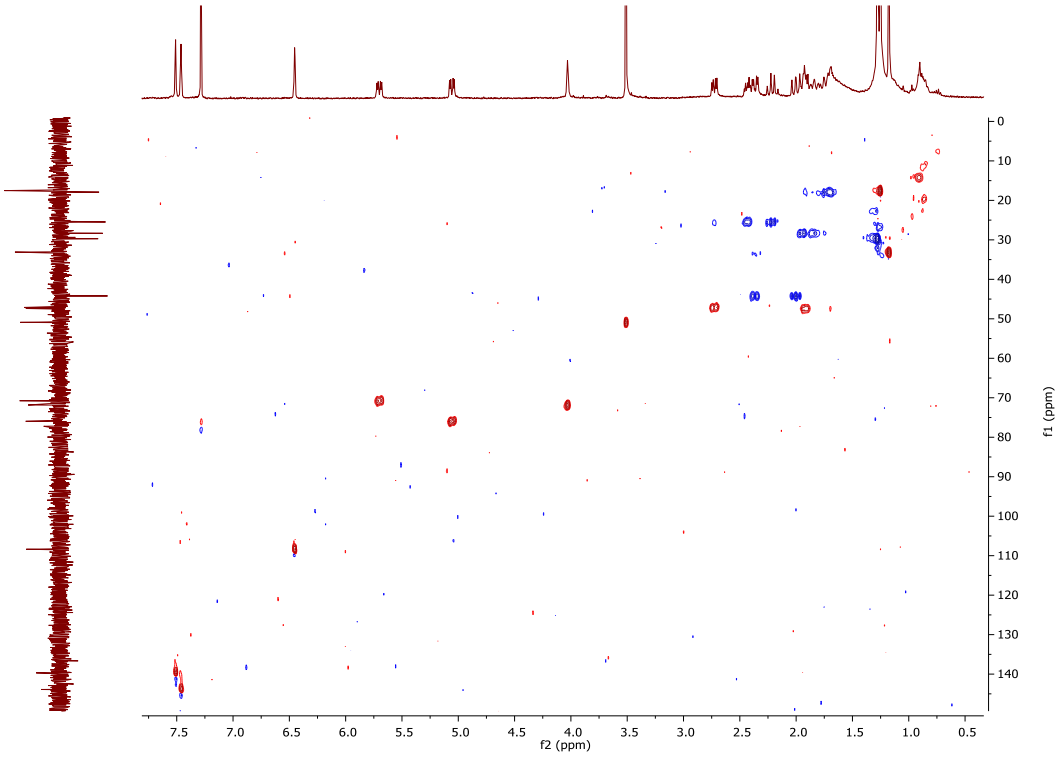
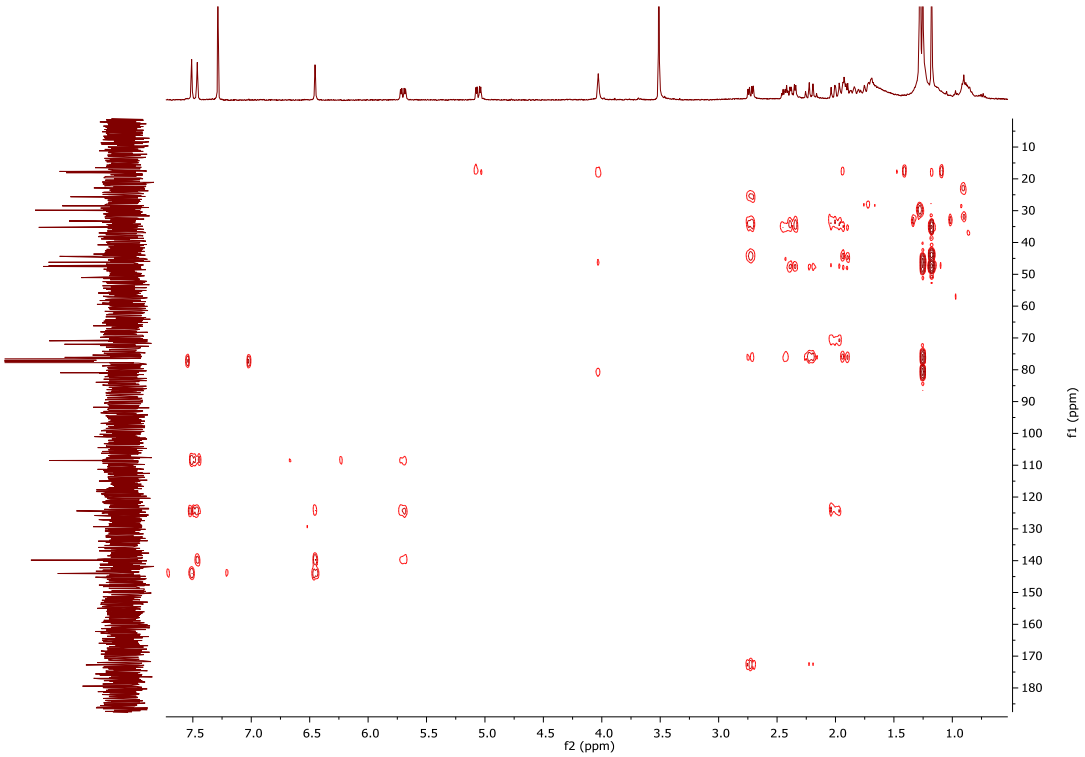


HSQC spectrum of Borapetol A



HMBC spectrum of Borapetol A

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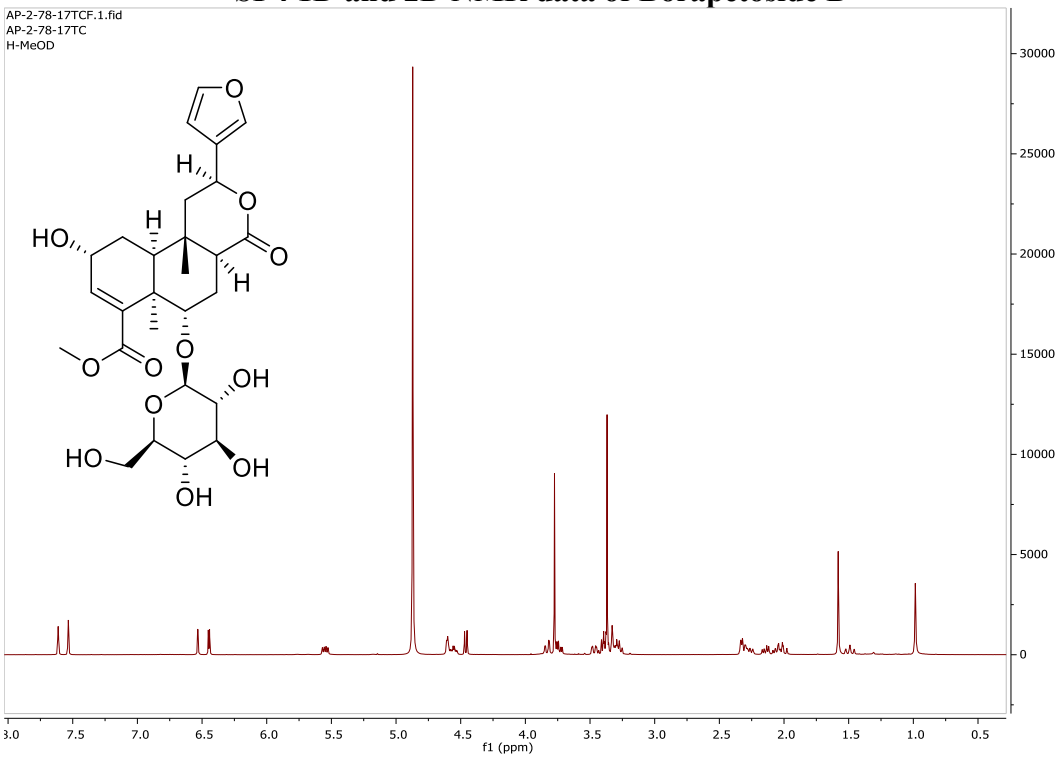
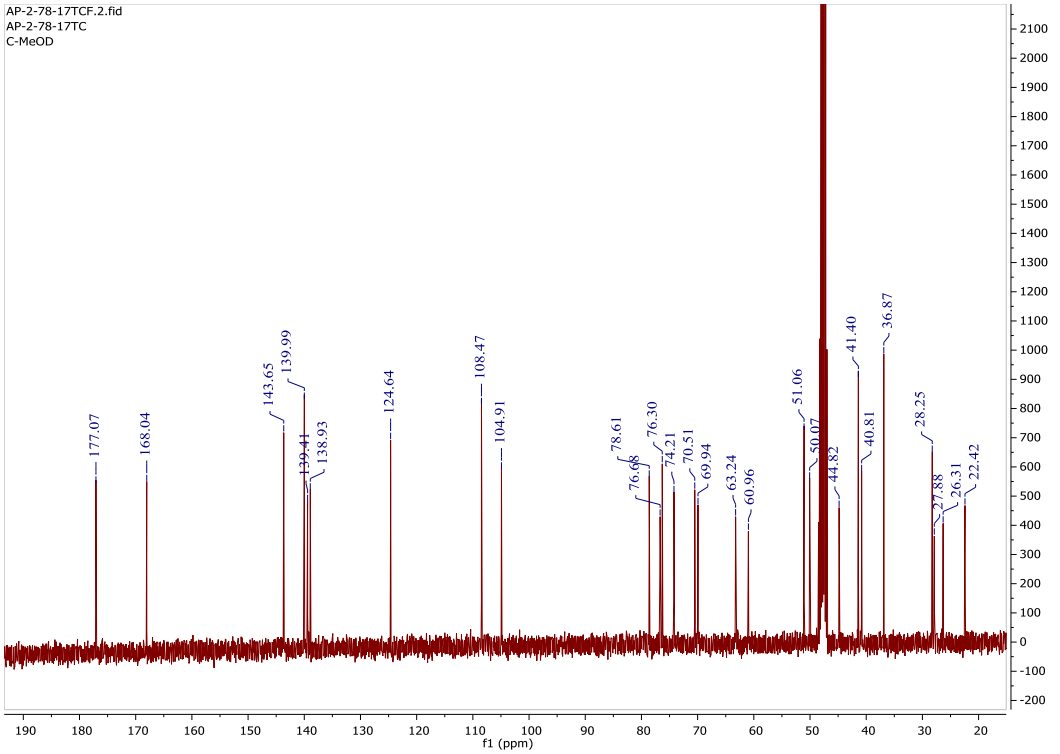


^{1}H-NMR spectrum of Borapetoside B



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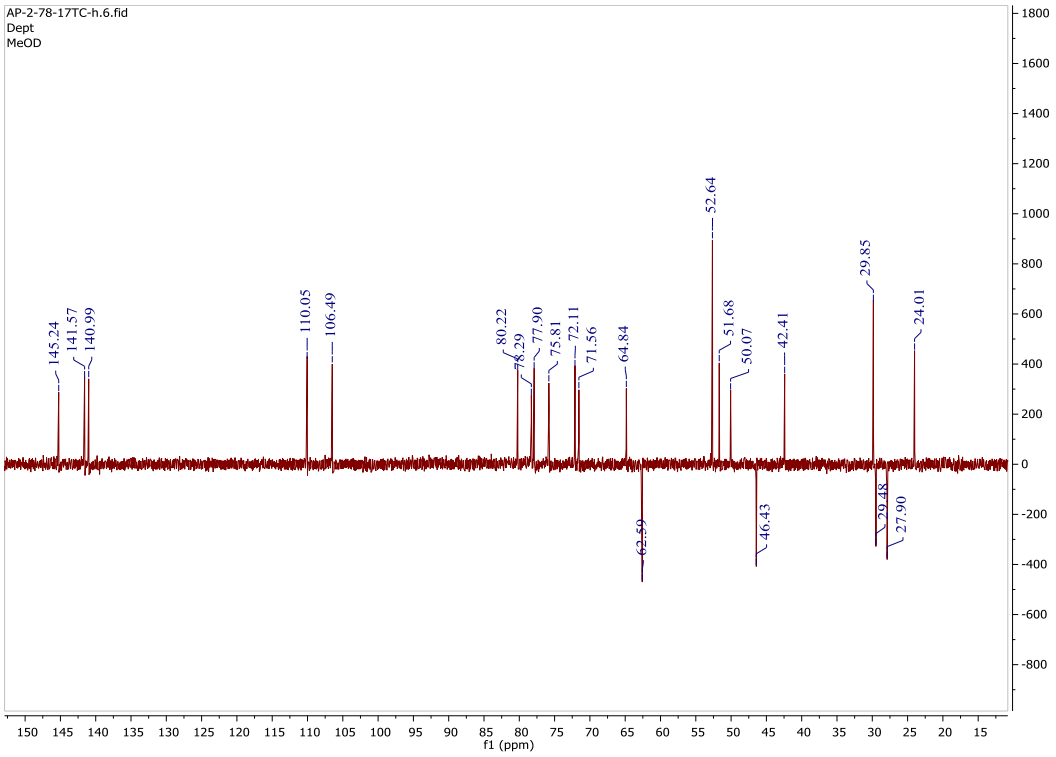
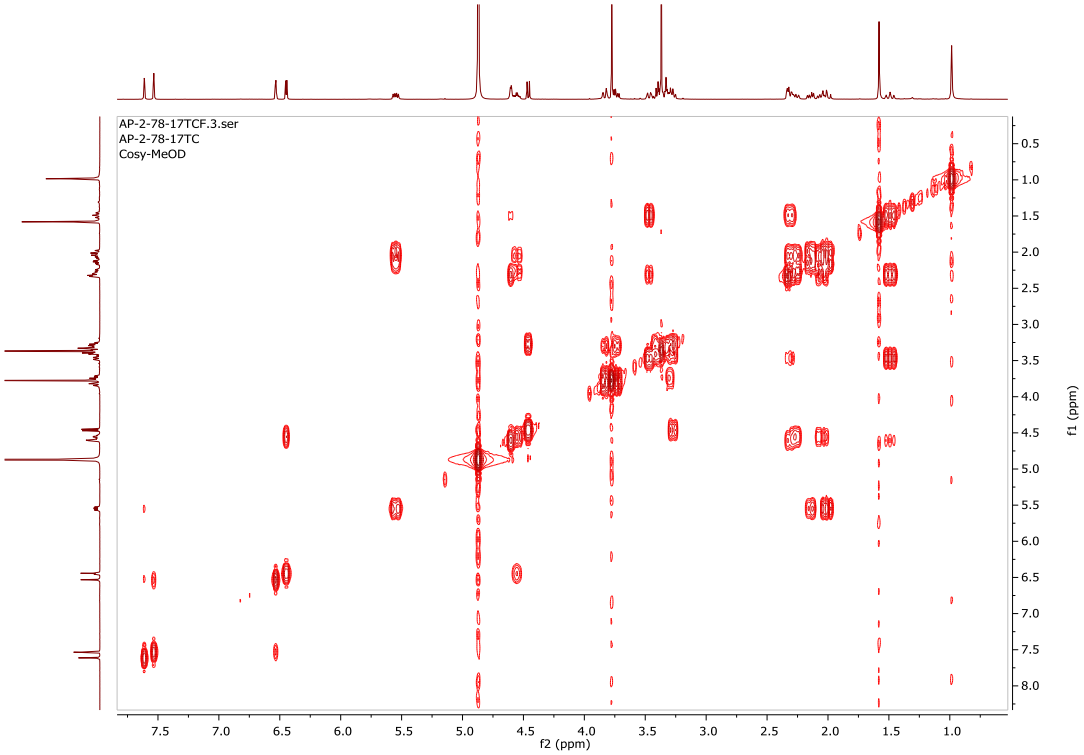


DEPT135 spectrum of Borapetoside B



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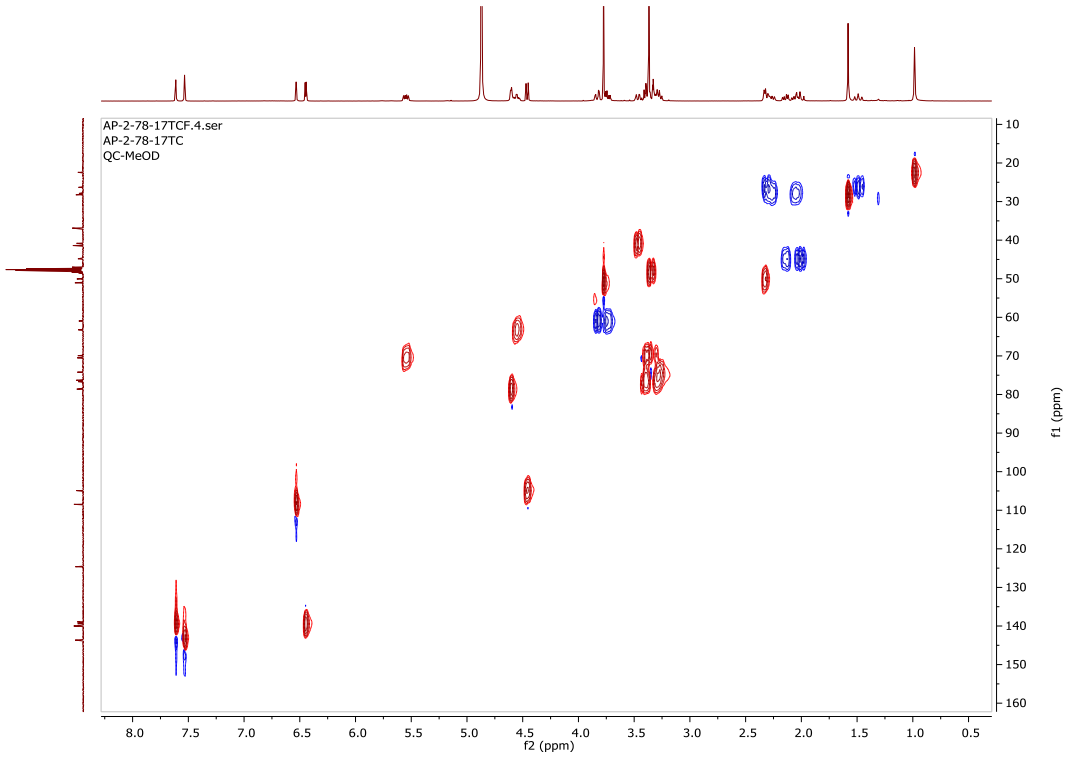
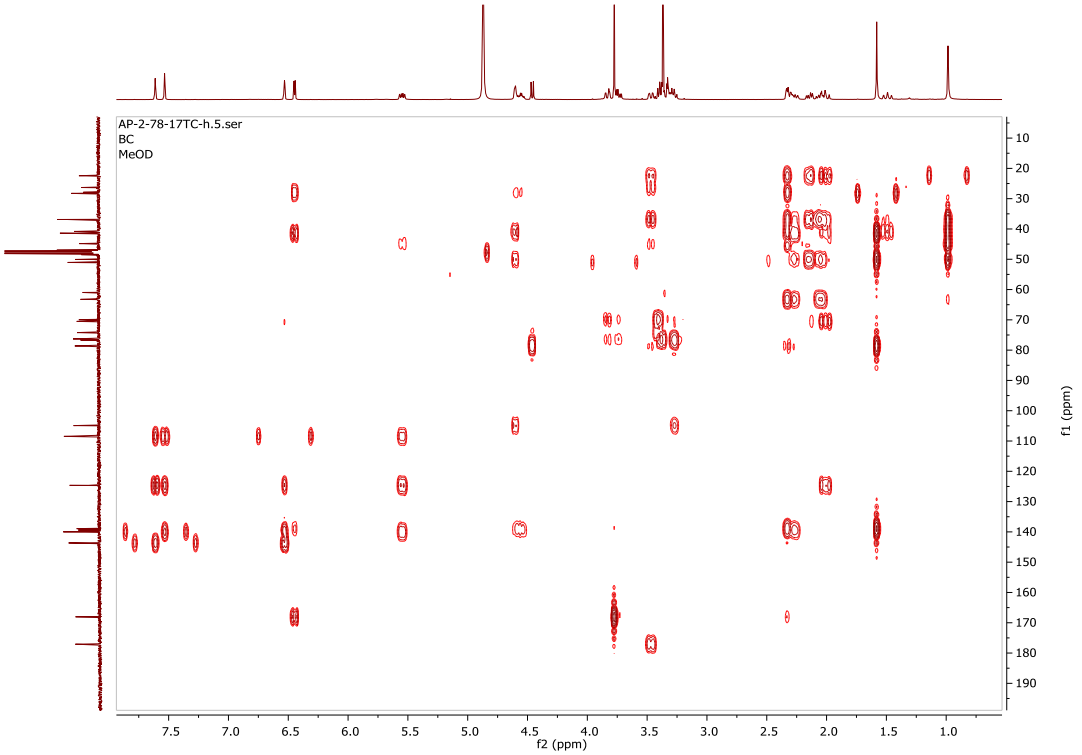
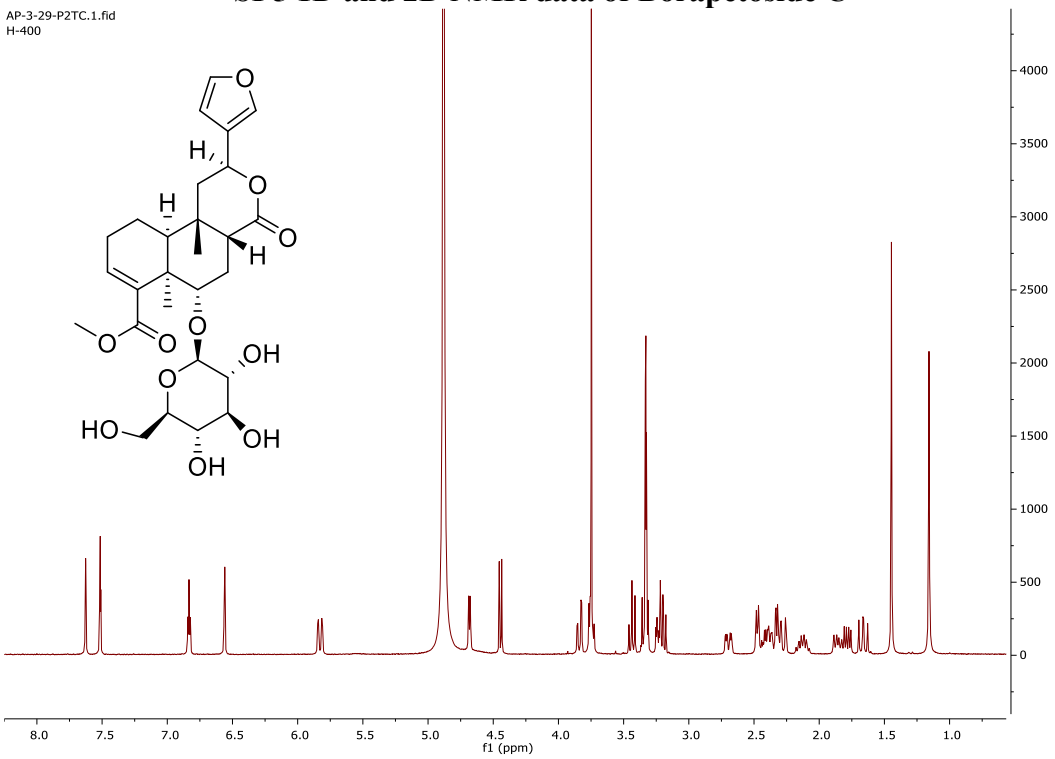


HSQC spectrum of Borapetoside B



HMBC spectrum of Borapetoside B

SI 5 1D and 2D NMR data of Borapetoside C



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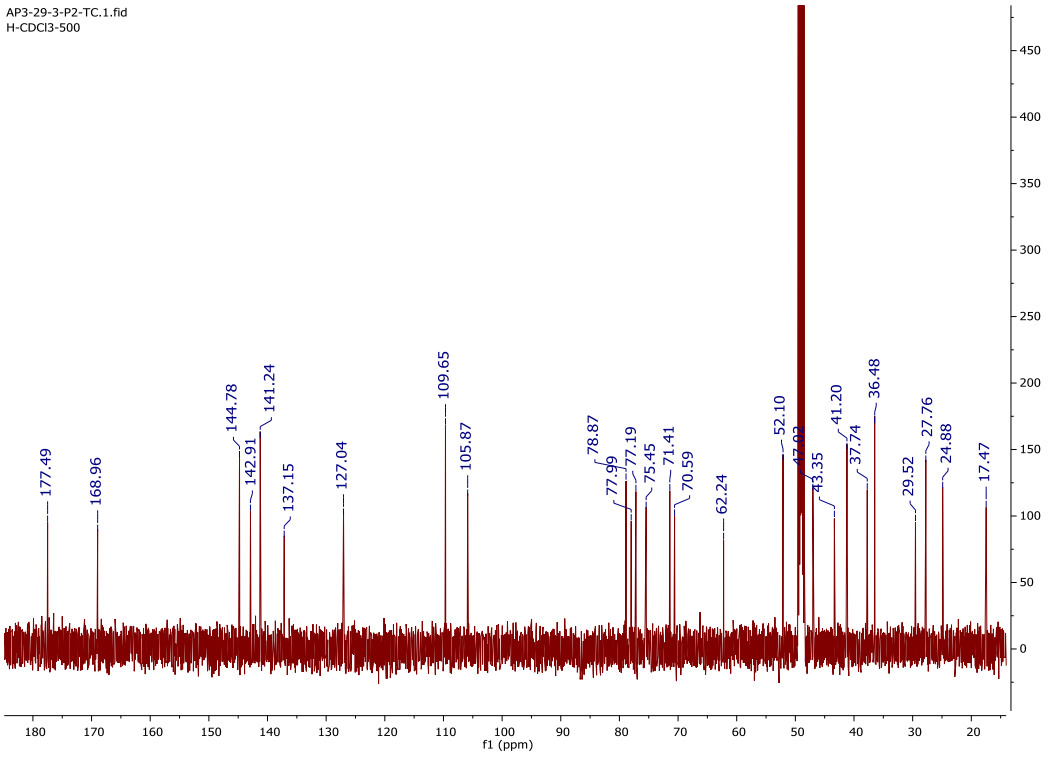

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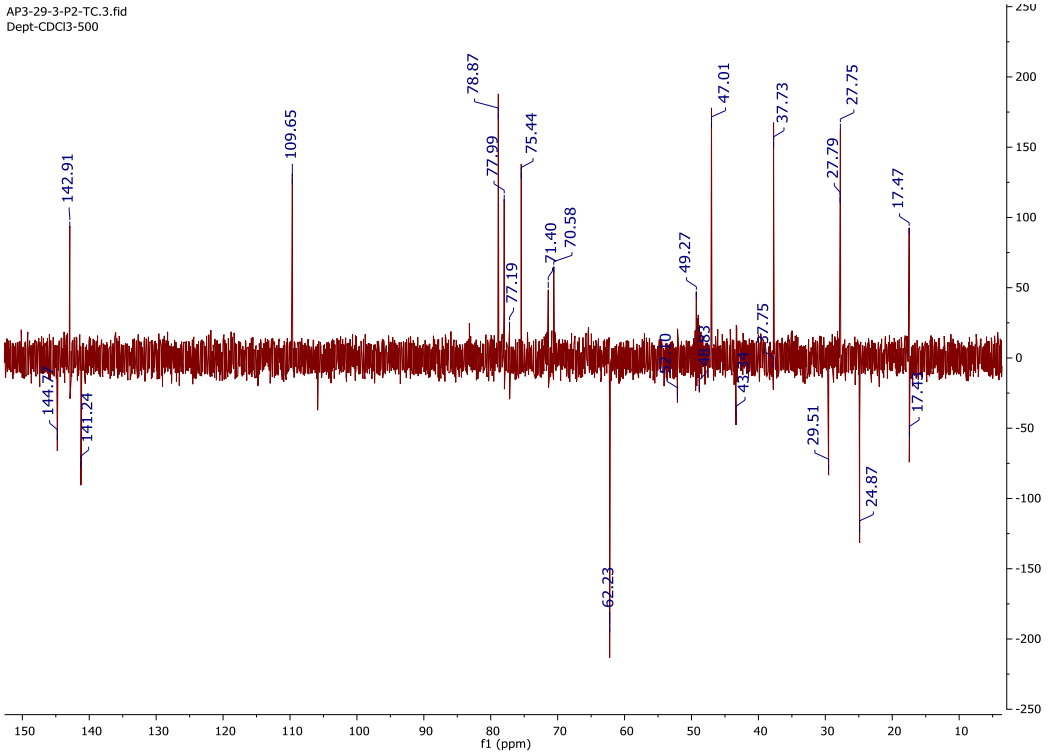

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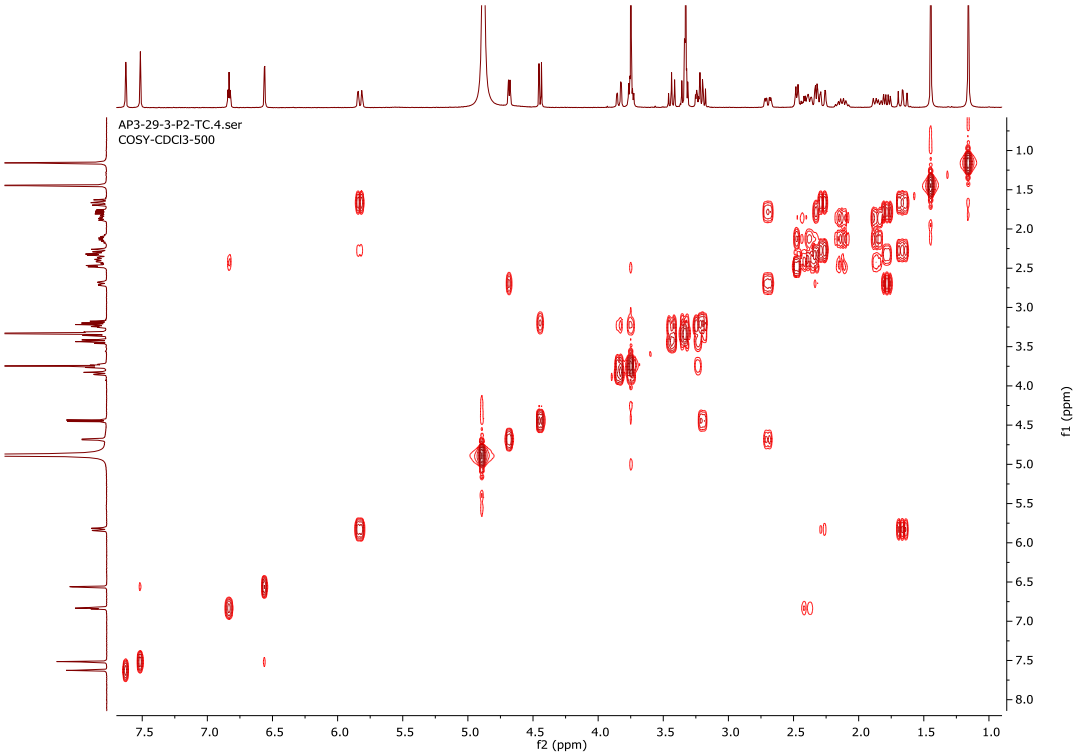

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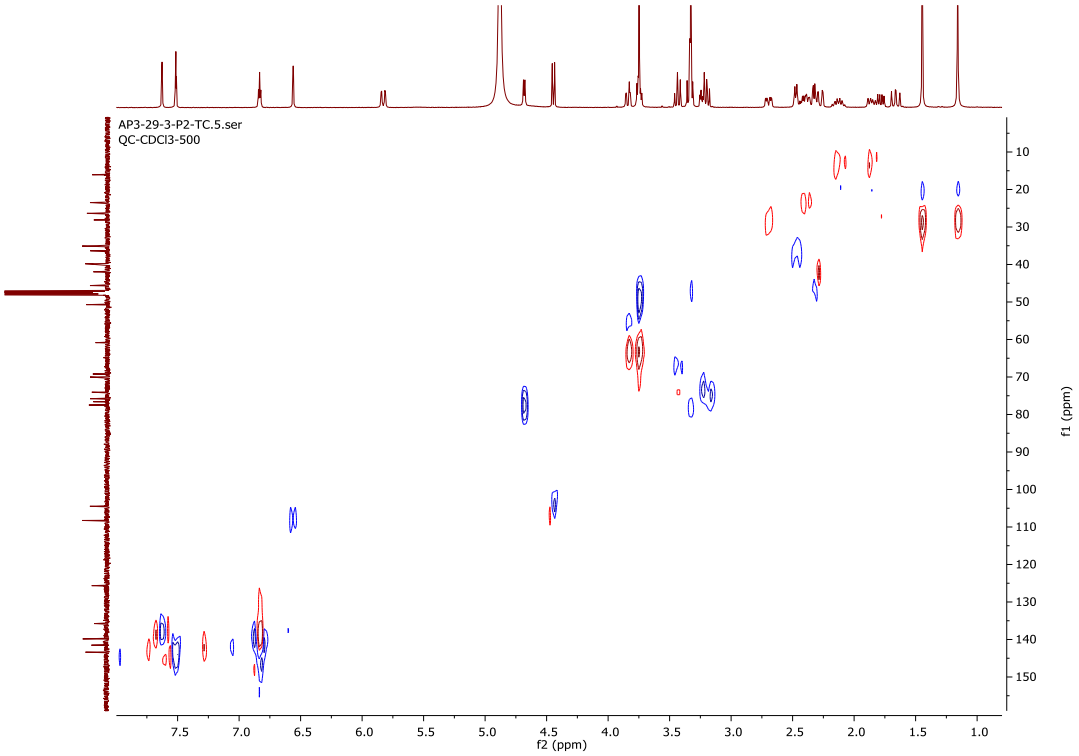
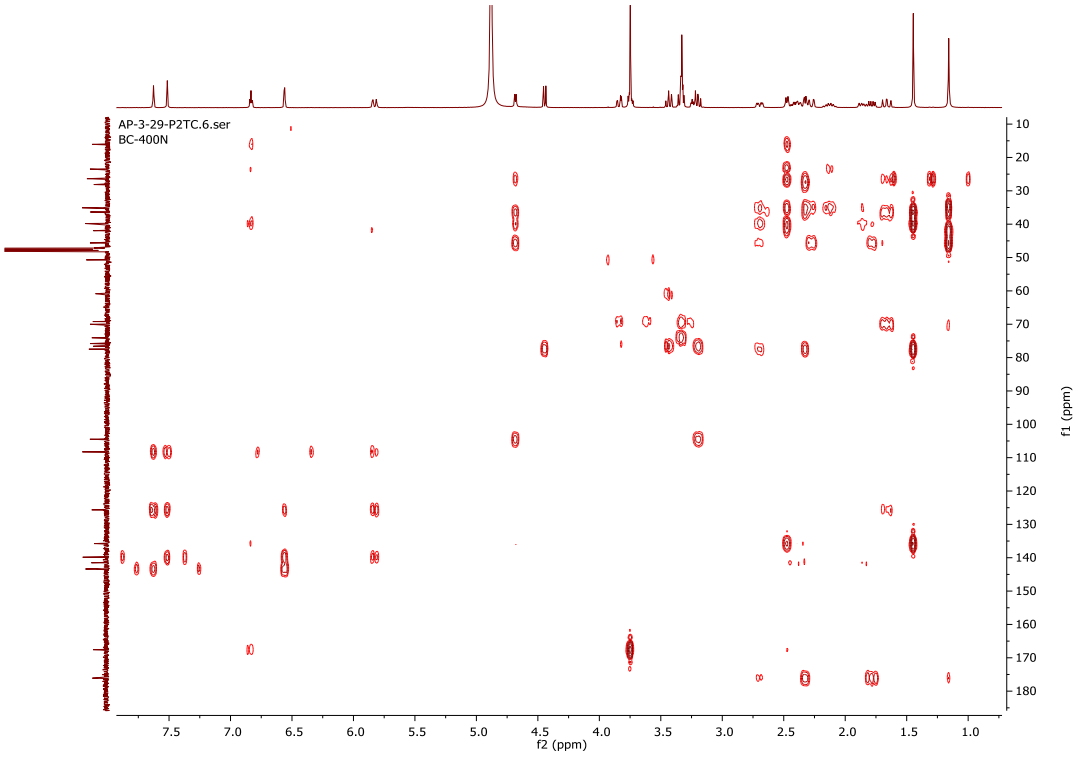
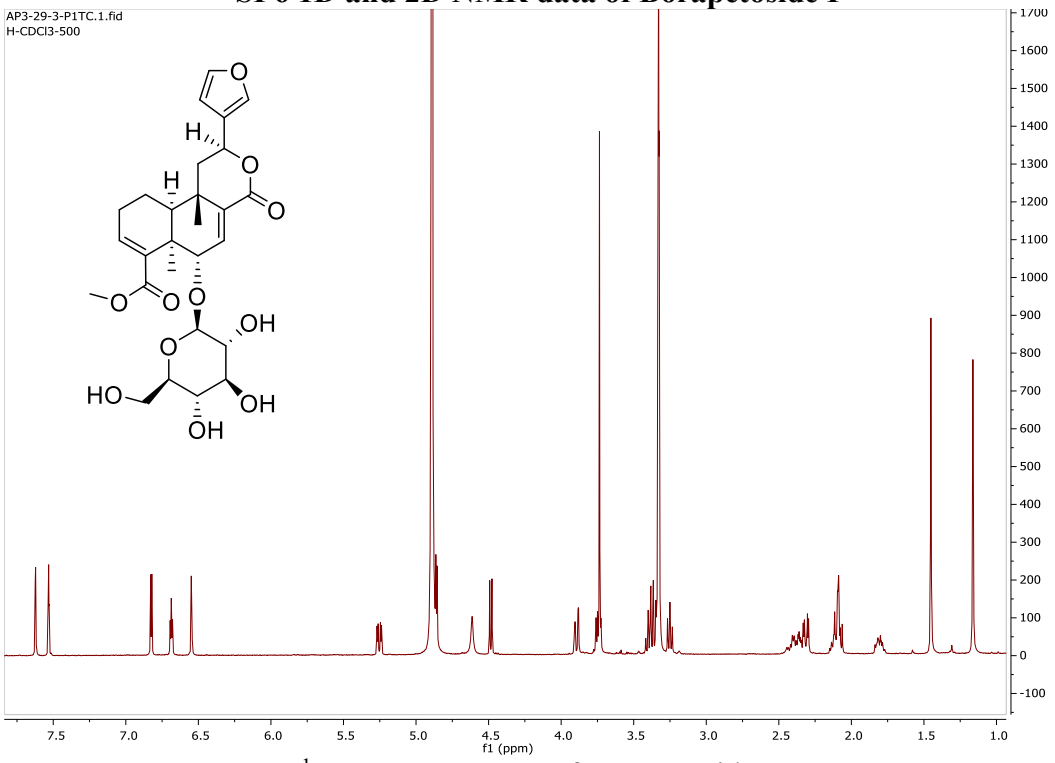


HSQC spectrum of Borapetoside C

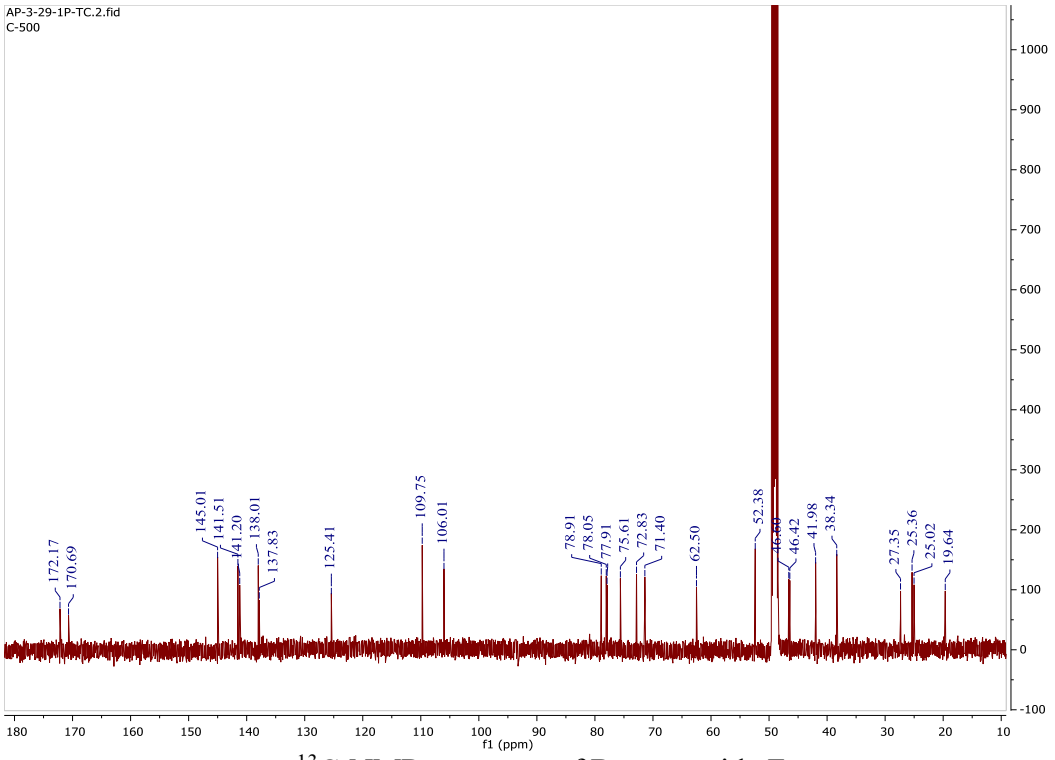


HMBC spectrum of Borapetoside C

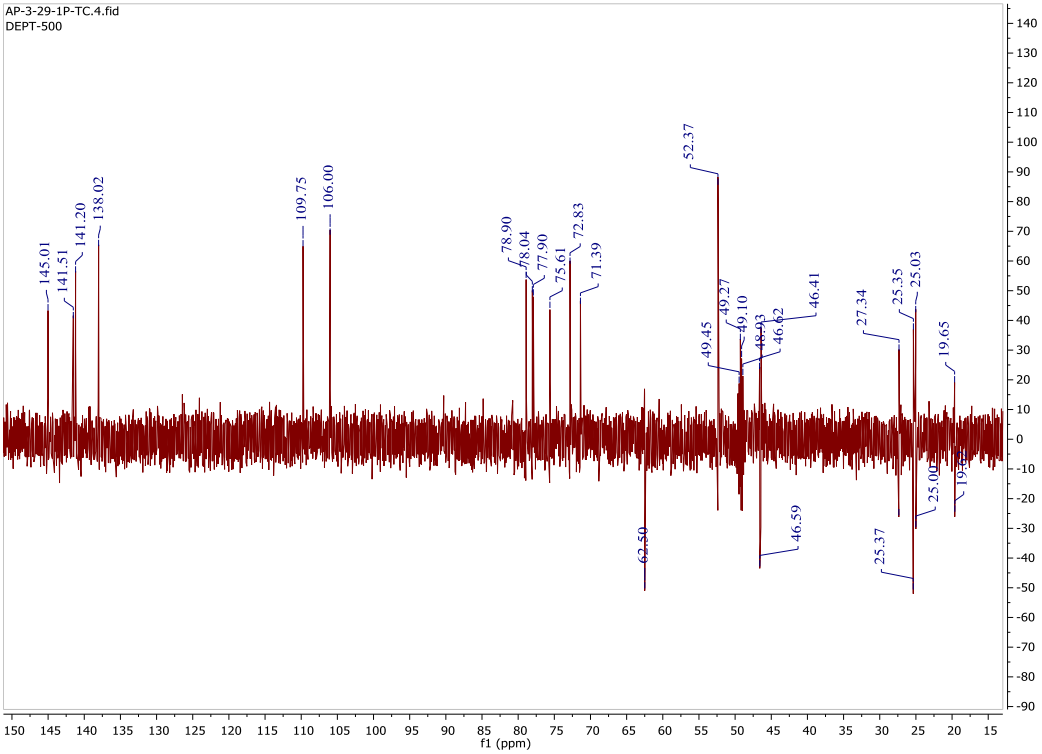
SI 6 1D and 2D NMR data of Borapetoside F



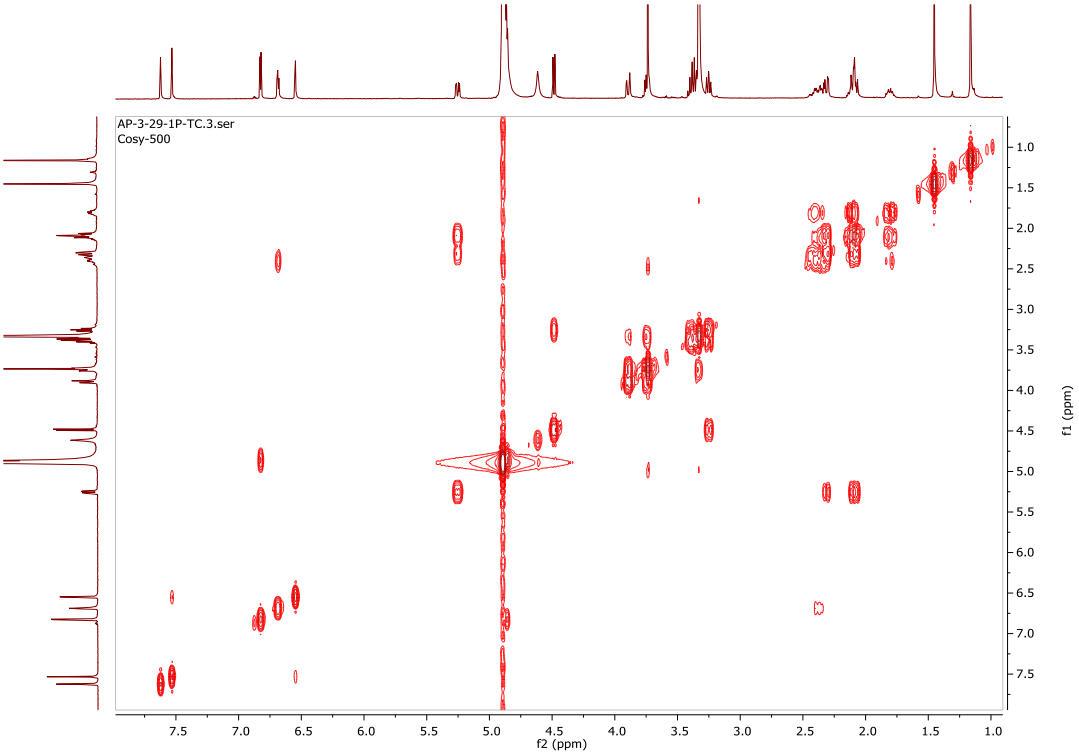
^{1}H-NMR spectrum of Borapetoside F



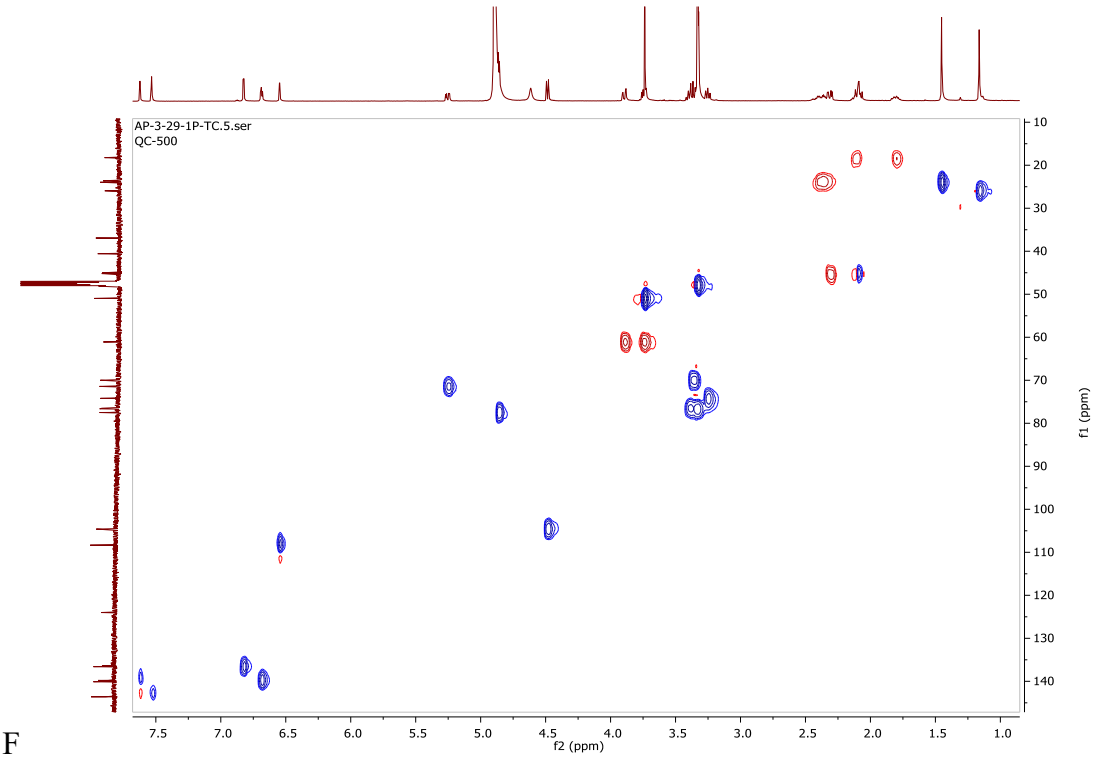
^{13}C-NMR spectrum of Borapetoside F



DEPT135 spectrum of Borapetoside F

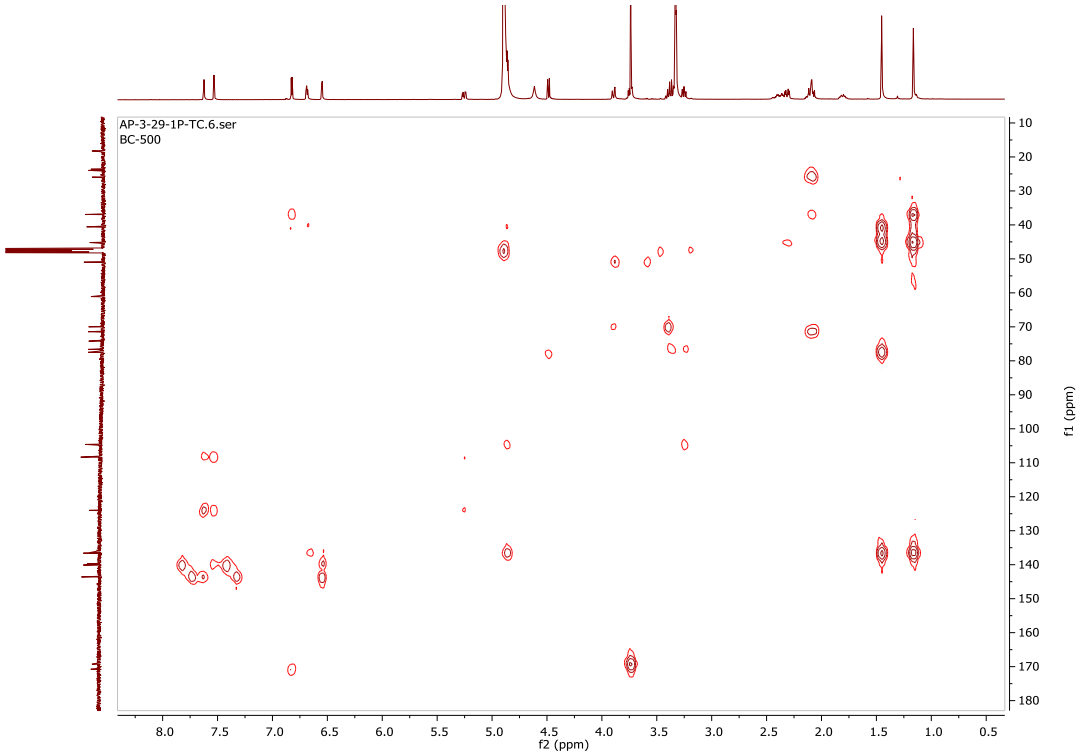


COSY spectrum of Borapetoside F



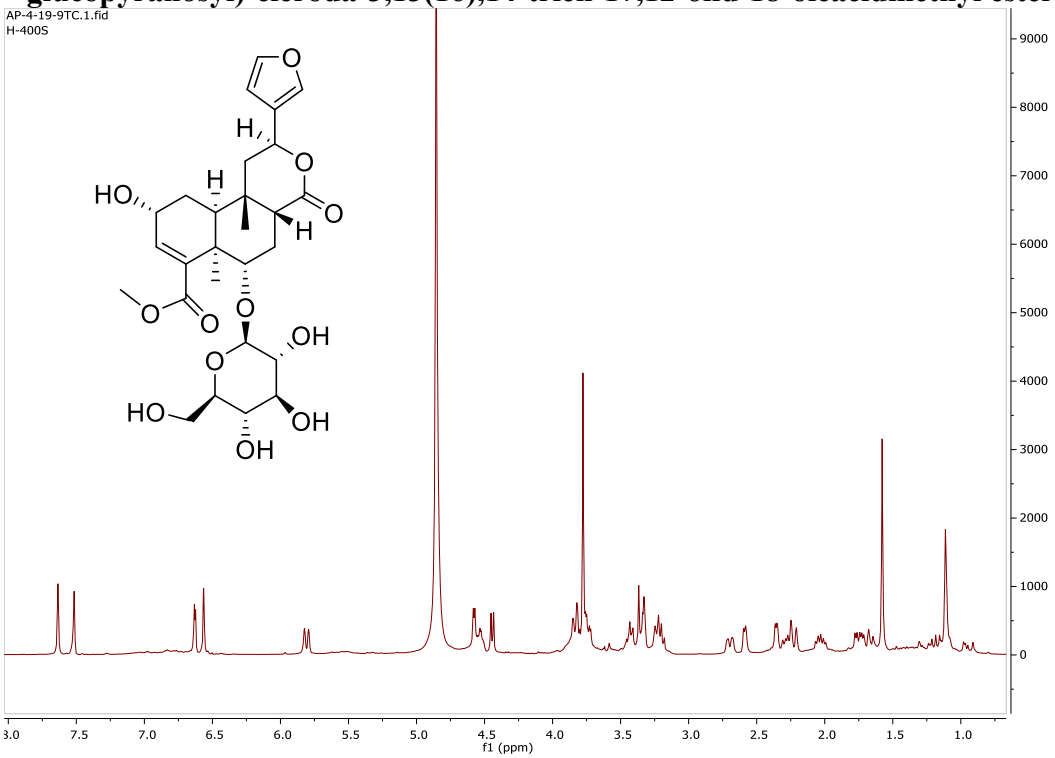
F

HSQC spectrum of Borapetoside F

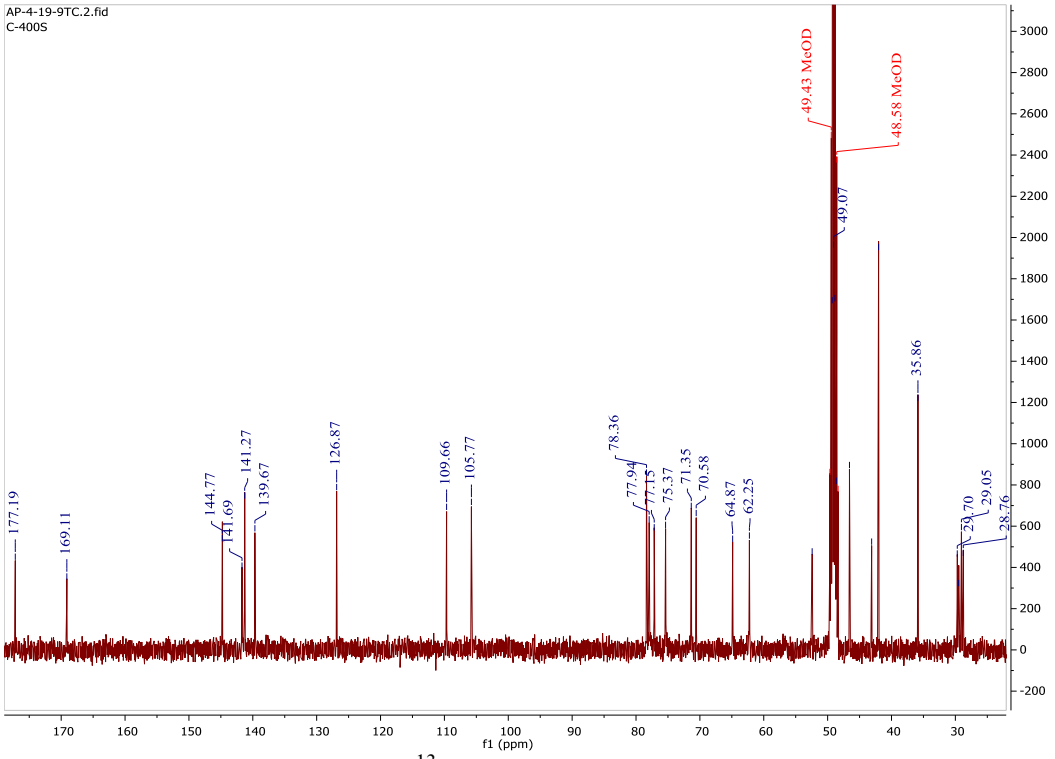


HMBC spectrum of Borapetoside F

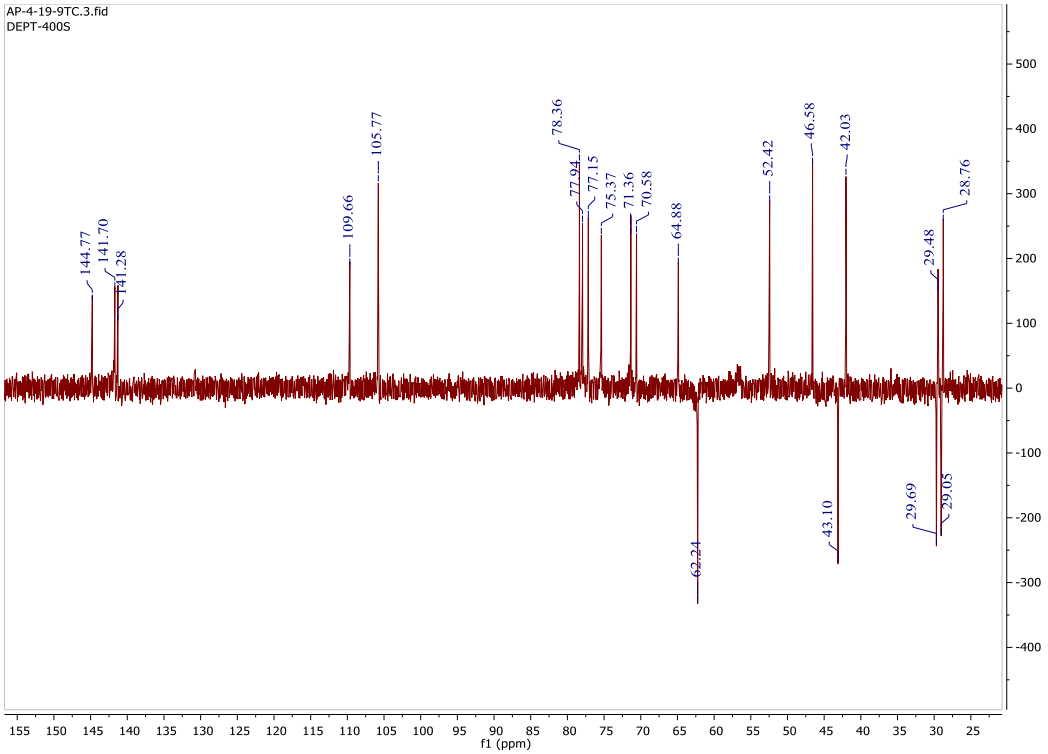
SI 7 1D and 2D NMR data of (2*R*,5*R*,6*R*,8*R*,9*S*,10*S*,12*S*)-15,16-epoxy-2-hydroxy-6-O-(β-D-glucopyranosyl)-cleroda-3,13(16),14-trien-17,12-olid-18-oicacidmethyl ester



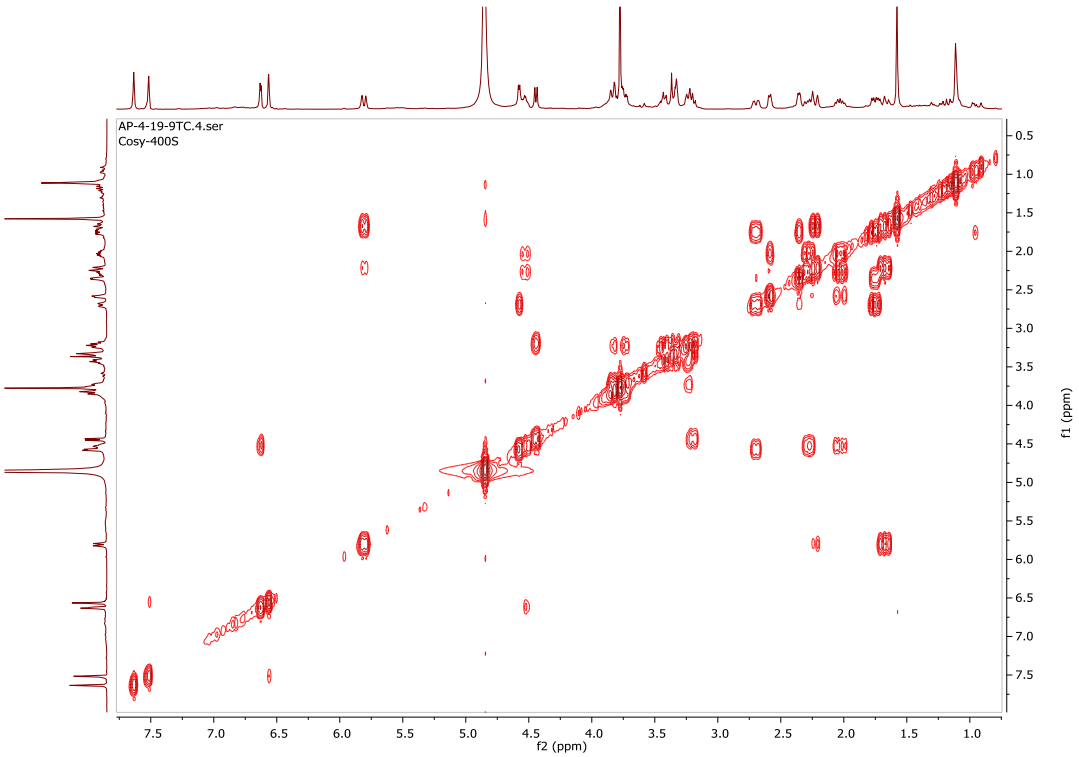
^{1}H-NMR spectrum



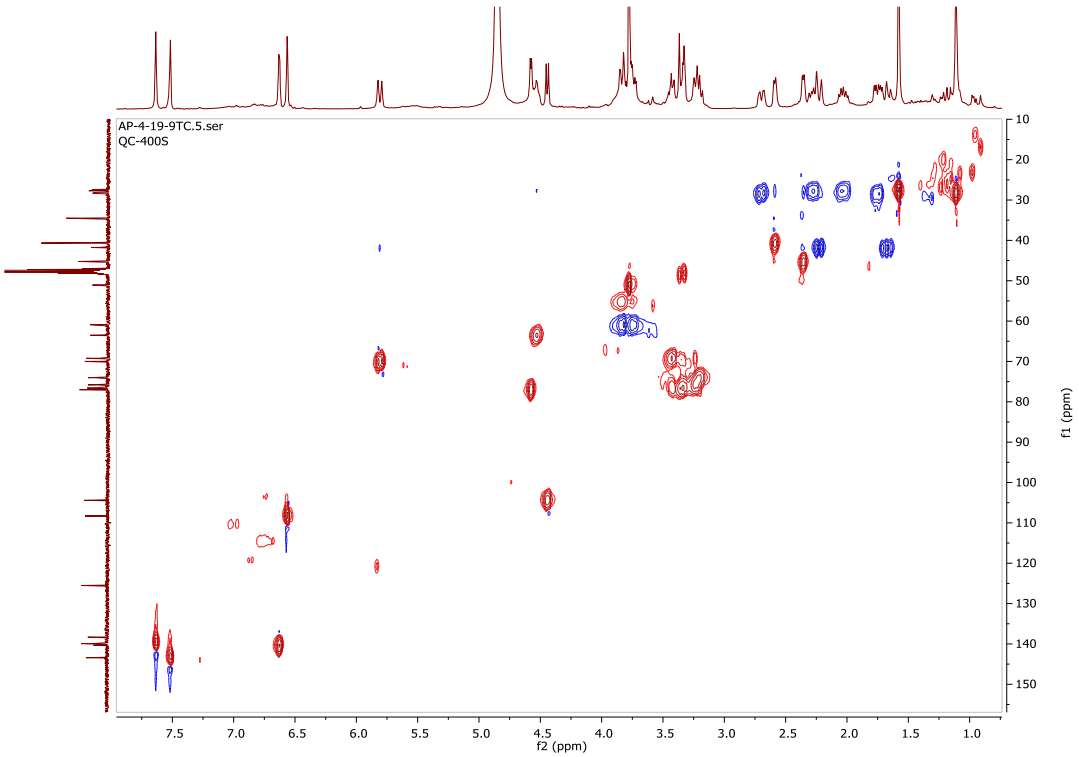
^{13}C-NMR spectrum



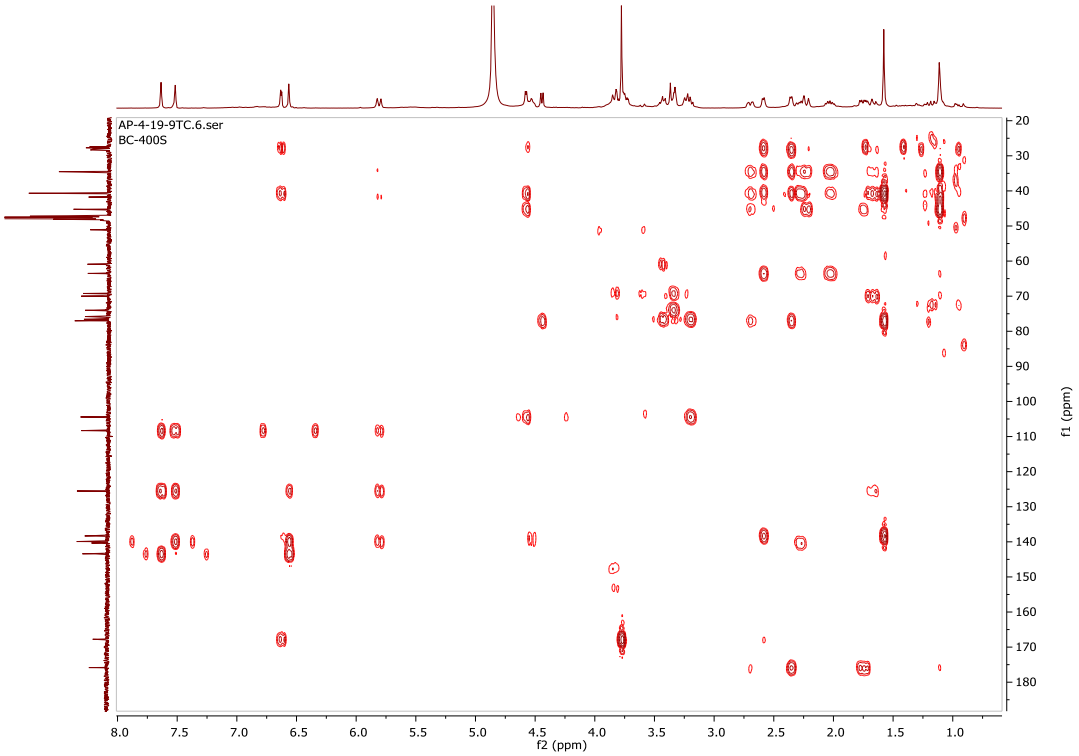
DEPT135 spectrum



COSY spectrum

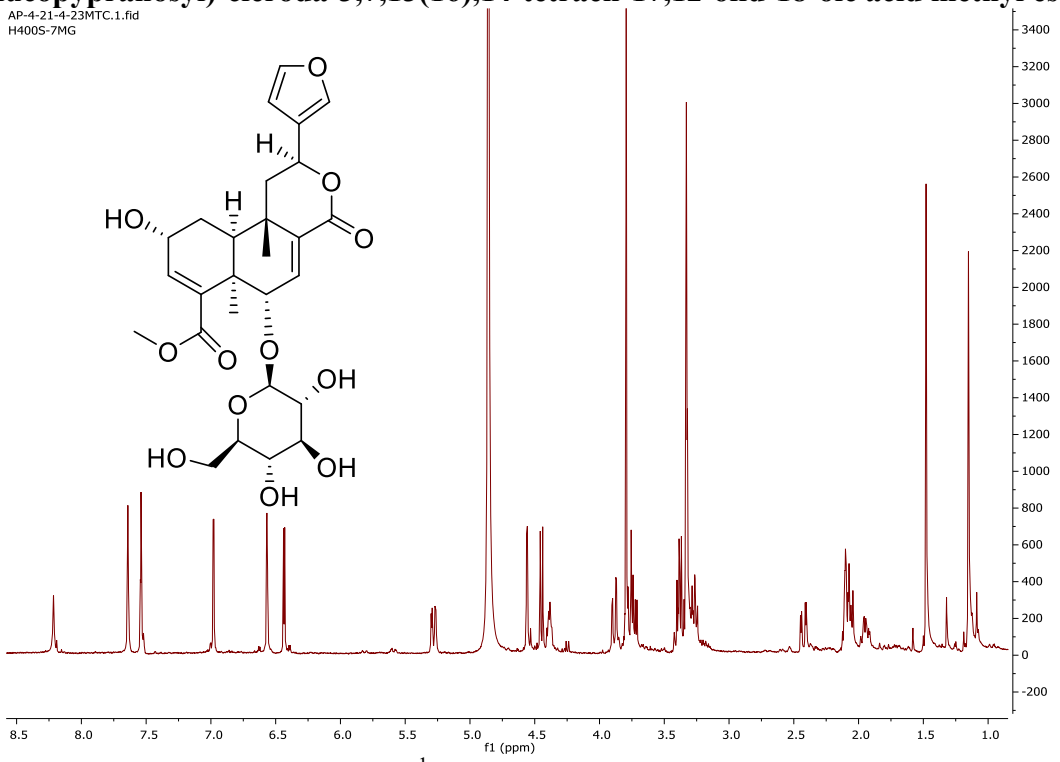


HSQC spectrum

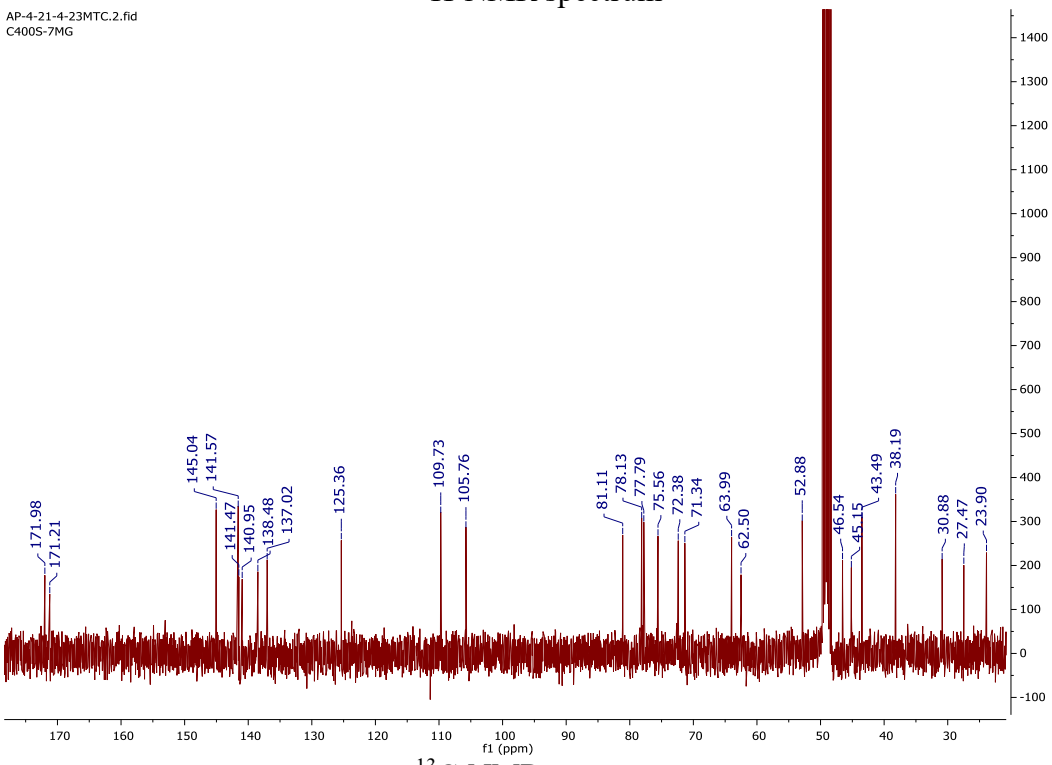


HMBC spectrum

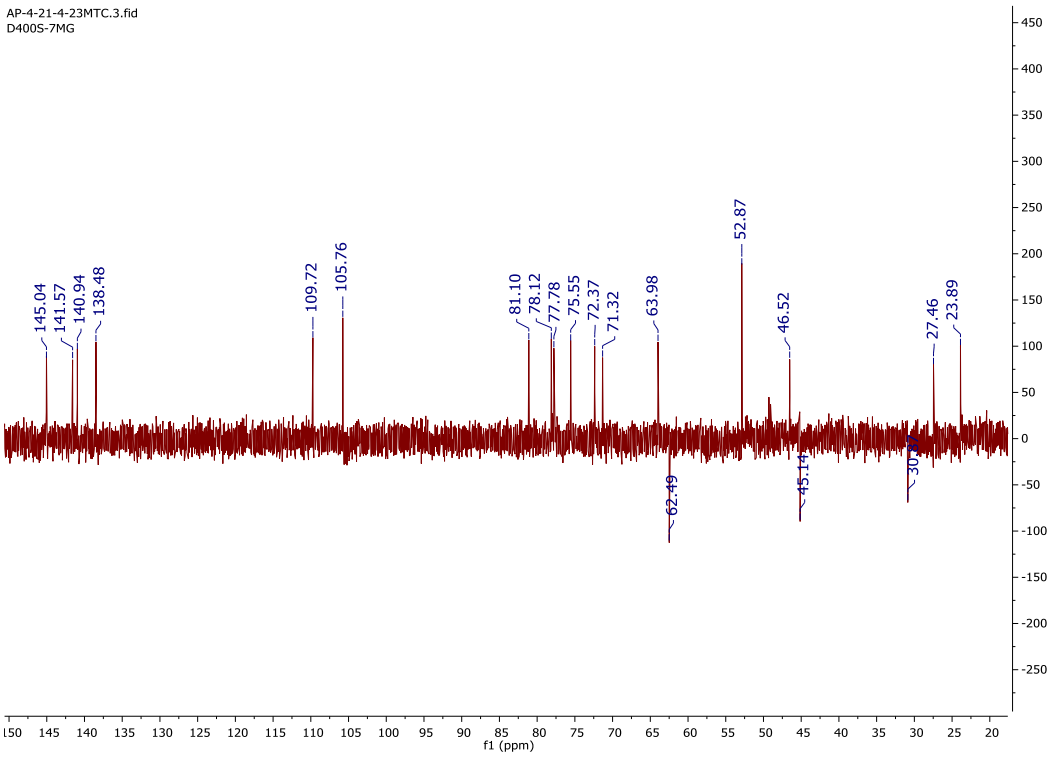
SI 8 1D and 2D NMR data of *(2R,5R,6S,9S,10S,12S)*-15,16-epoxy-2-hydroxy-6-O- (β-D-glucopypranosyl)-cleroda-3,7,13(16),14-tetraen-17,12-olid-18-oic acid methyl ester



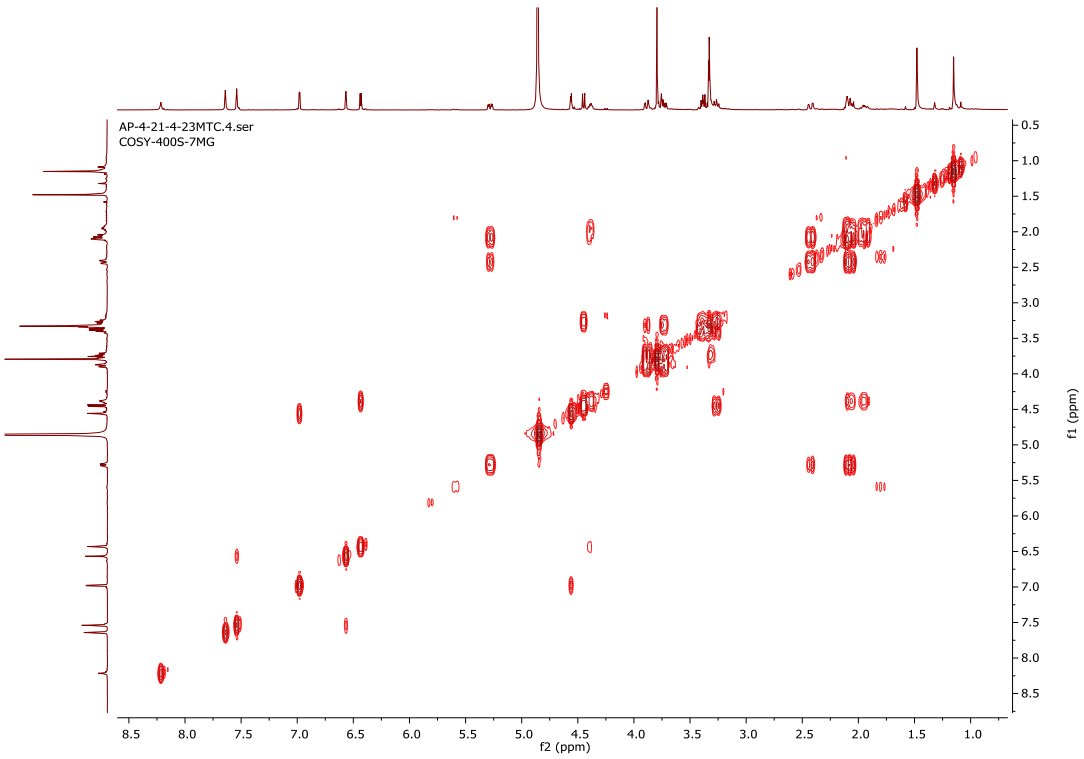
^{1}H-NMR spectrum



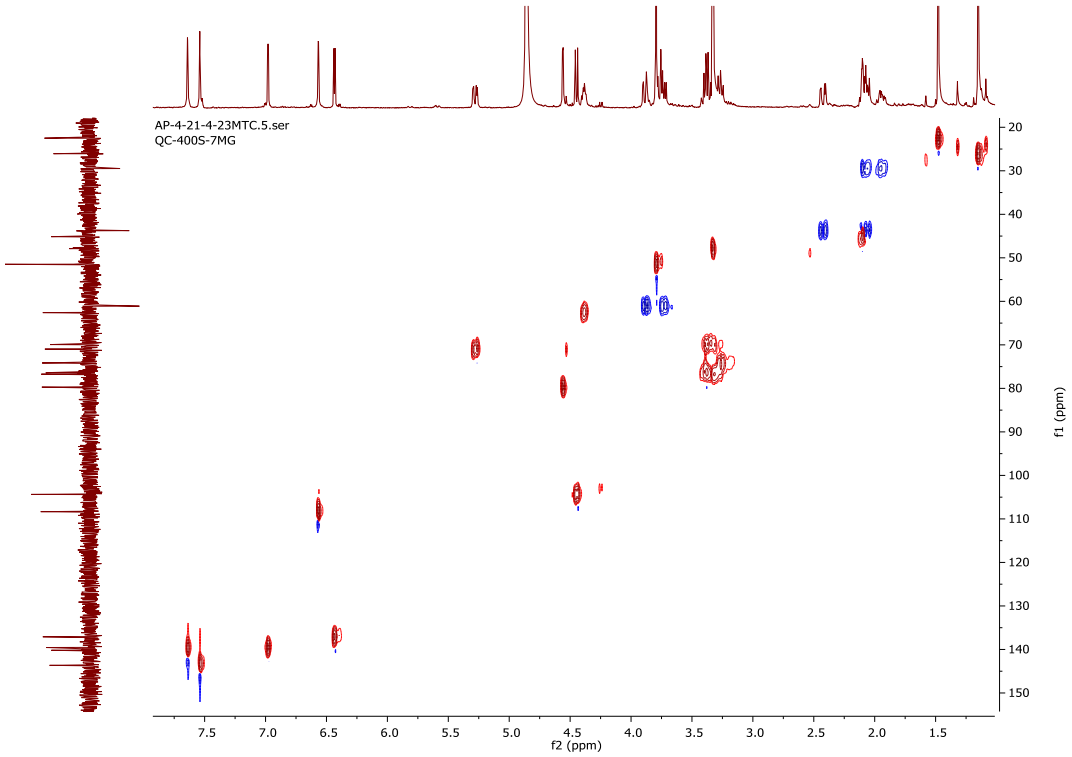
^{13}C-NMR spectrum



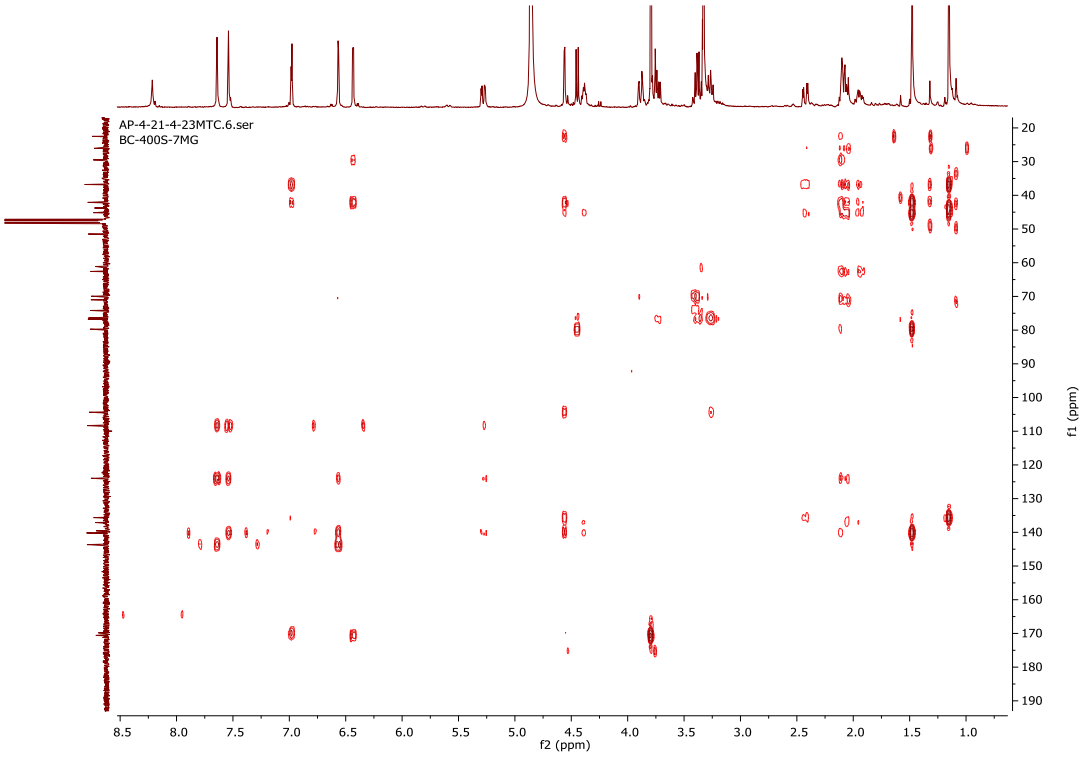
DEPT135 spectrum



COSY spectrum

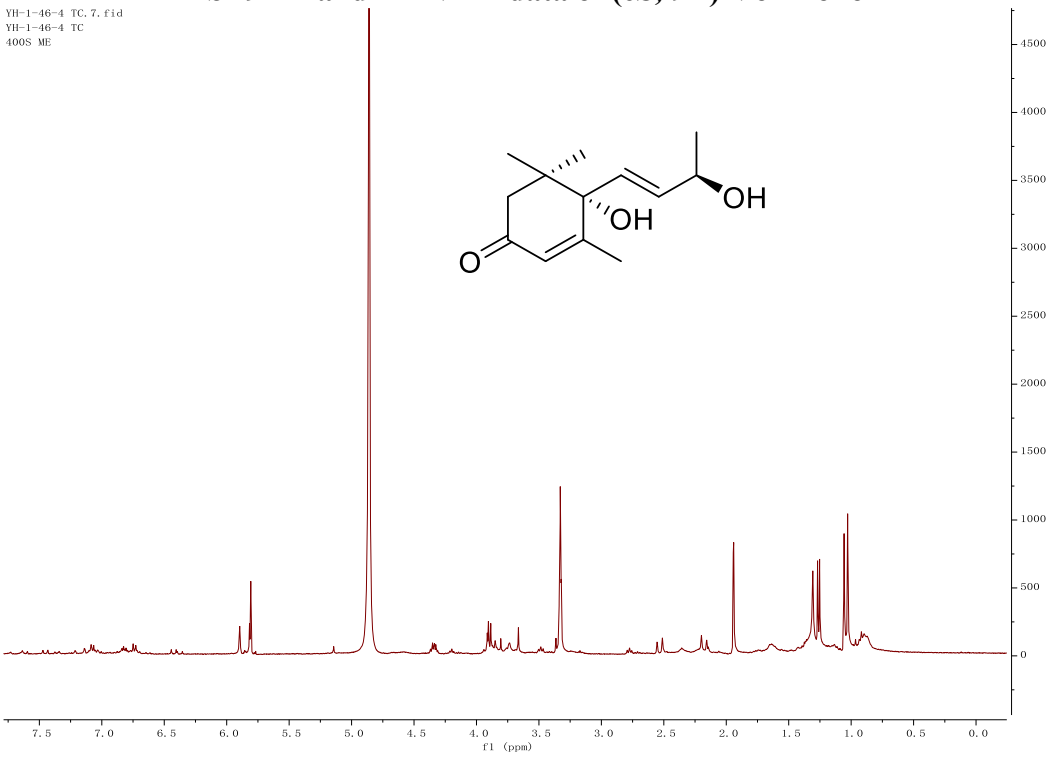


HSQC spectrum

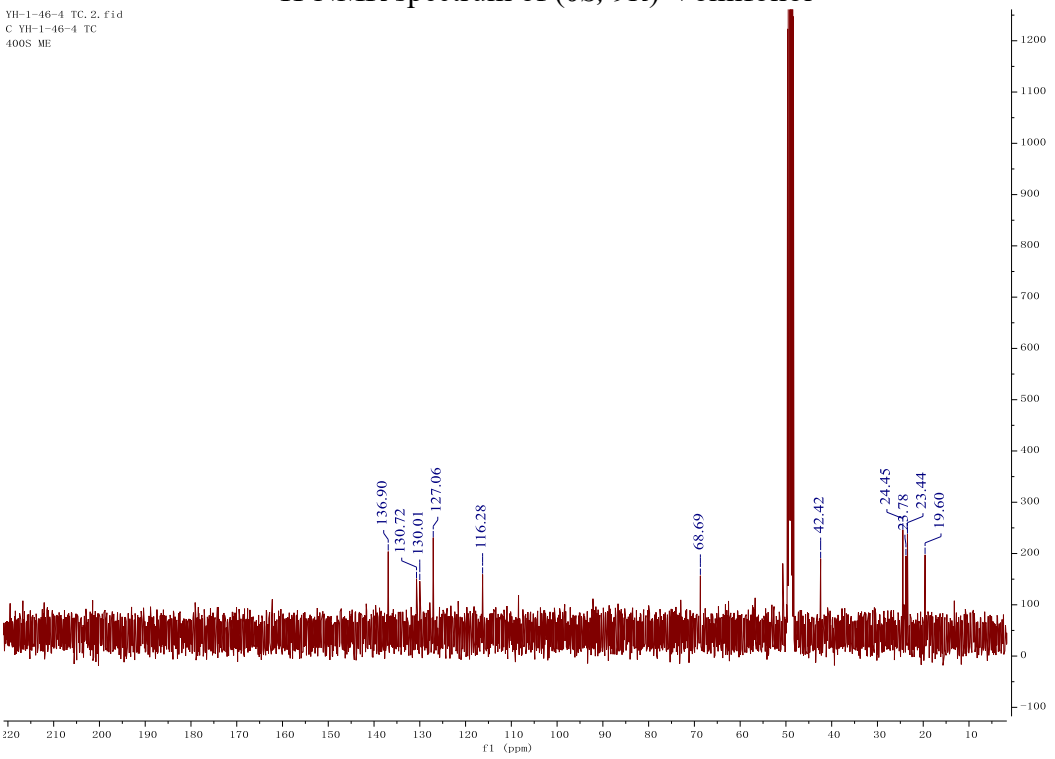


HMBC spectrum

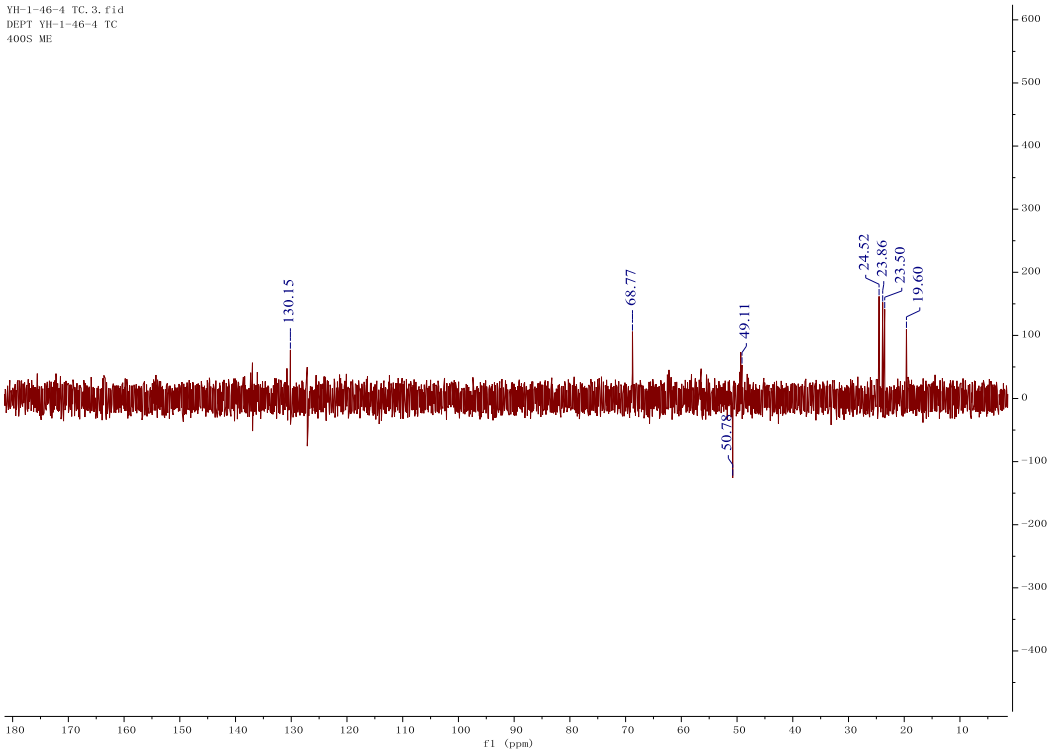
SI 9 1D and 2D NMR data of (*6S, 9R*)-Vomifoliol



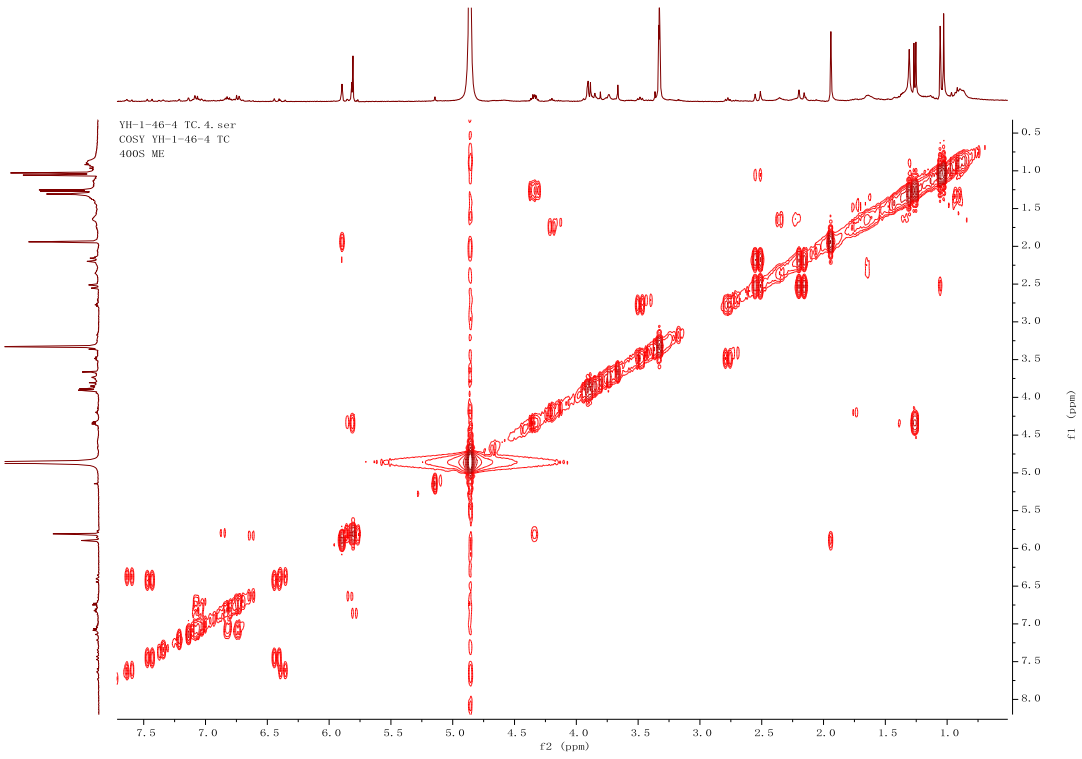
^{1}H-NMR spectrum of (*6S, 9R*)-Vomifoliol



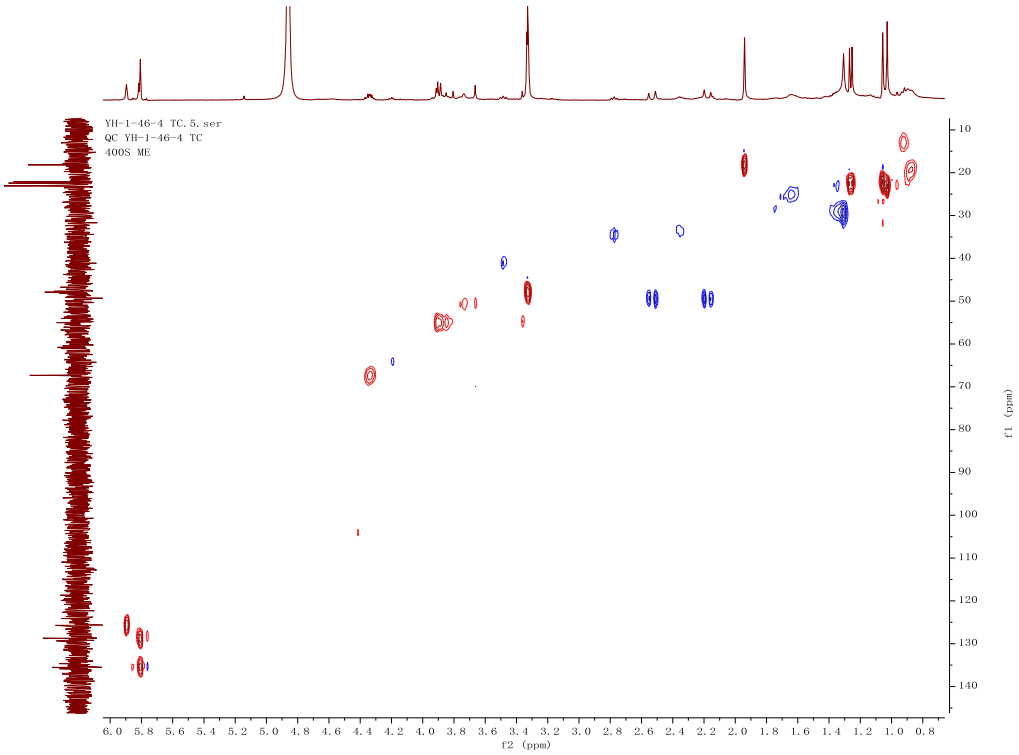
^{13}C-NMR spectrum of (*6S, 9R*)-Vomifoliol



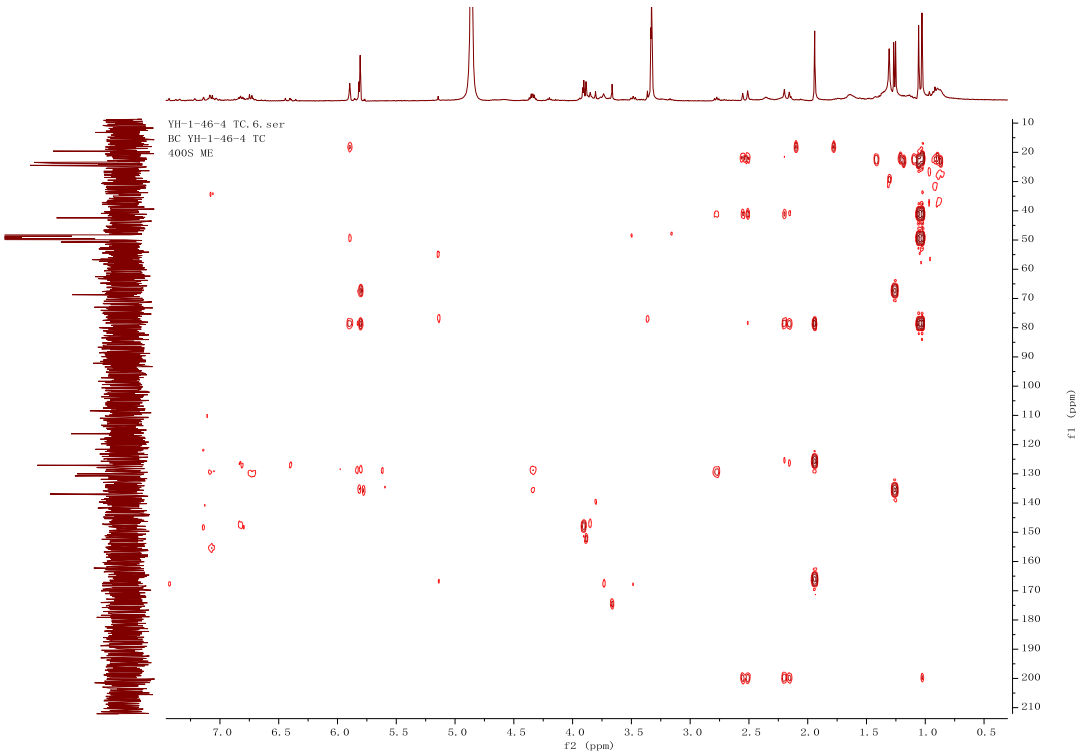
DEPT135 spectrum of (*6S, 9R*)-Vomifoliol



COSY spectrum of (*6S, 9R*)-Vomifoliol

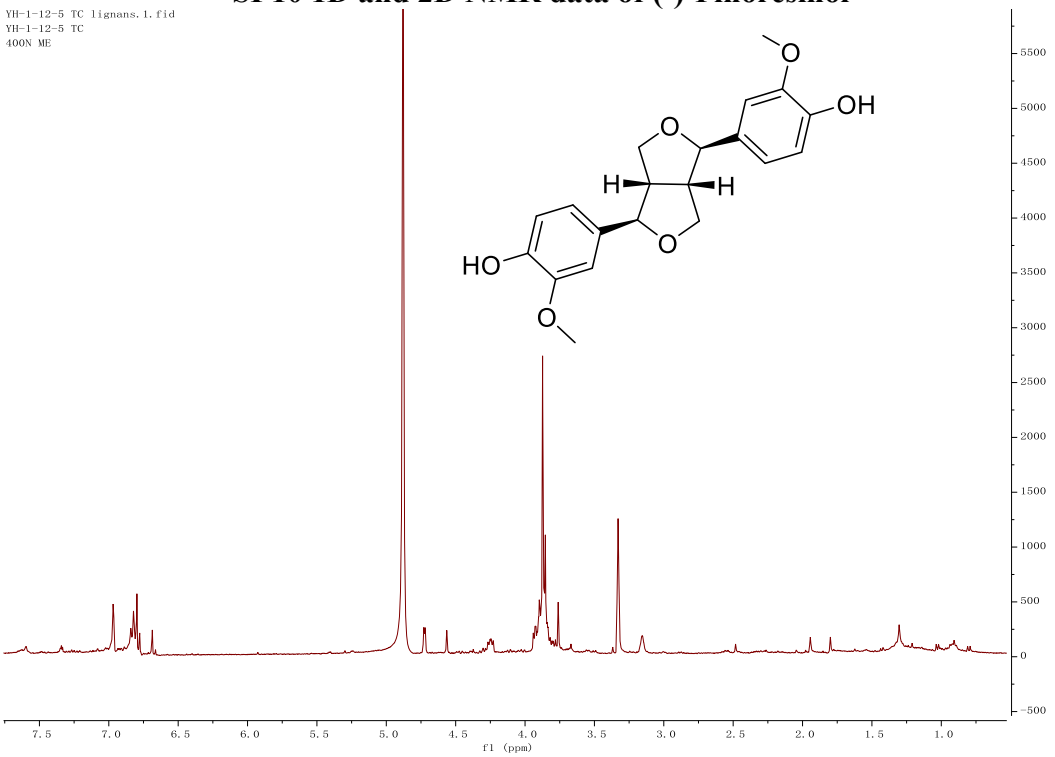


HSQC spectrum of (*6S, 9R*)-Vomifoliol

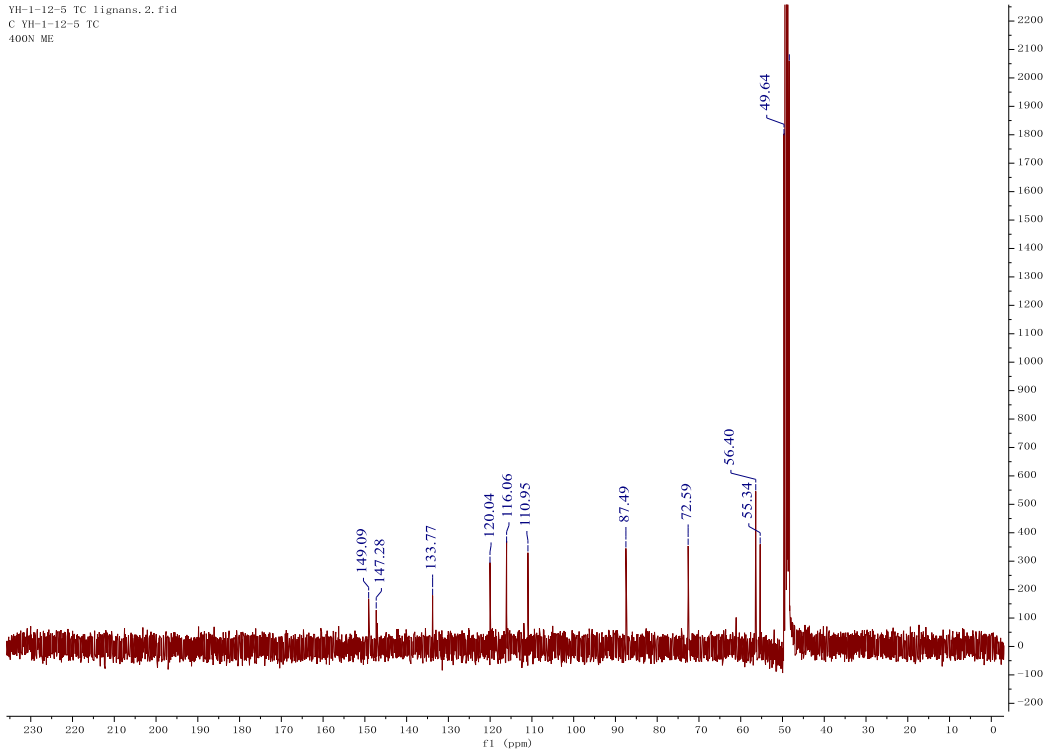


HMBC spectrum of (*6S, 9R*)-Vomifoliol

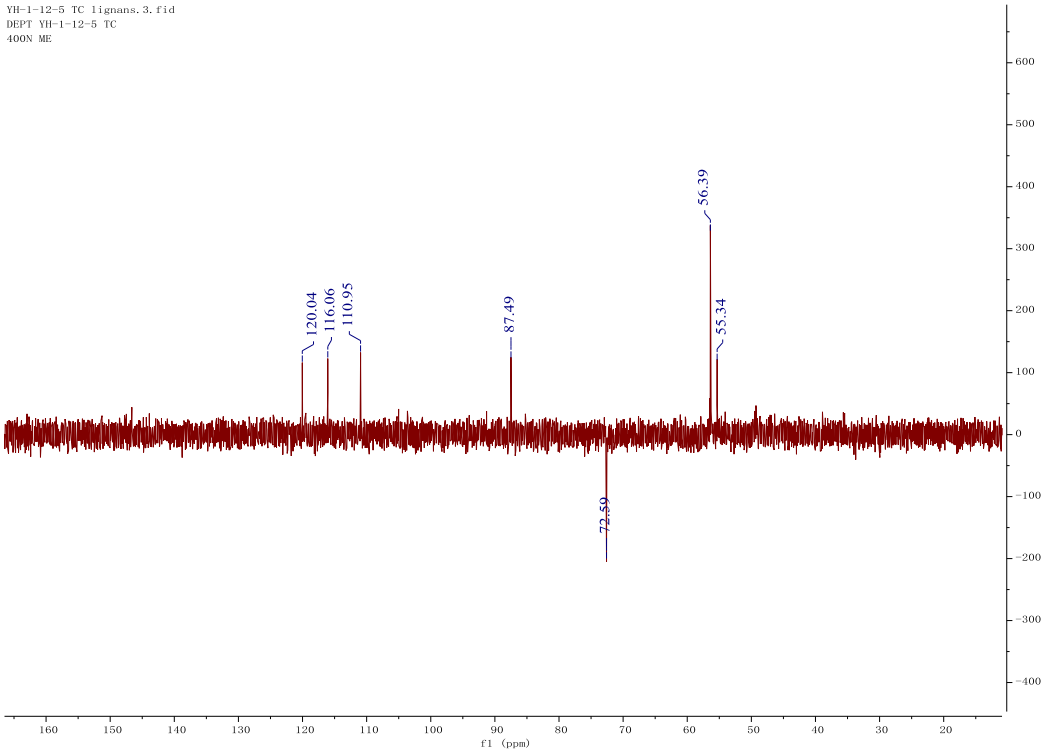
SI 10 1D and 2D NMR data of (-)-Pinoresinol



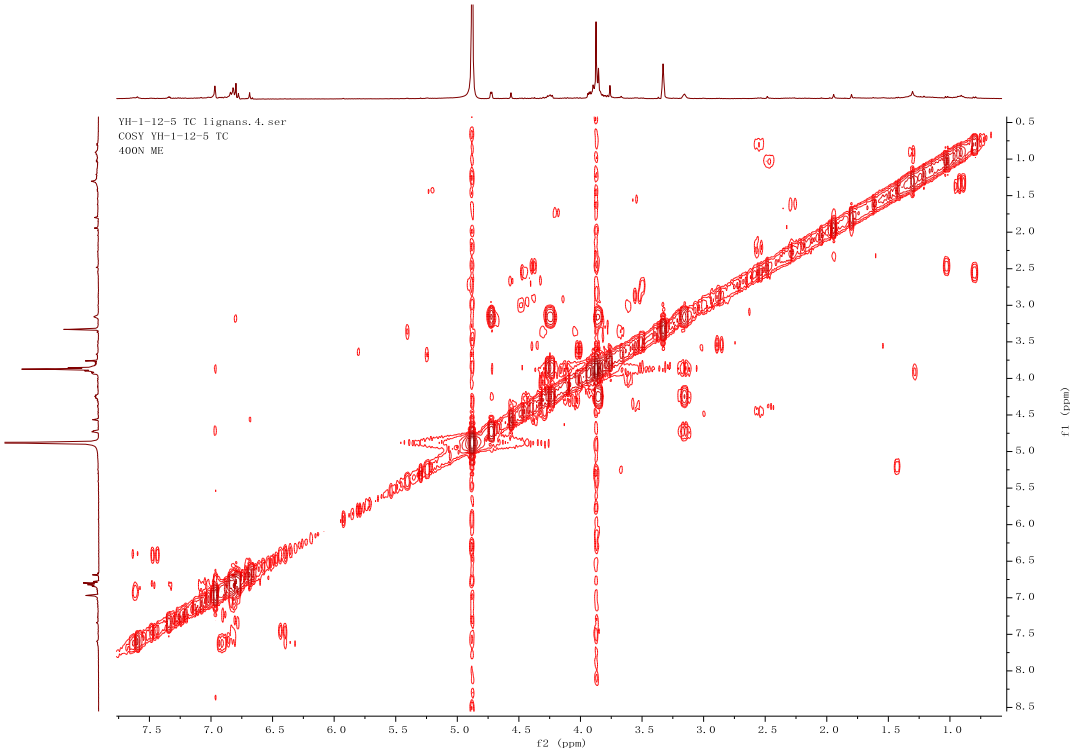
^{1}H-NMR spectrum of (-)-Pinoresinol



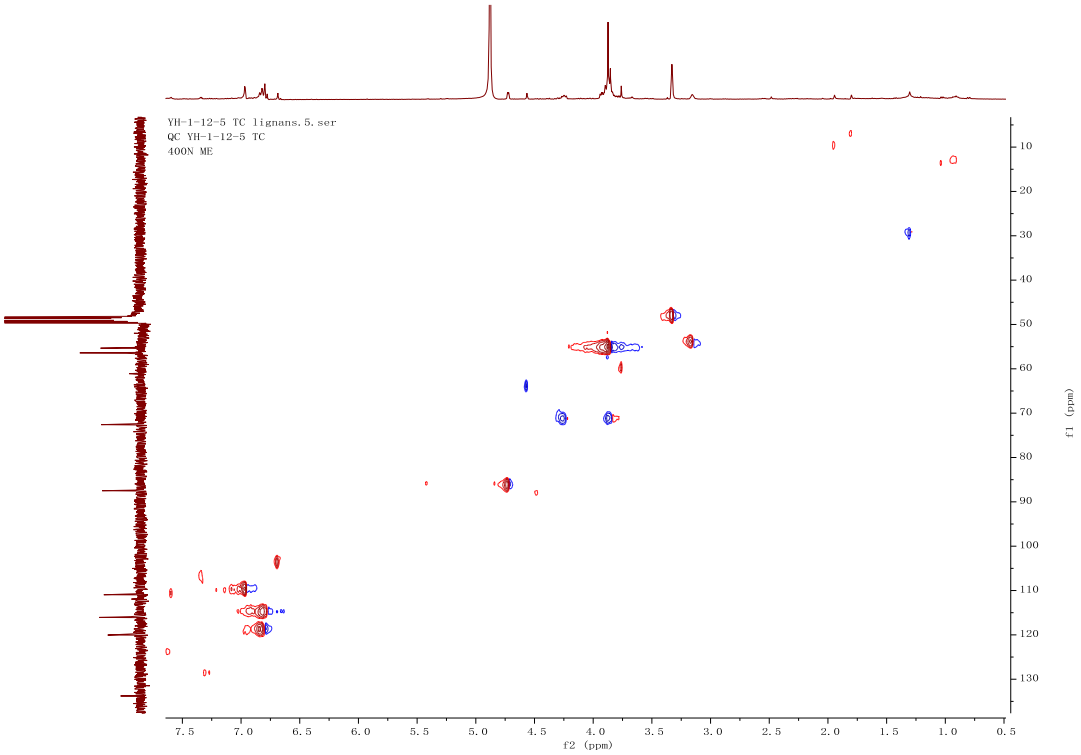
^{13}C-NMR spectrum of (-)-Pinoresinol



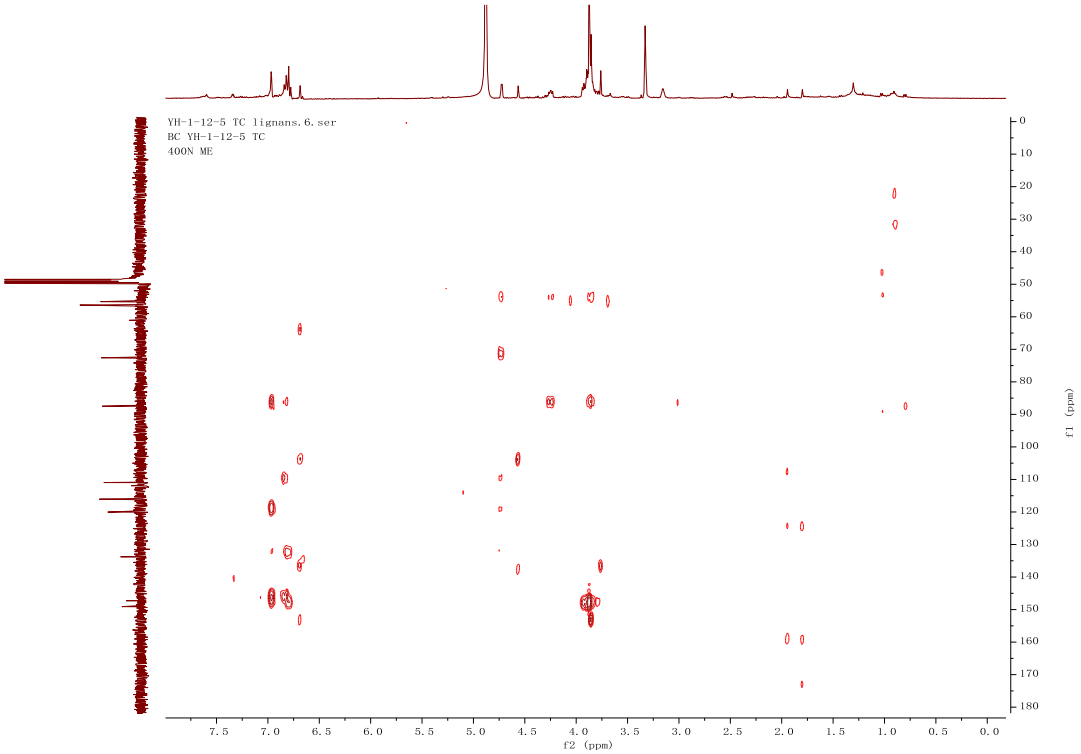
DEPT135 spectrum of (-)-Pinoresinol



COSY spectrum of (-)-Pinoresinol

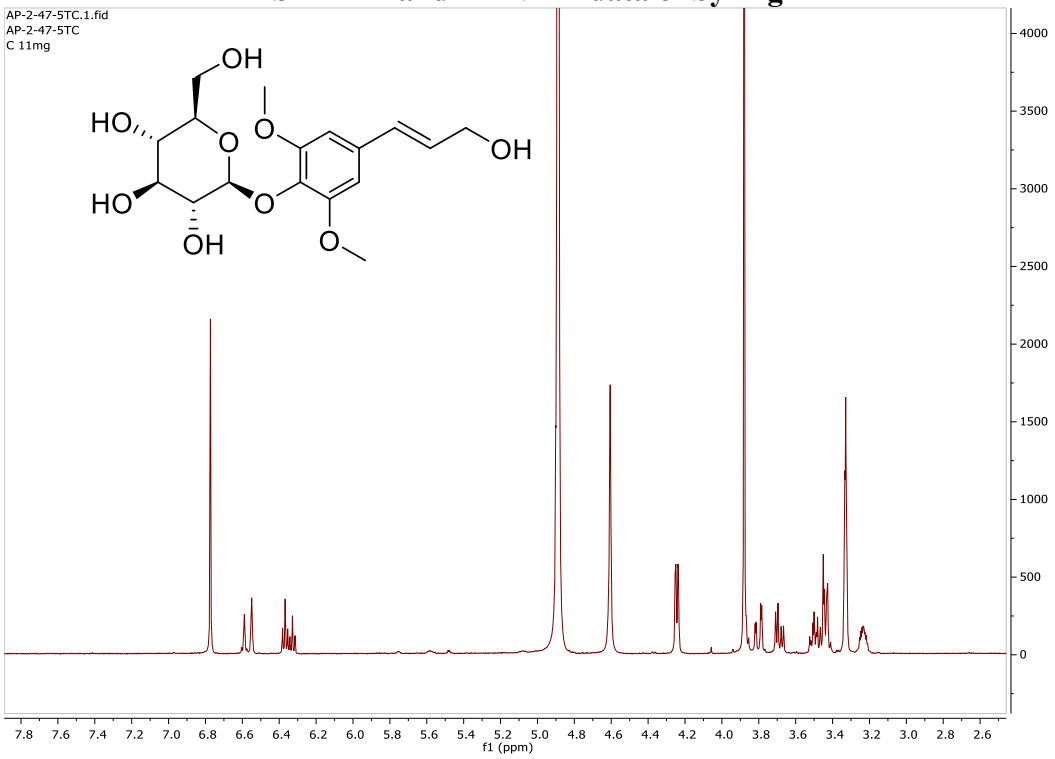


HSQC spectrum of (-)-Pinoresinol

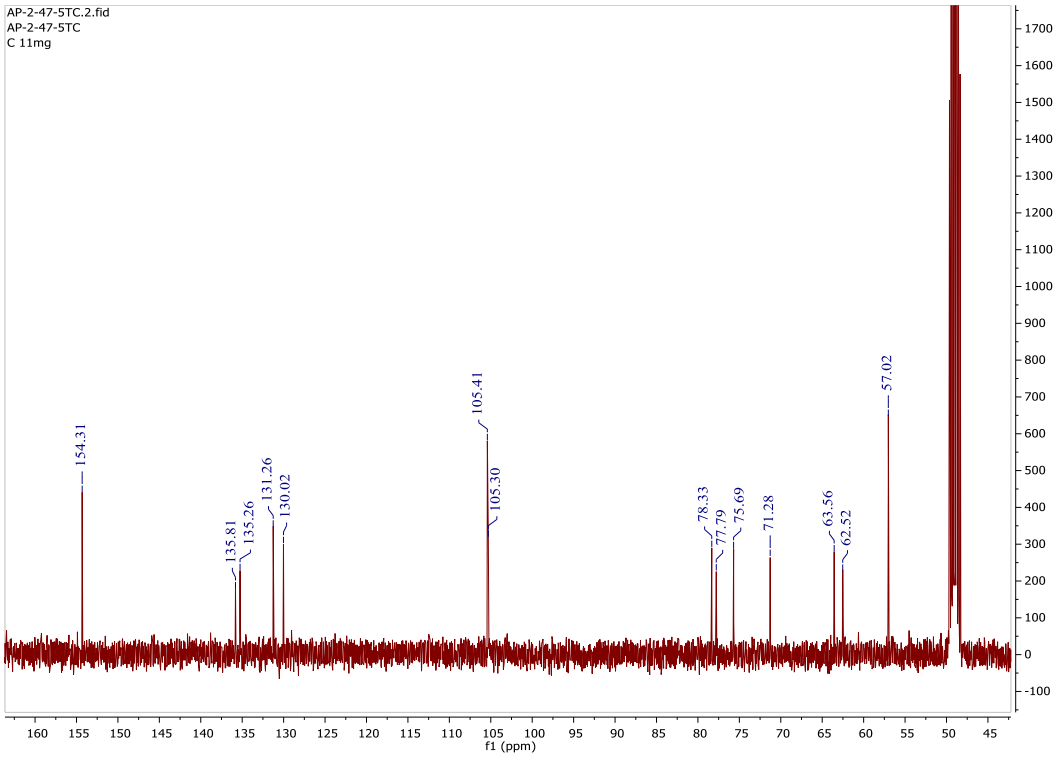


HMBC spectrum of (-)-Pinoresinol

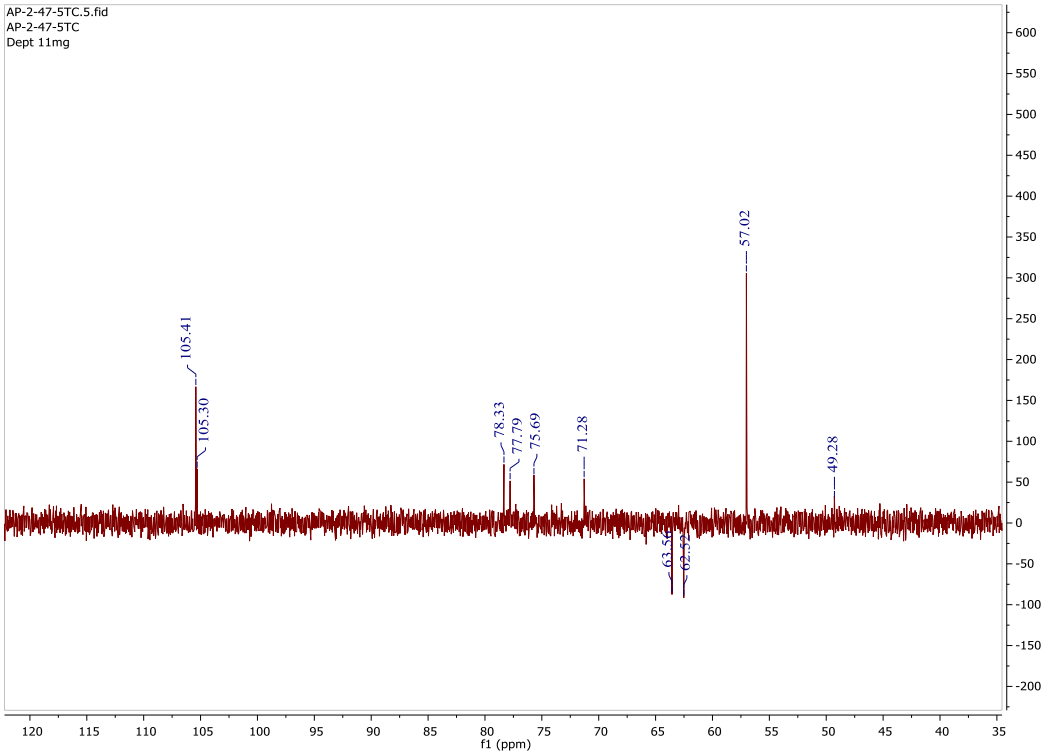
SI 11 1D and 2D NMR data of Syringin



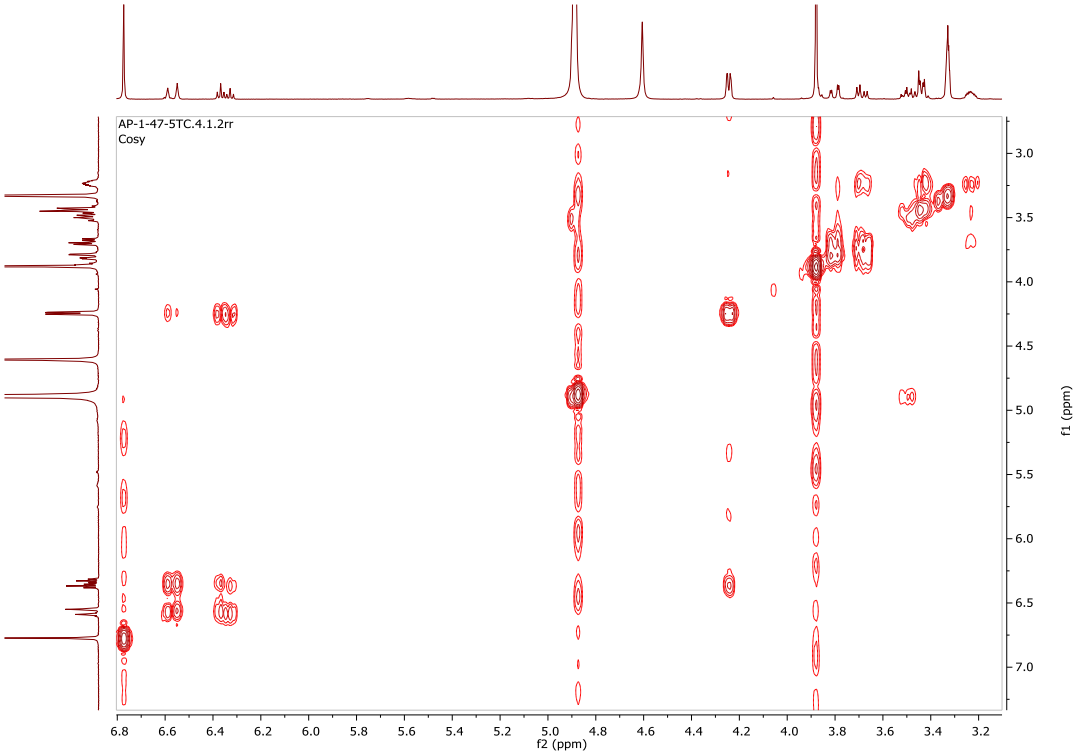
^{1}H-NMR spectrum of Syringin



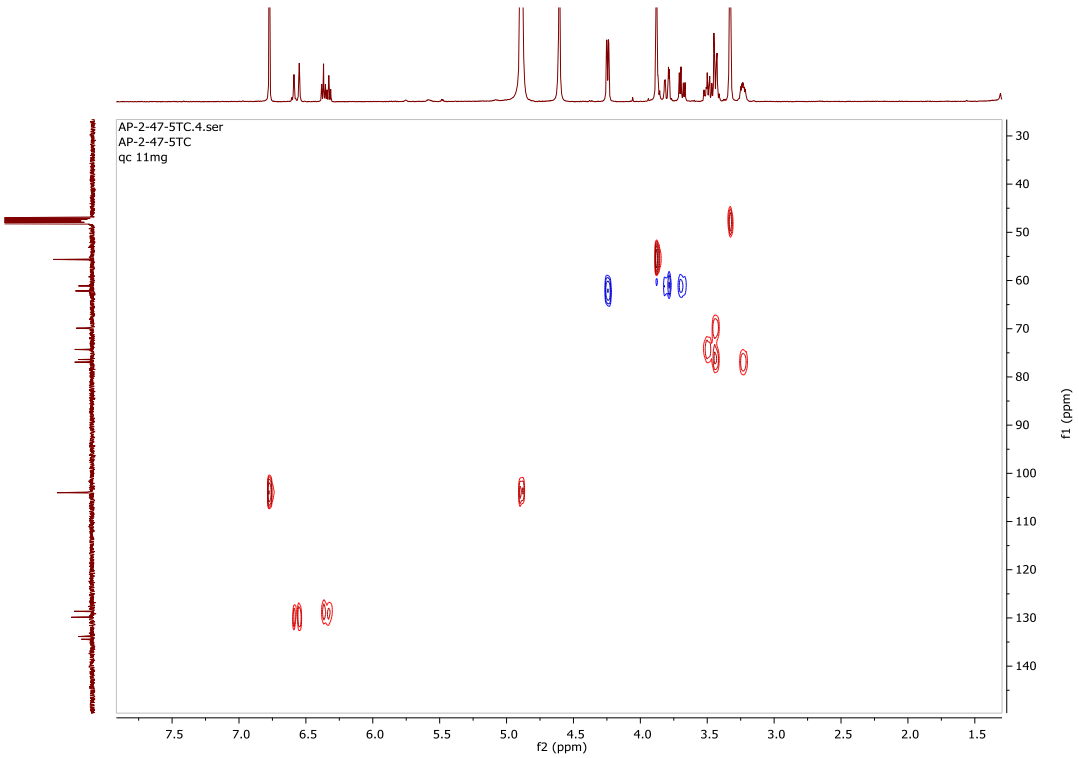
^{13}C-NMR spectrum of Syringin



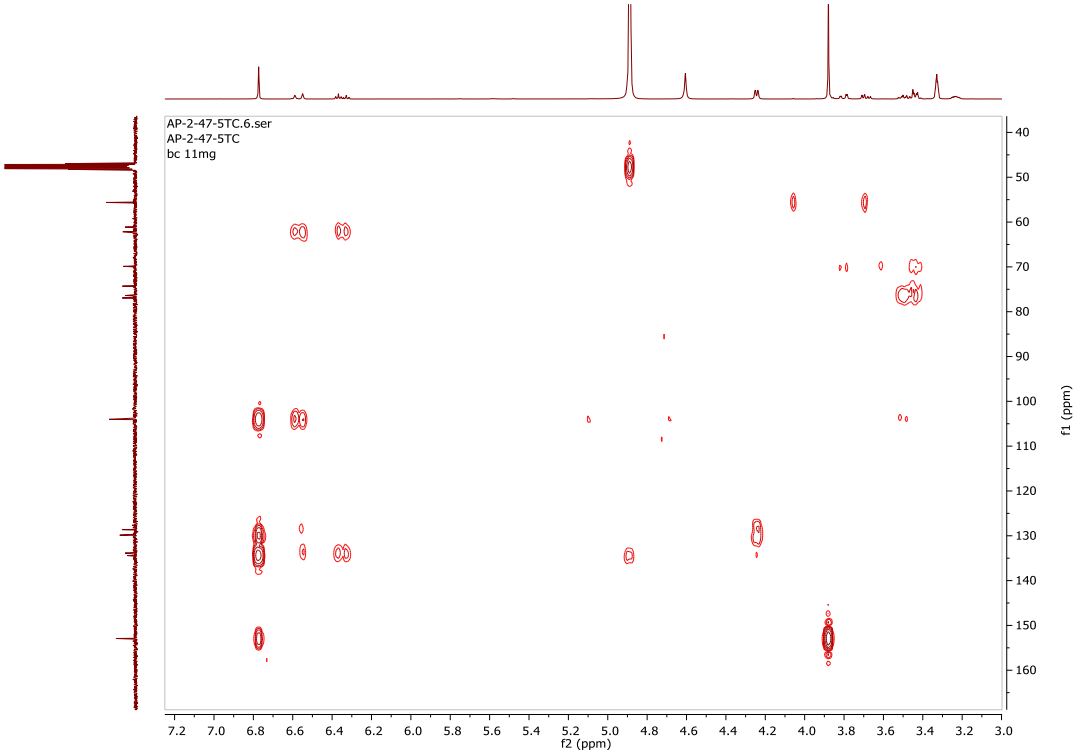
DEPT135 spectrum of Syringin



COSY spectrum of Syringin

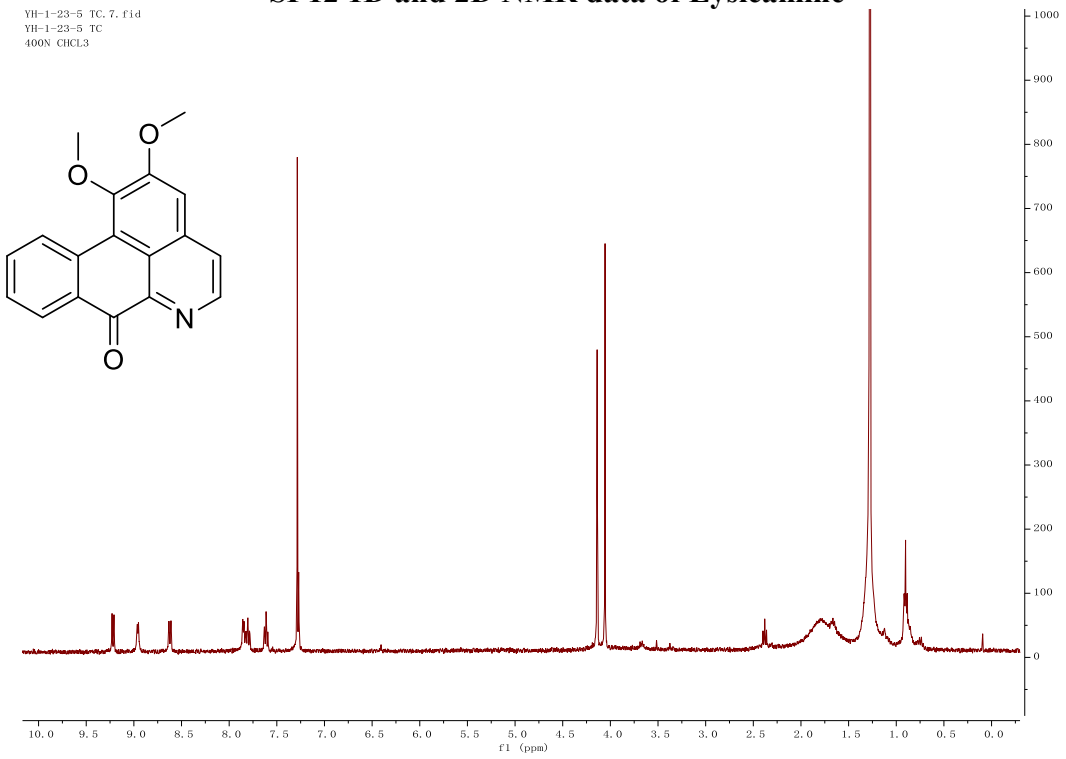


HSQC spectrum of Syringin

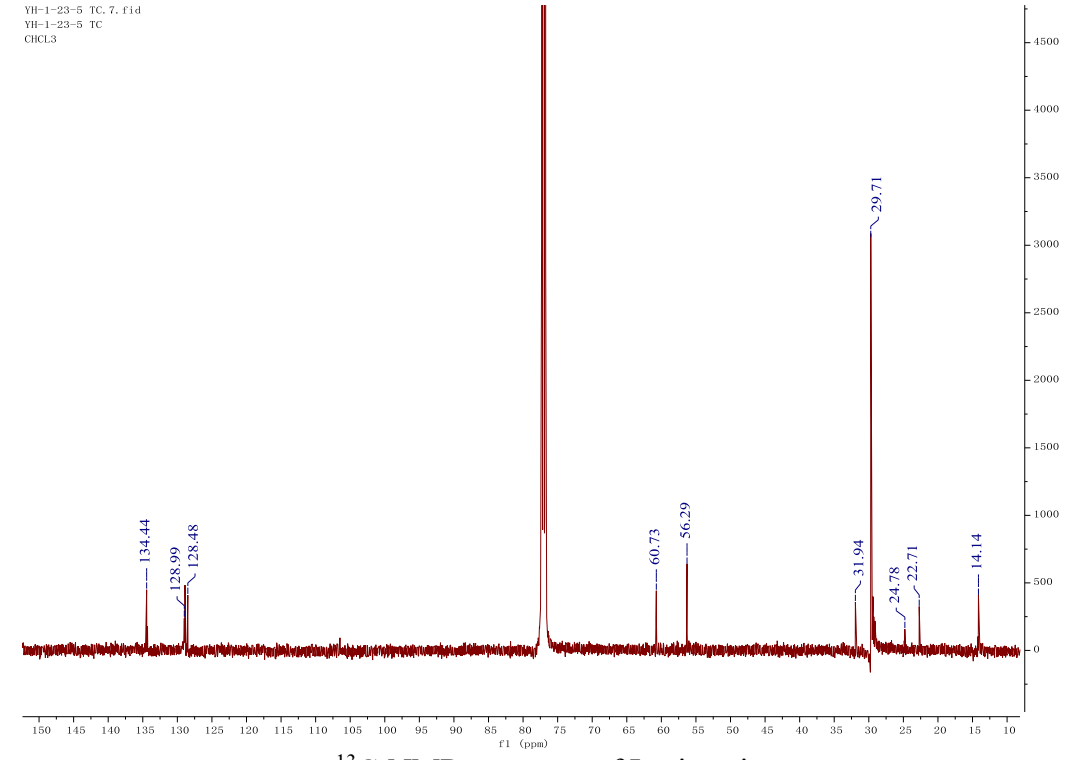


HMBC spectrum of Syringin

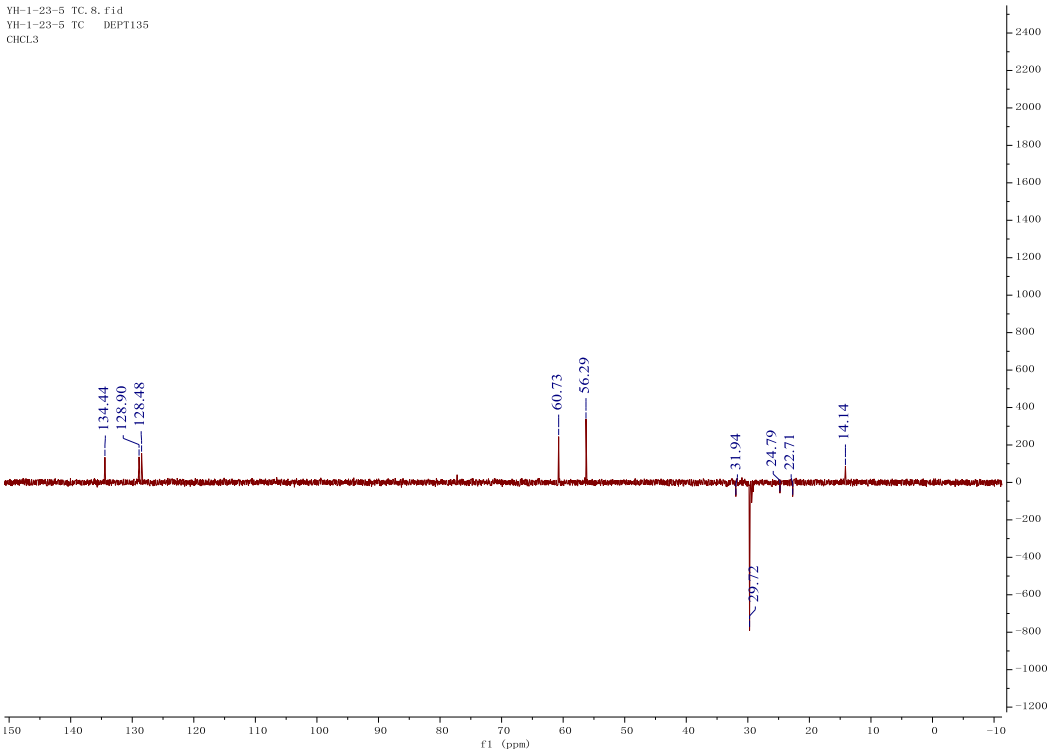
SI 12 1D and 2D NMR data of Lysicamine



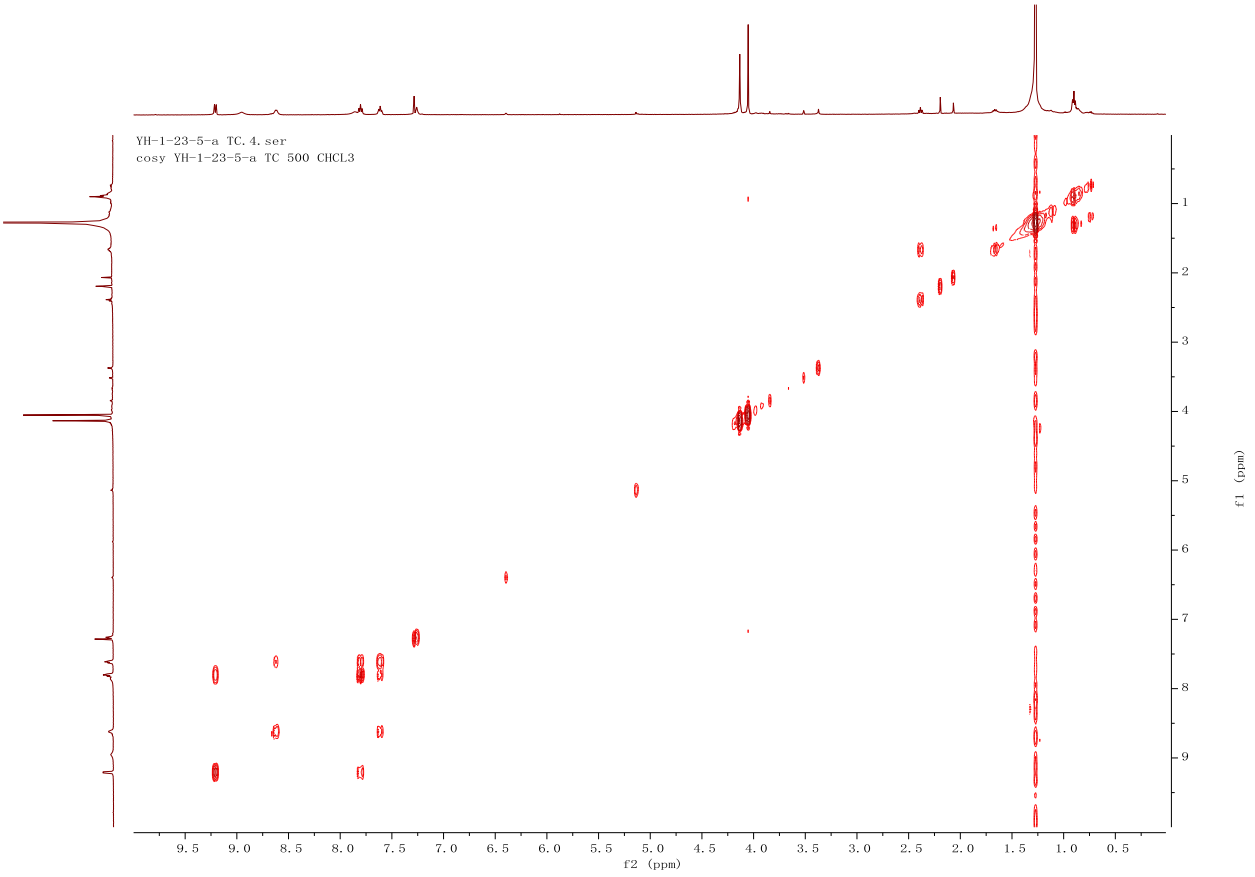
^{1}H-NMR spectrum of Lysicamine



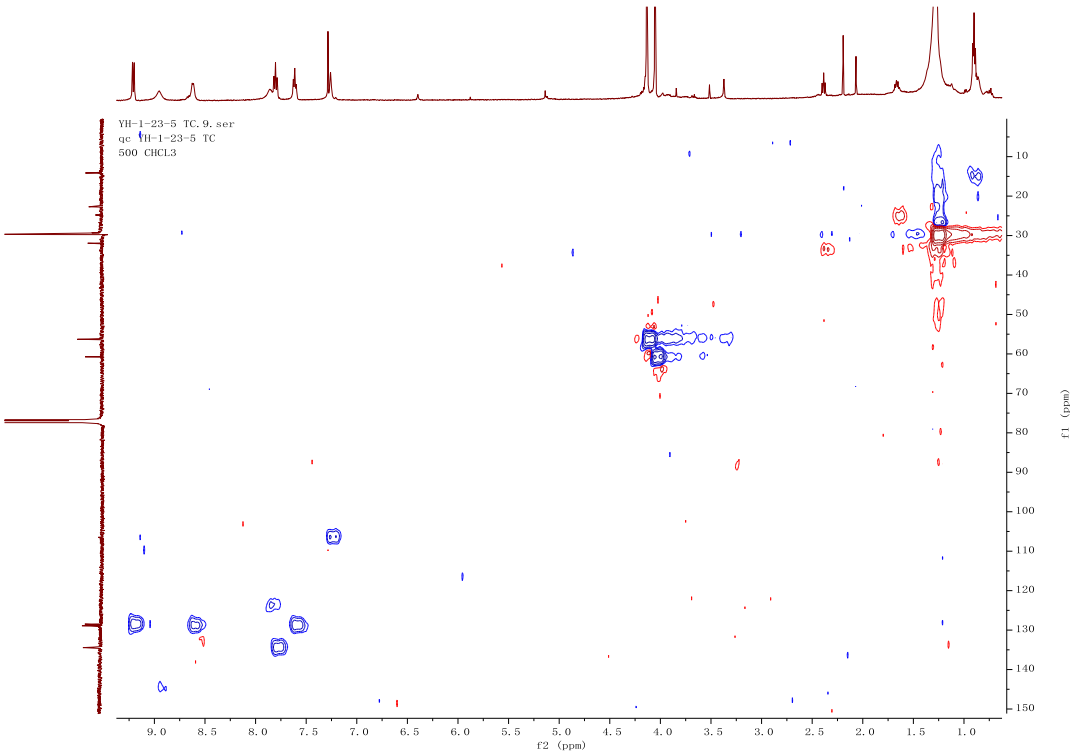
^{13}C-NMR spectrum of Lysicamine



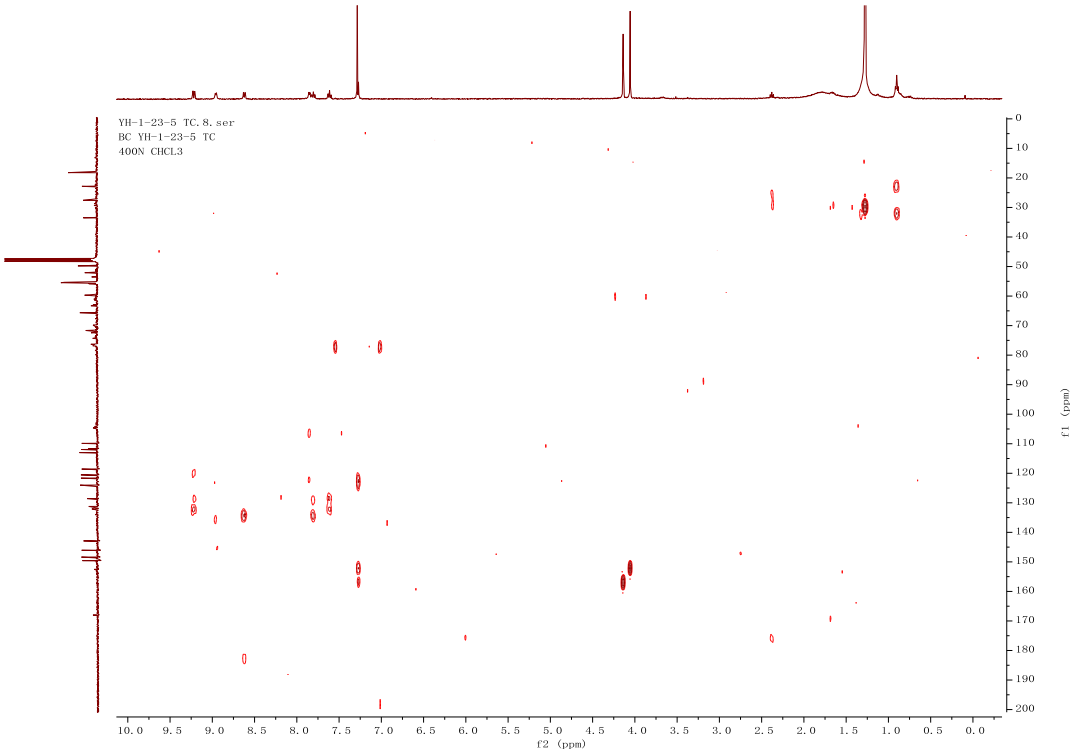
DEPT135 spectrum of Lysicamine



COSY spectrum of Lysicamine

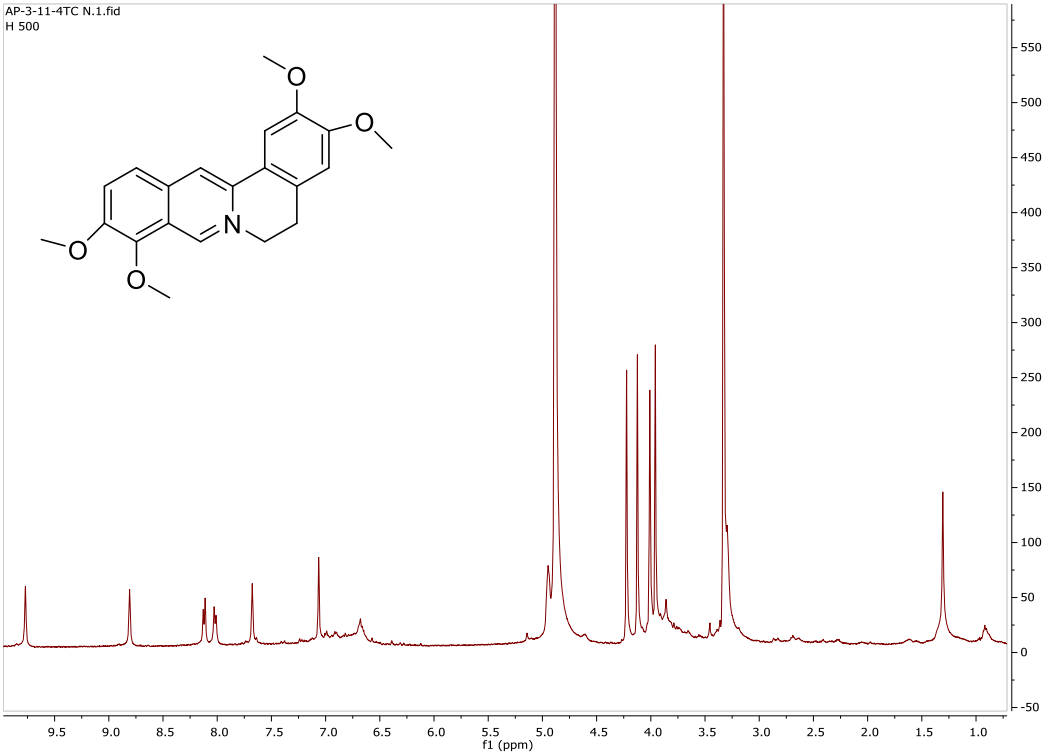


HMQC spectrum of Lysicamine

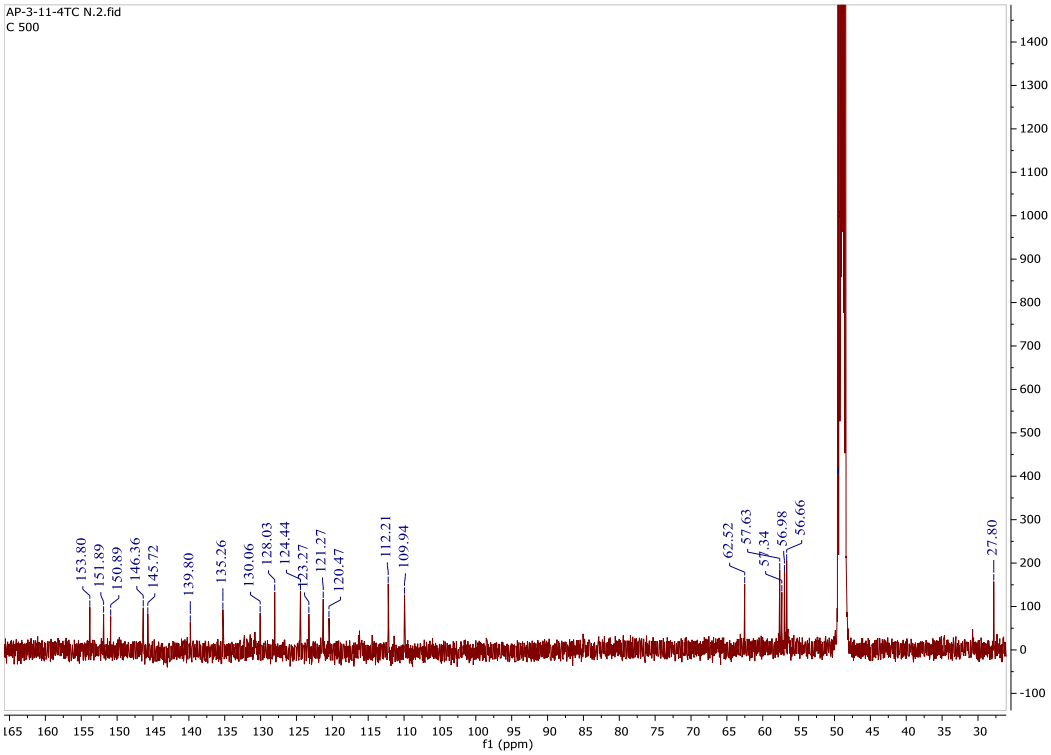


HMBC spectrum of Lysicamine

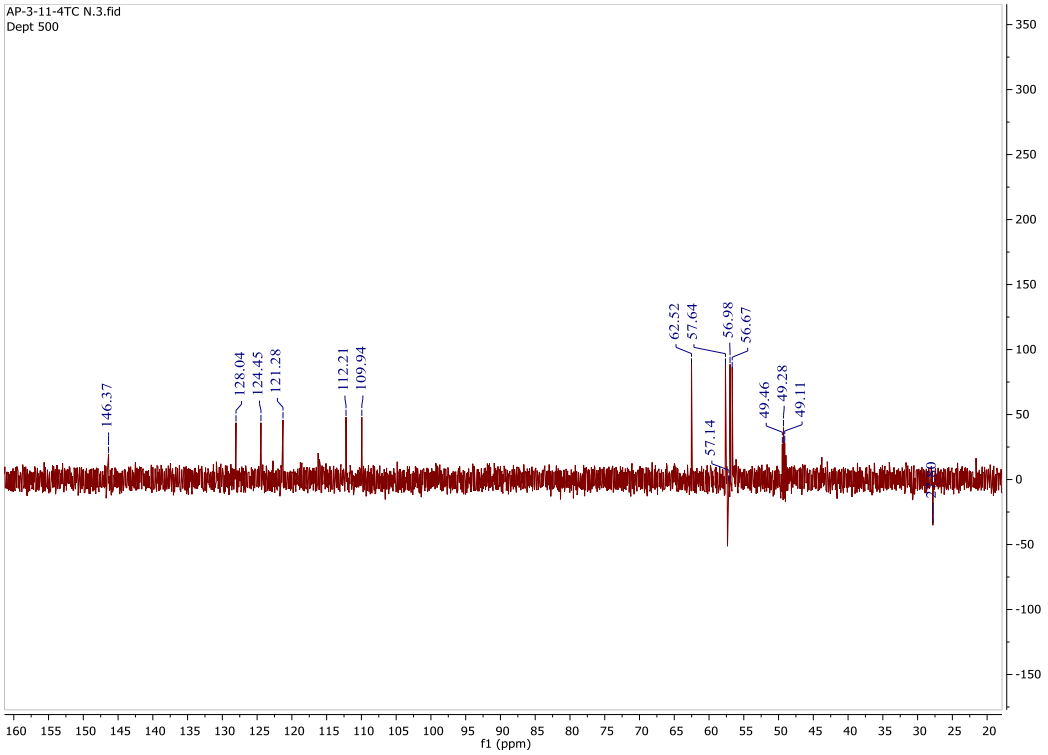
SI 13 1D and 2D NMR data of Palmatine



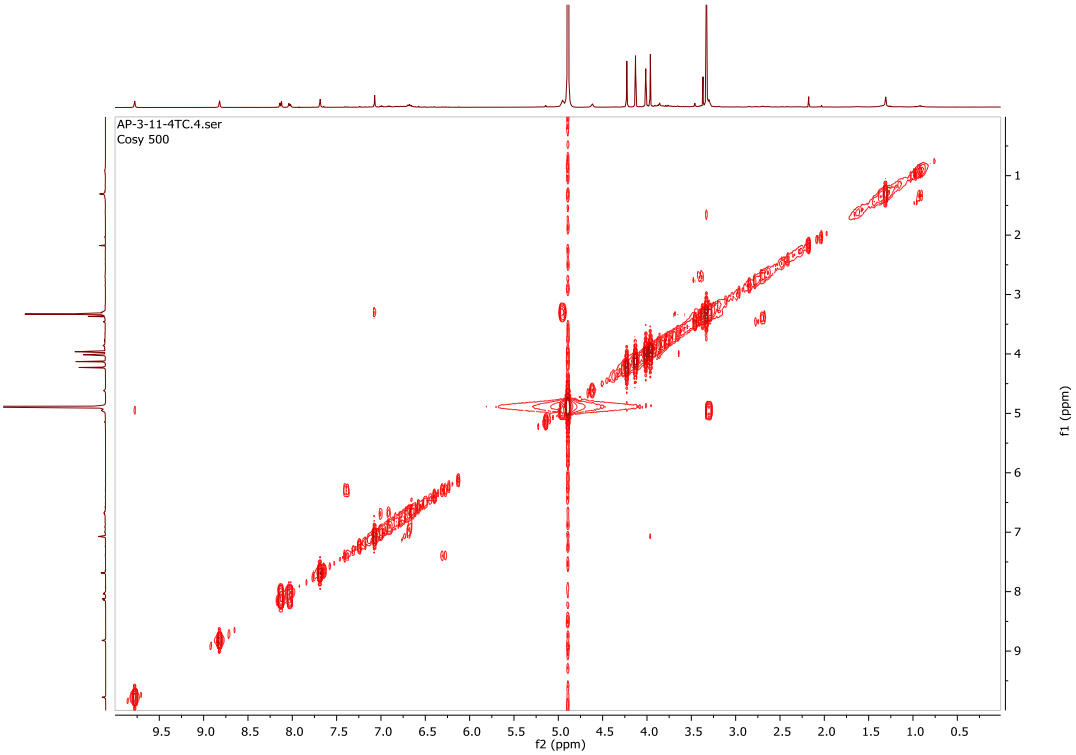
^{1}H-NMR spectrum of Palmatine



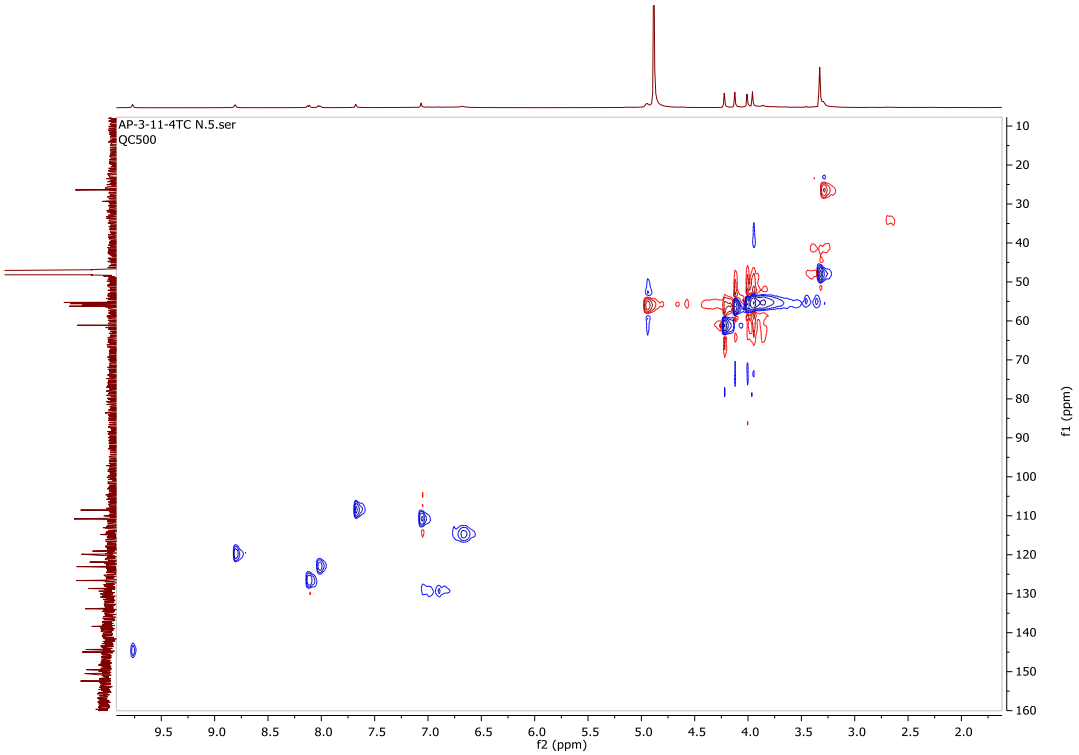
^{13}C-NMR spectrum of Palmatine



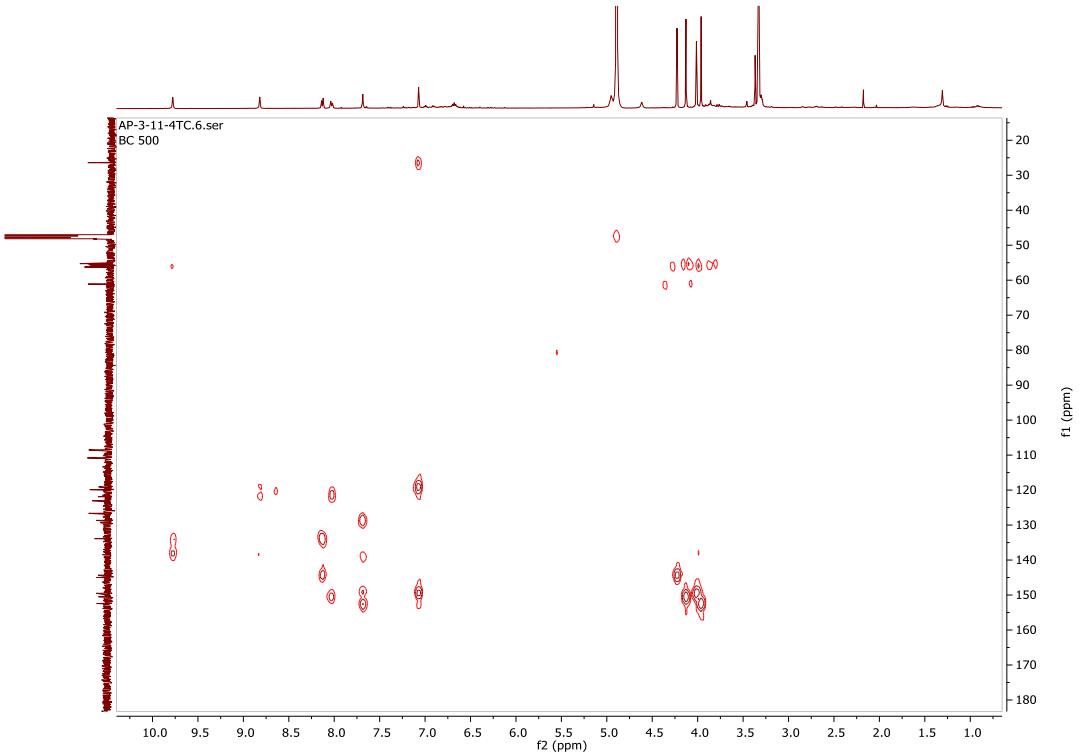
DEPT135 spectrum of Palmatine



COSY spectrum of Palmatine

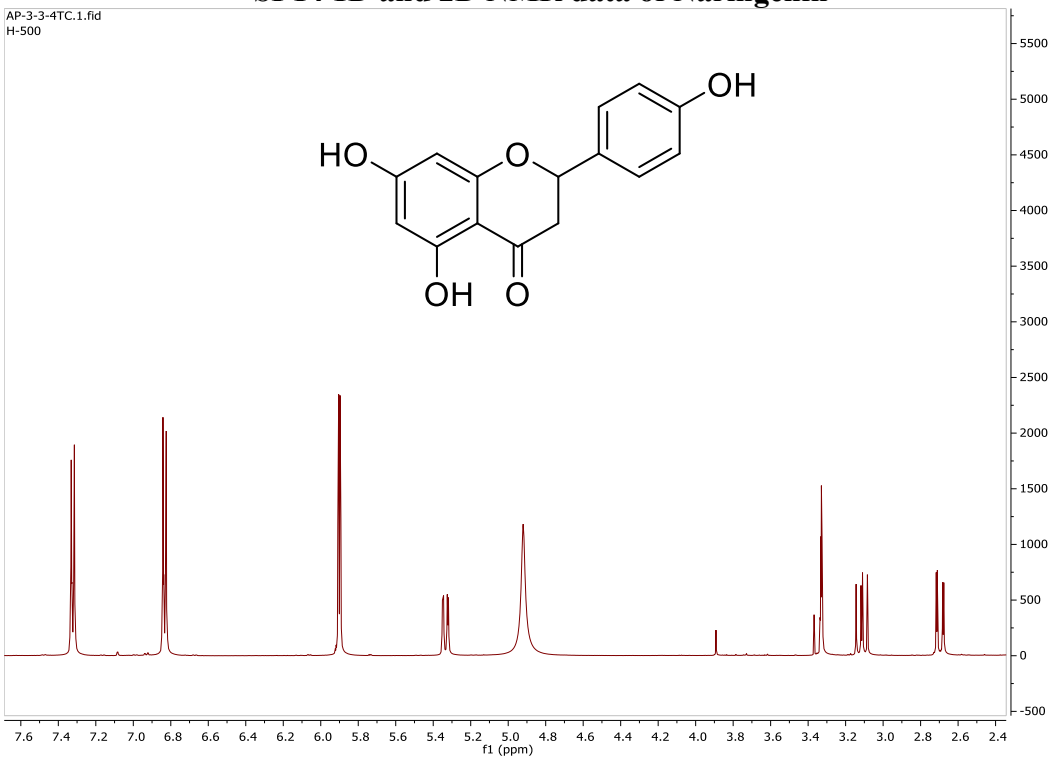


HSQC spectrum of Palmatine

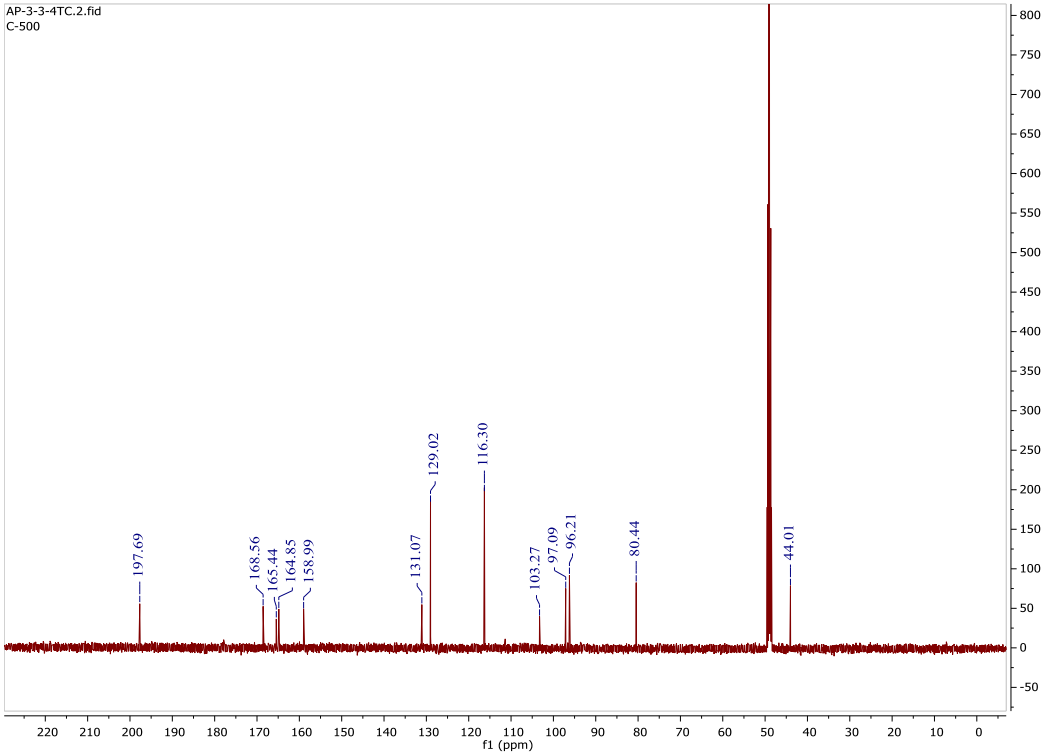


HMBC spectrum of Palmatine

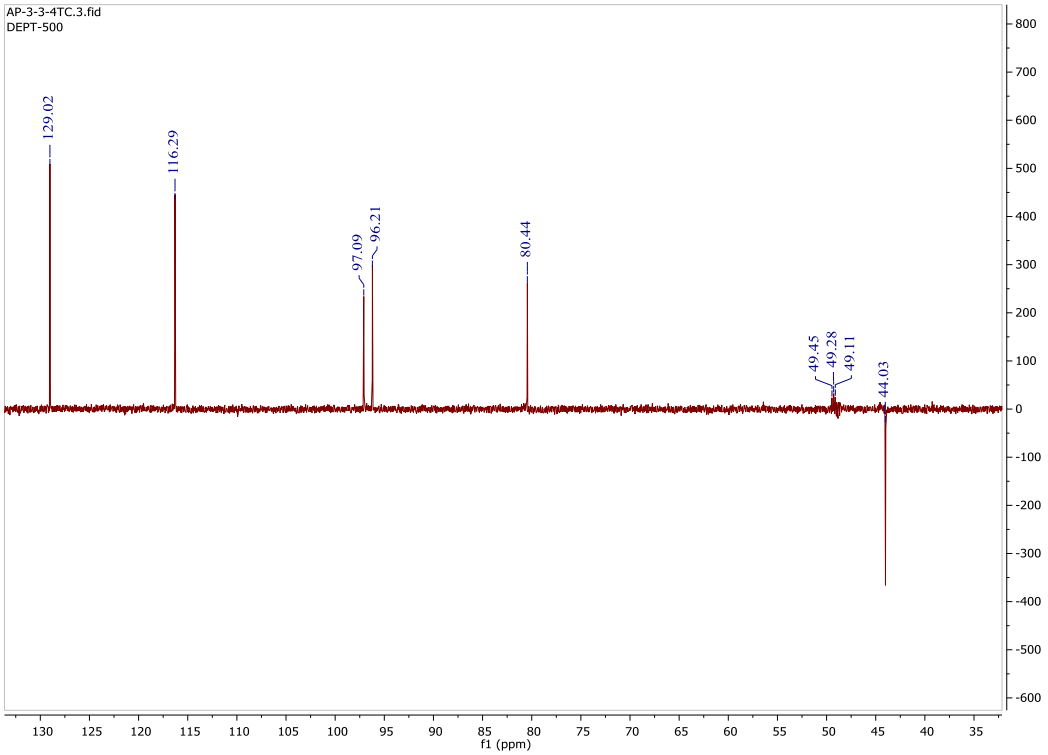
SI 14 1D and 2D NMR data of Naringenin



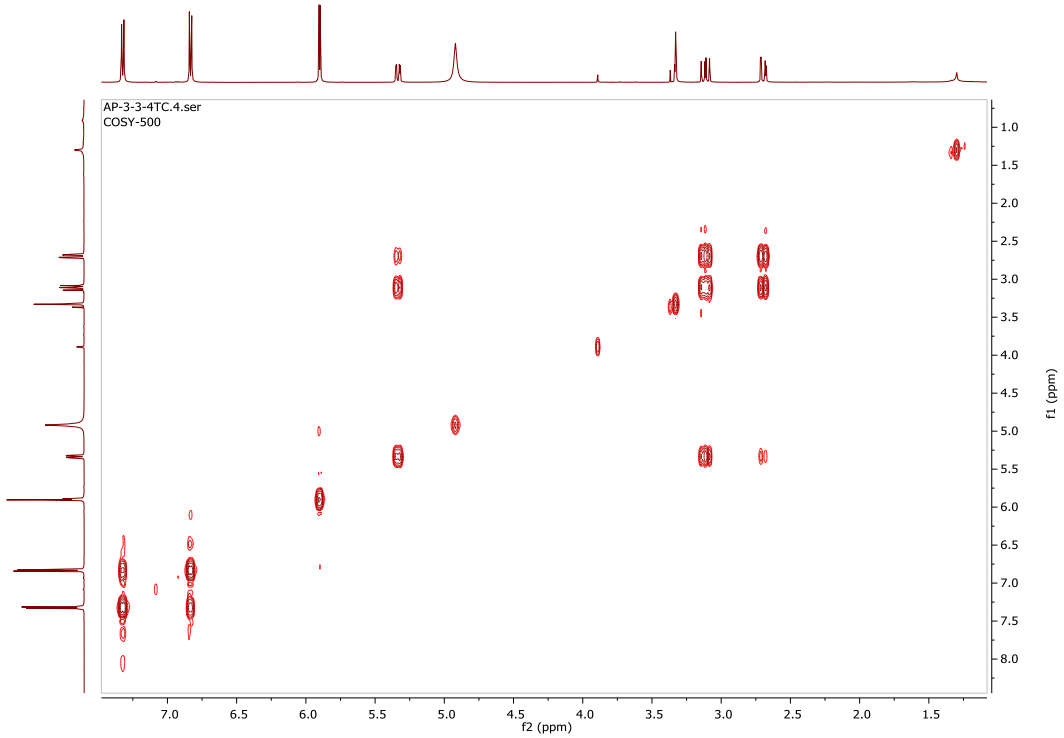
^{1}H-NMR spectrum of Naringenin



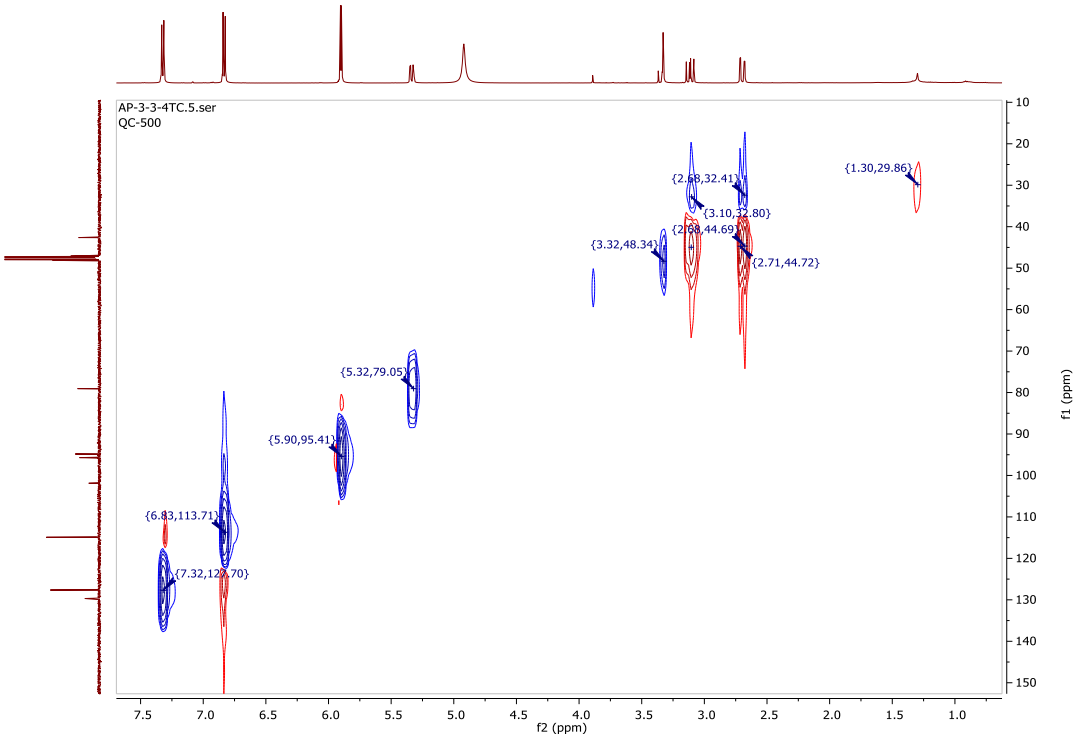
^{13}C- NMR spectrum of Naringenin



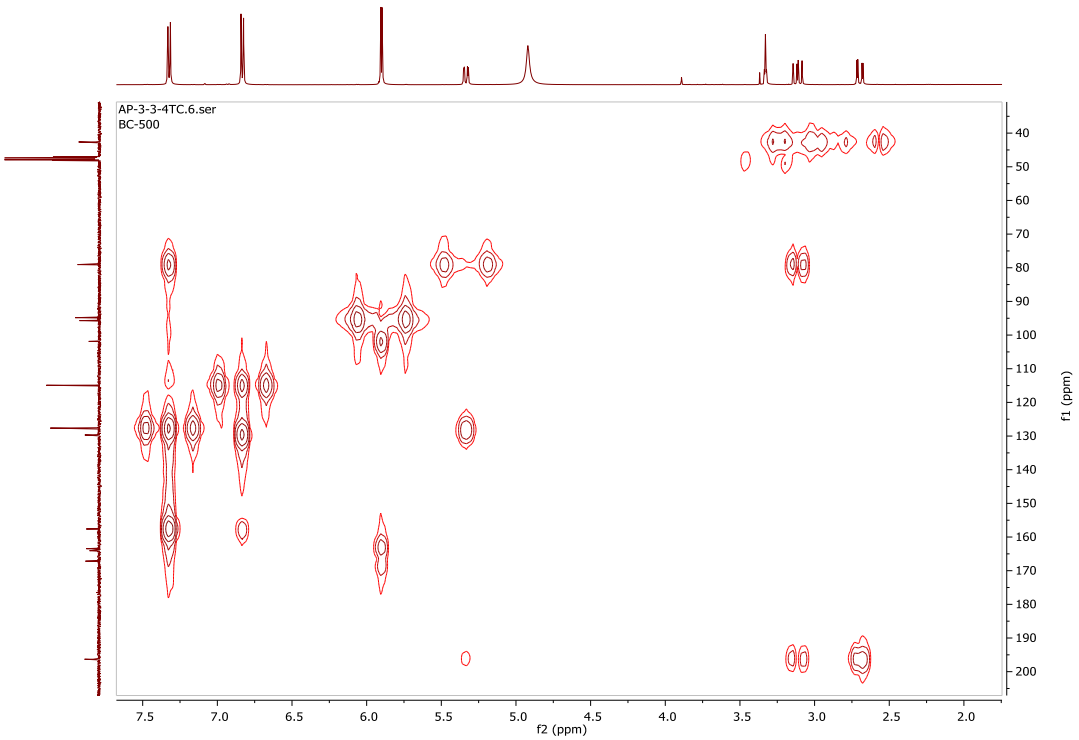
DEPT135 spectrum of Naringenin



COSY spectrum of Naringenin

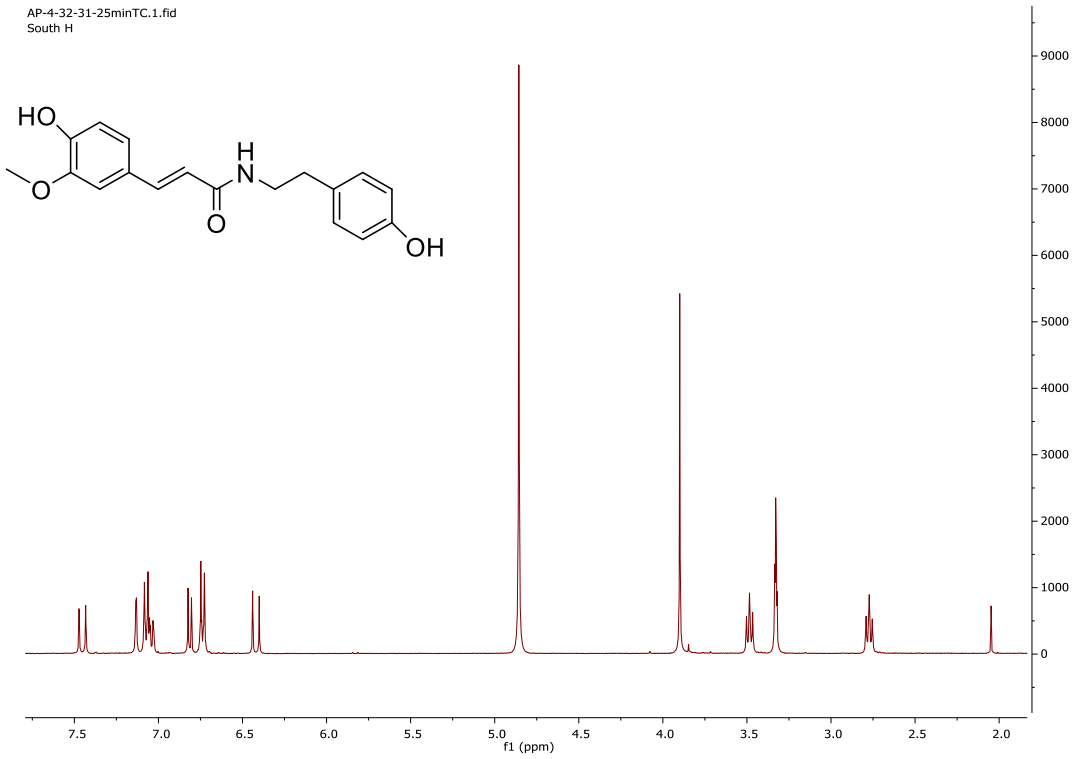


HSQC spectrum of Naringenin

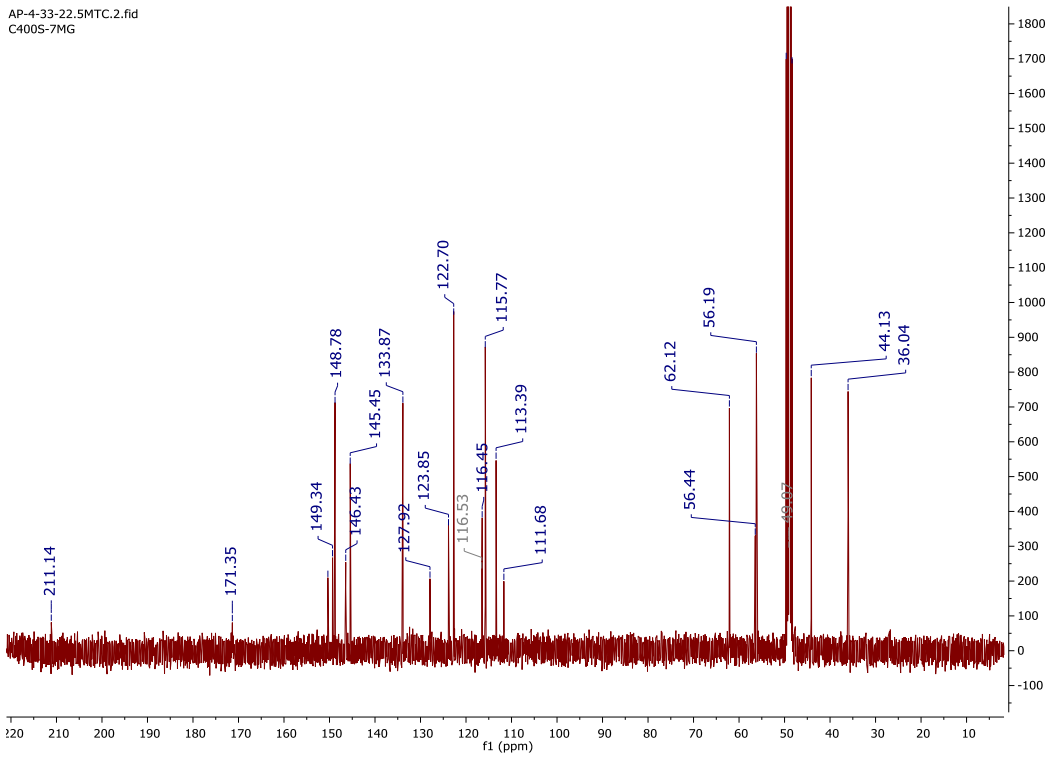


HMBC spectrum of Naringenin

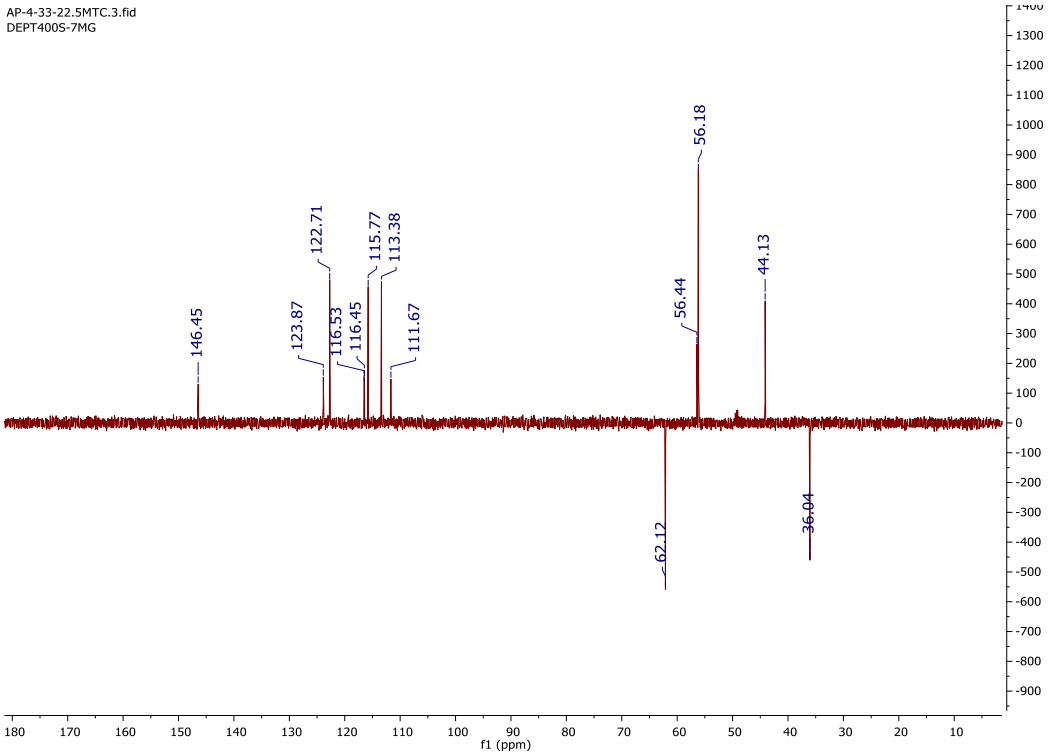
SI 15 1D and 2D NMR data of N-trans feruloyl tyramine



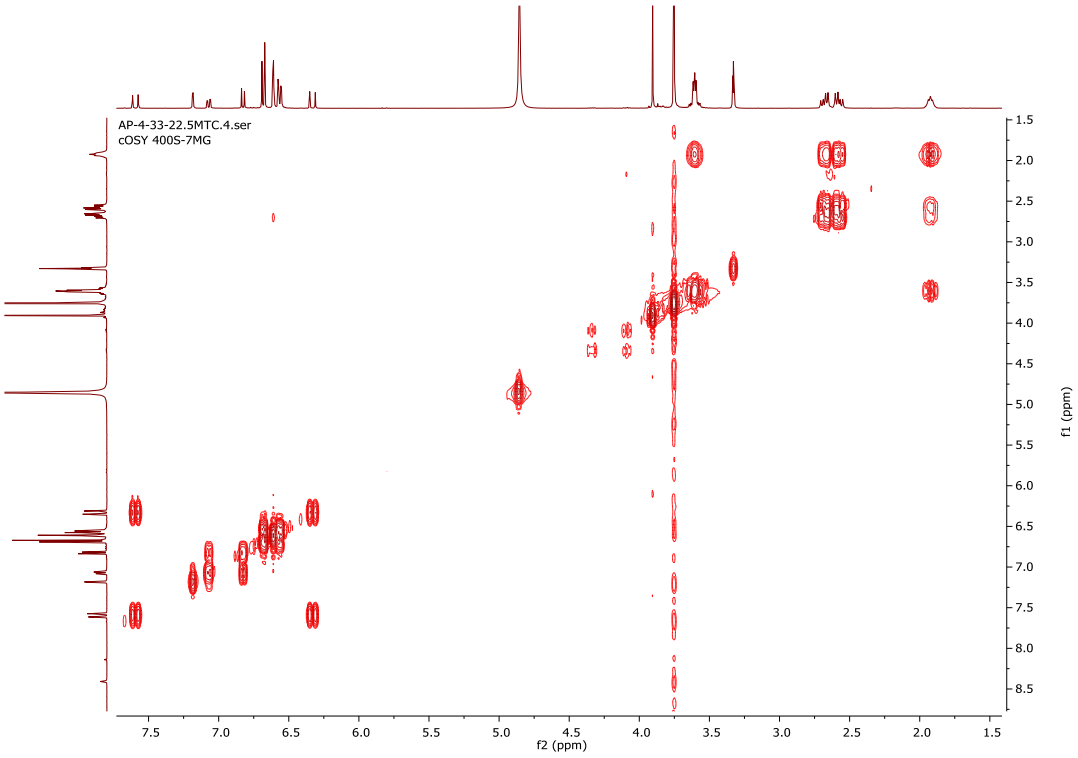
^{1}H-NMR spectrum of N-trans feruloyl tyramine



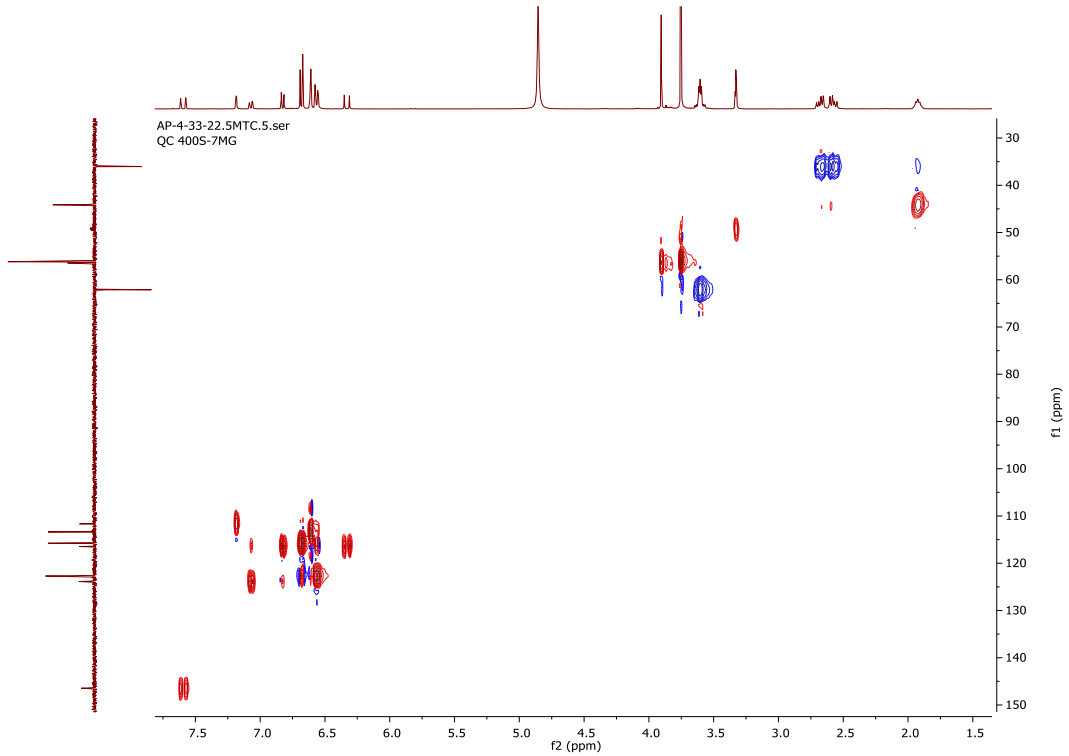
^{13}C-NMR spectrum of N-trans feruloyl tyramine



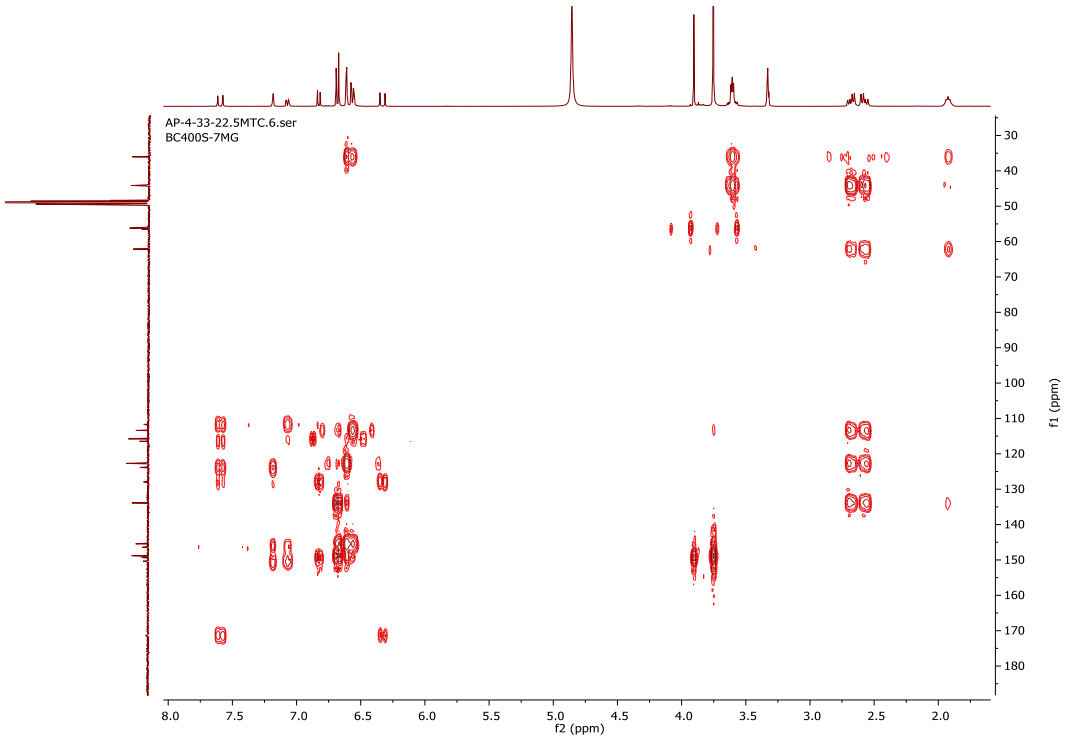
DEPT135 spectrum of N-trans feruloyl tyramine



COSY spectrum of N-trans feruloyl tyramine

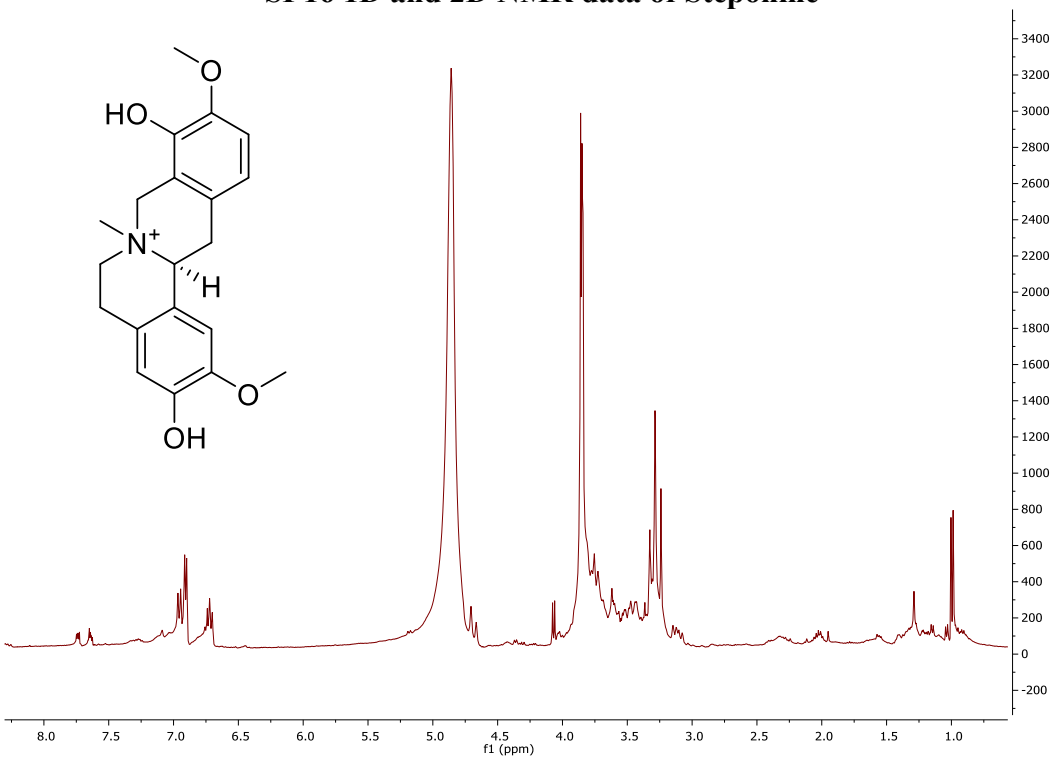


HSQC spectrum of N-trans feruloyl tyramine

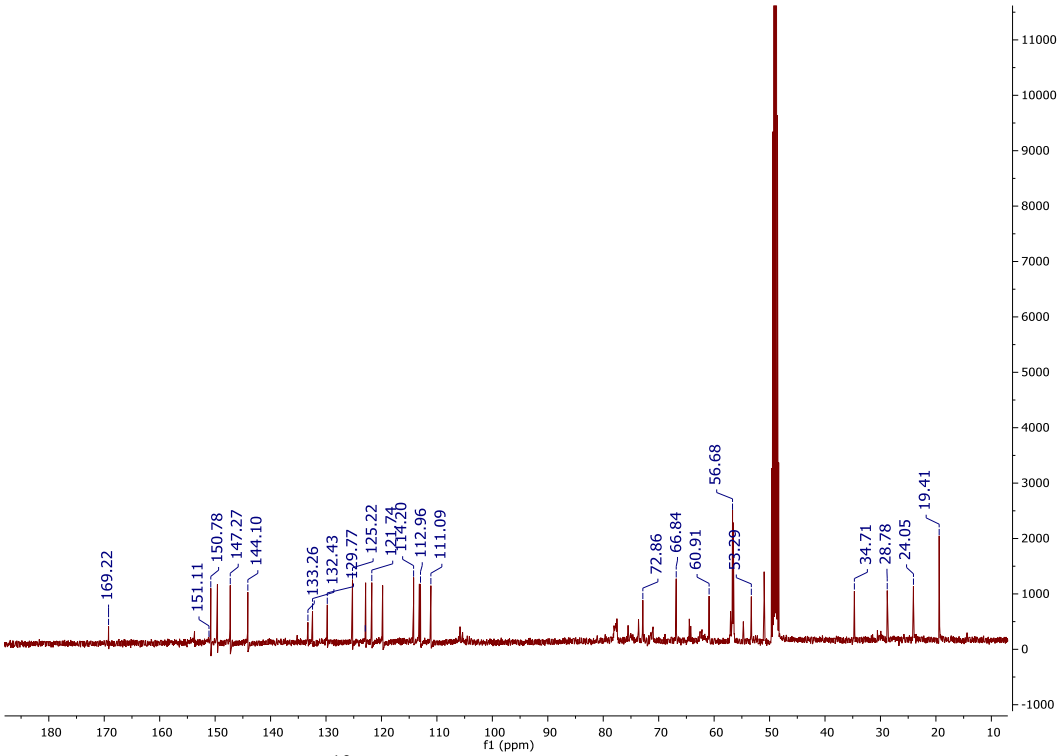


HMBC spectrum of N-trans feruloyl tyramine

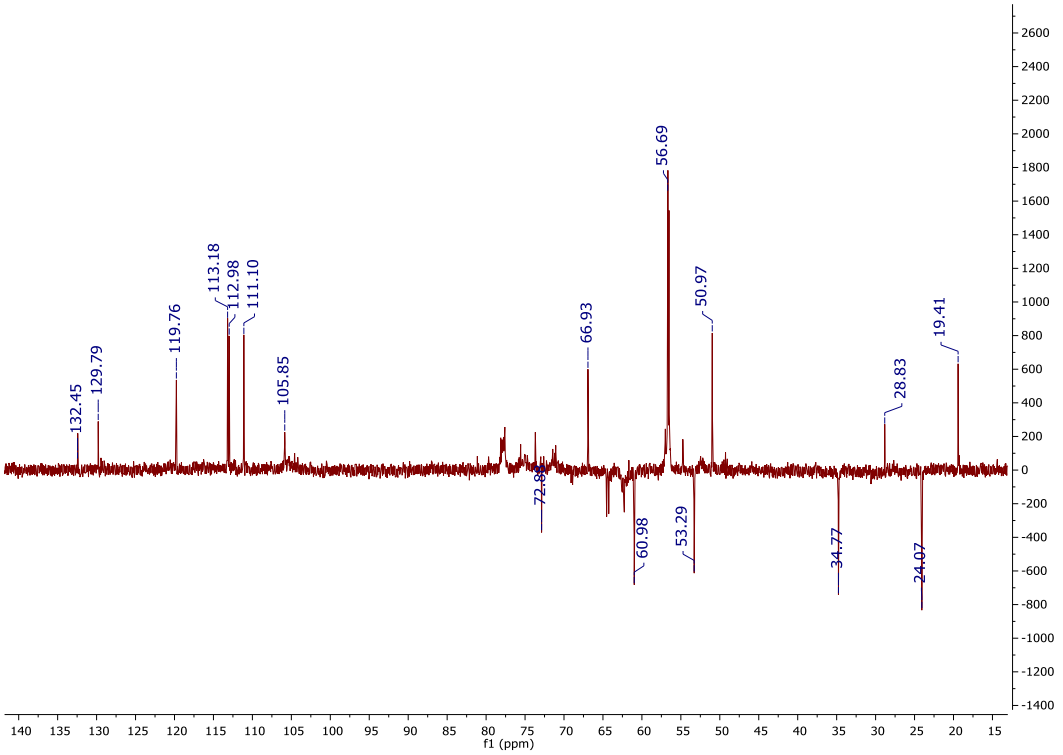
SI 16 1D and 2D NMR data of Steponine



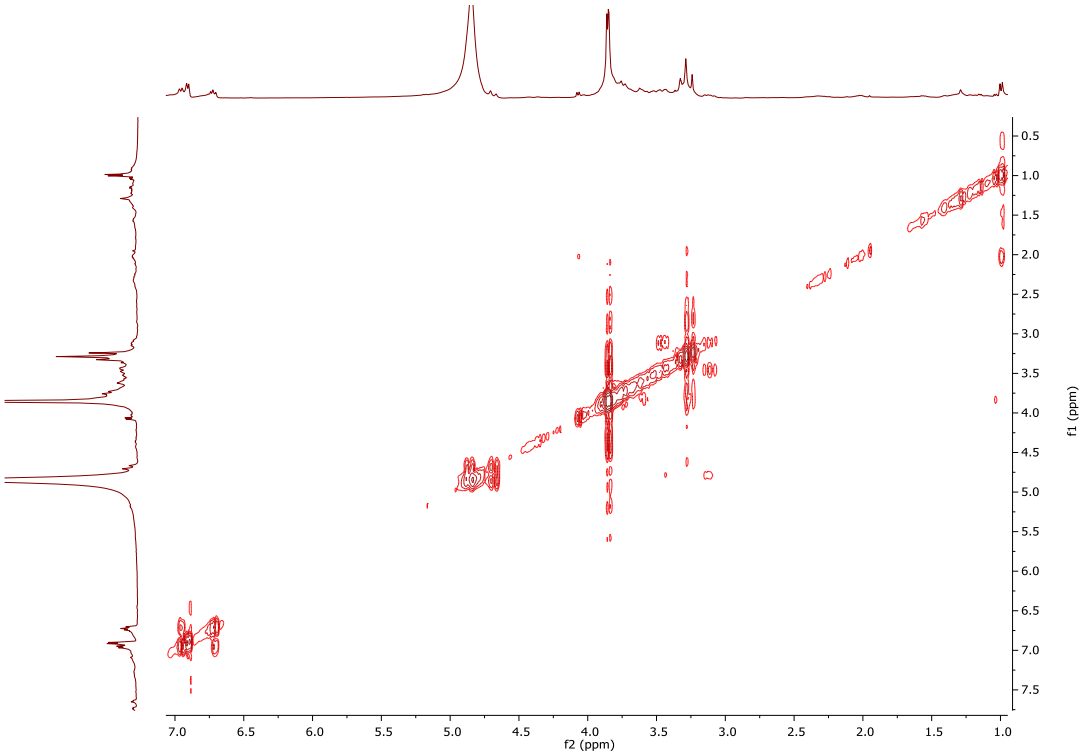
^{1}H-NMR spectrum of Steponine



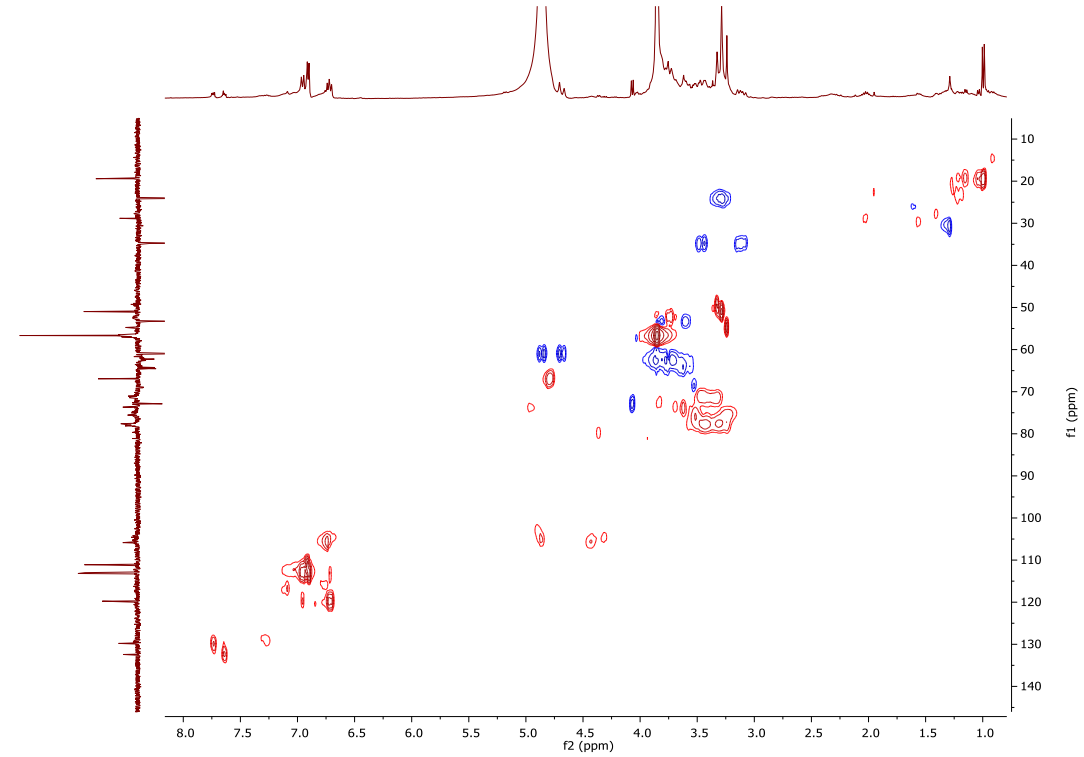
^{13}C-NMR spectrum of Steponine



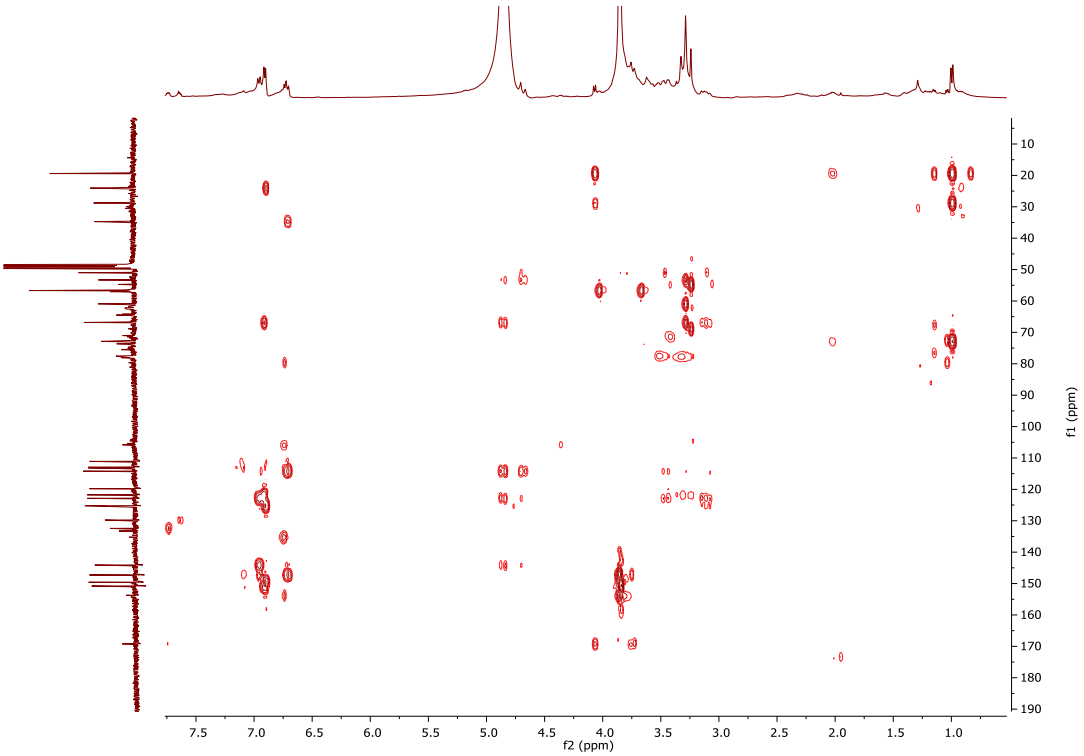
DEPT135 spectrum of Steponine



COSY spectrum of Steponine



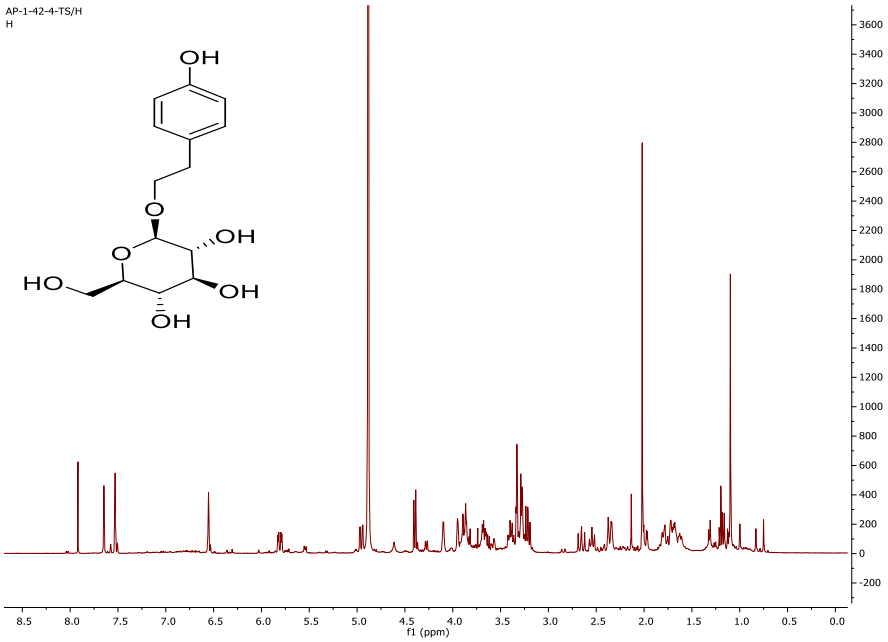
HSQC spectrum of Steponine



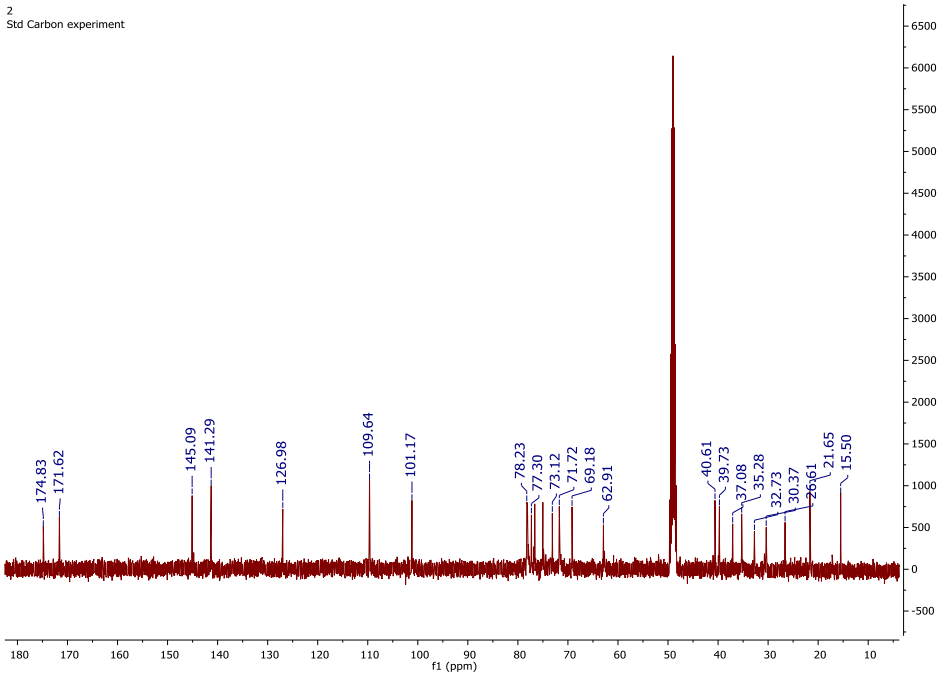
HMBC spectrum of Steponine

APPENDIX II-SUPPLEMENTARY INFORMATION-CHAPTER 3

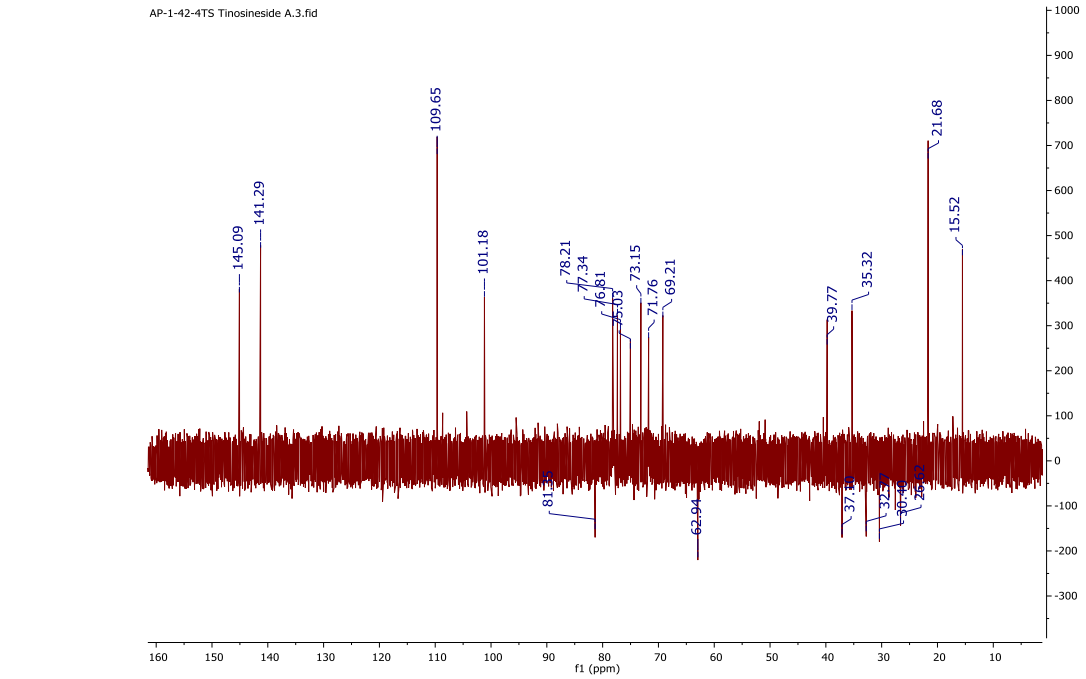
SI 17 1D and 2D NMR spectra of Tinosineside A



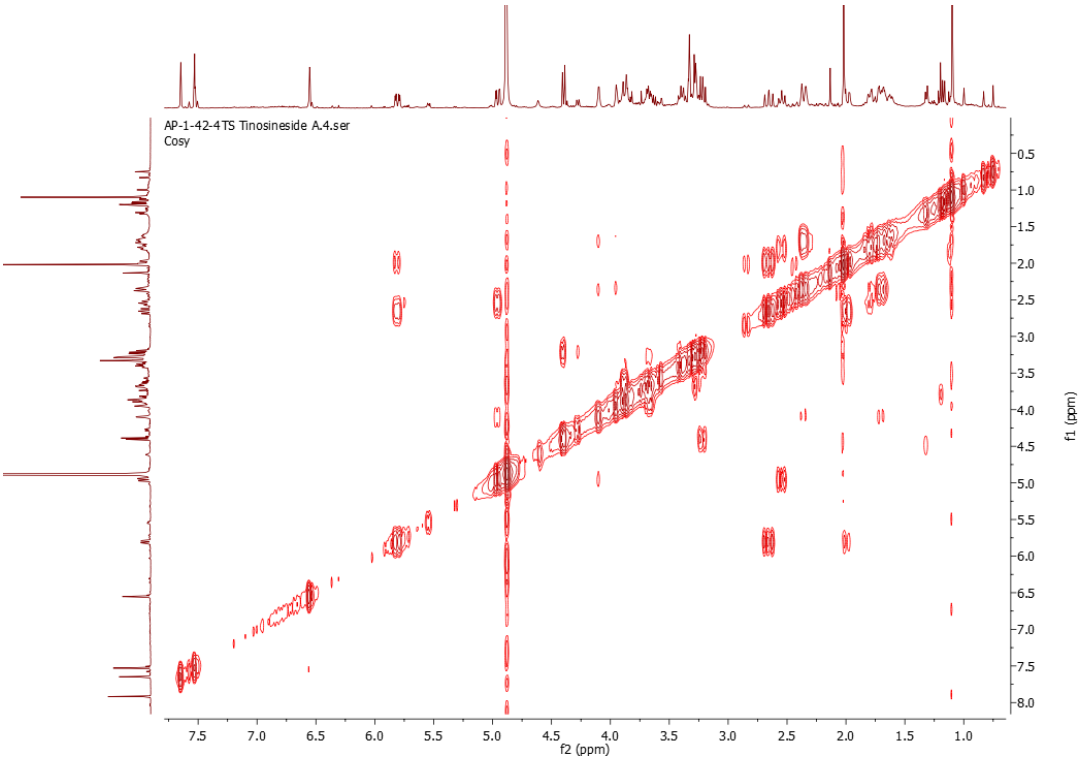
^{1}H-NMR spectrum of Tinosineside A



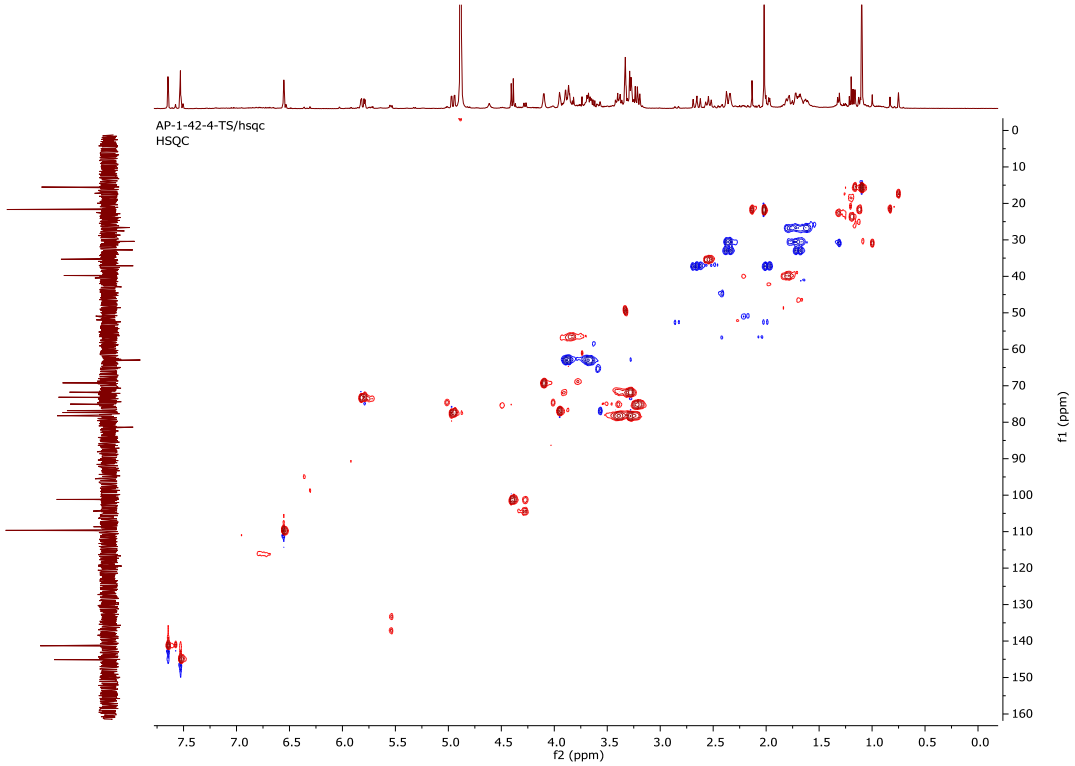
^{13}C-NMR spectrum of Tinosineside A



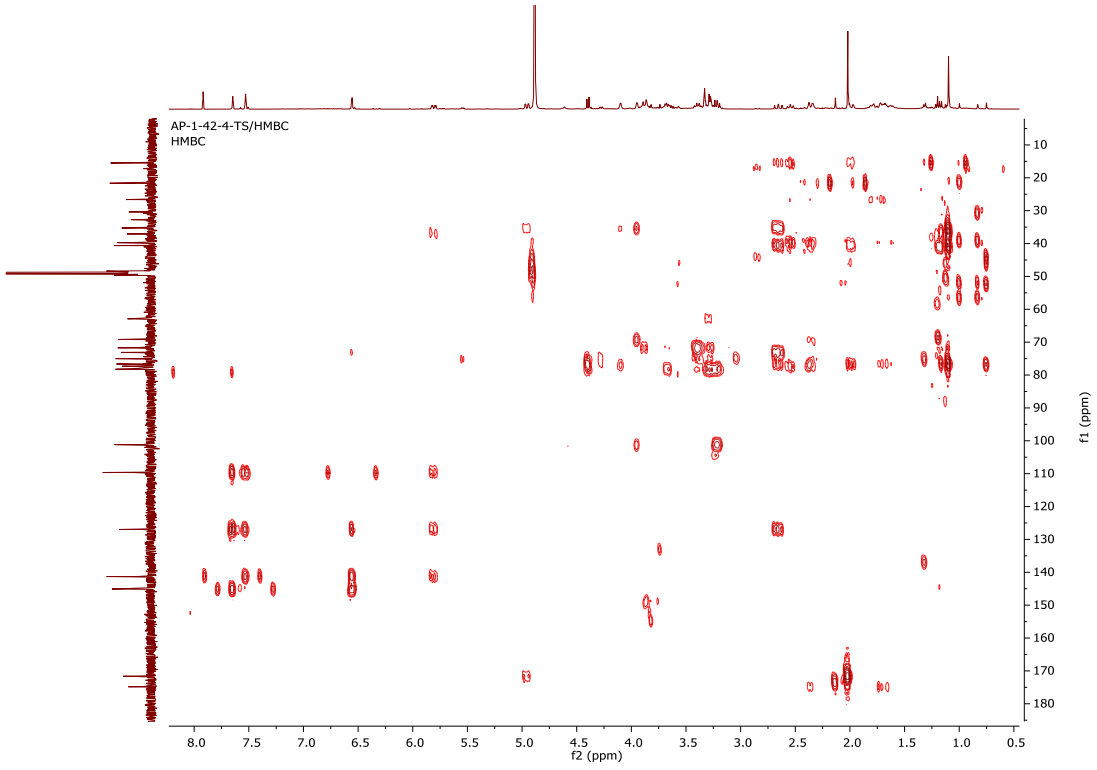
DEPT135 spectrum of Tinosineside A



COSY spectrum of Tinosineside A

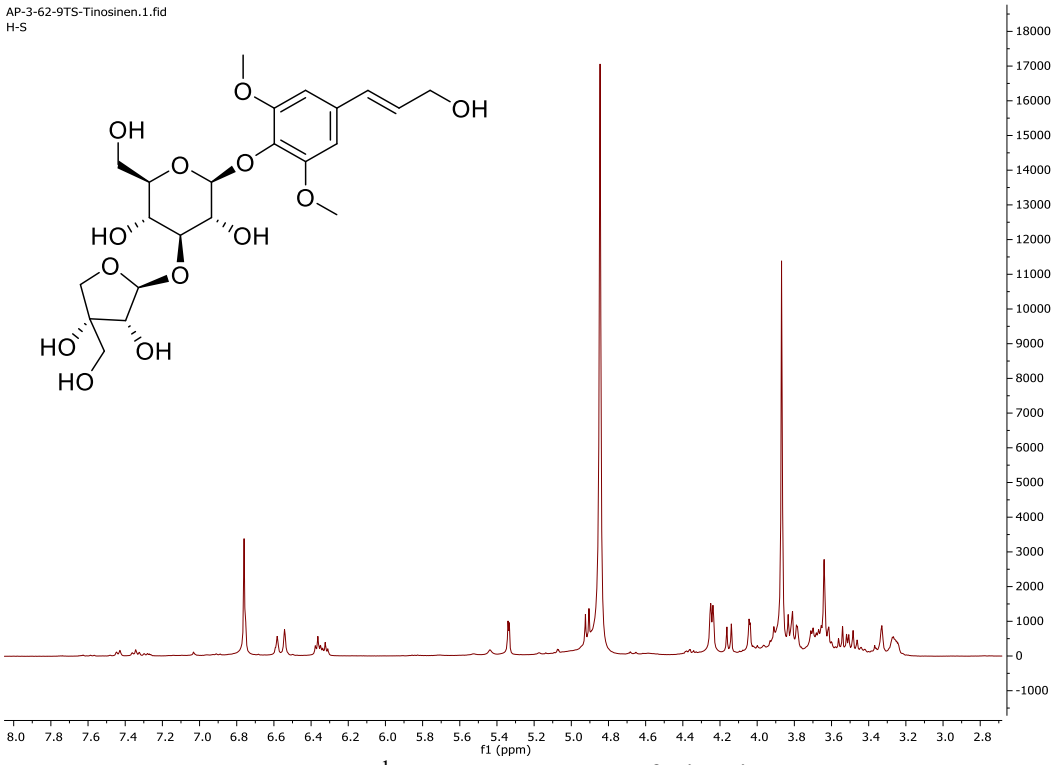


HSQC spectrum of Tinosineside A

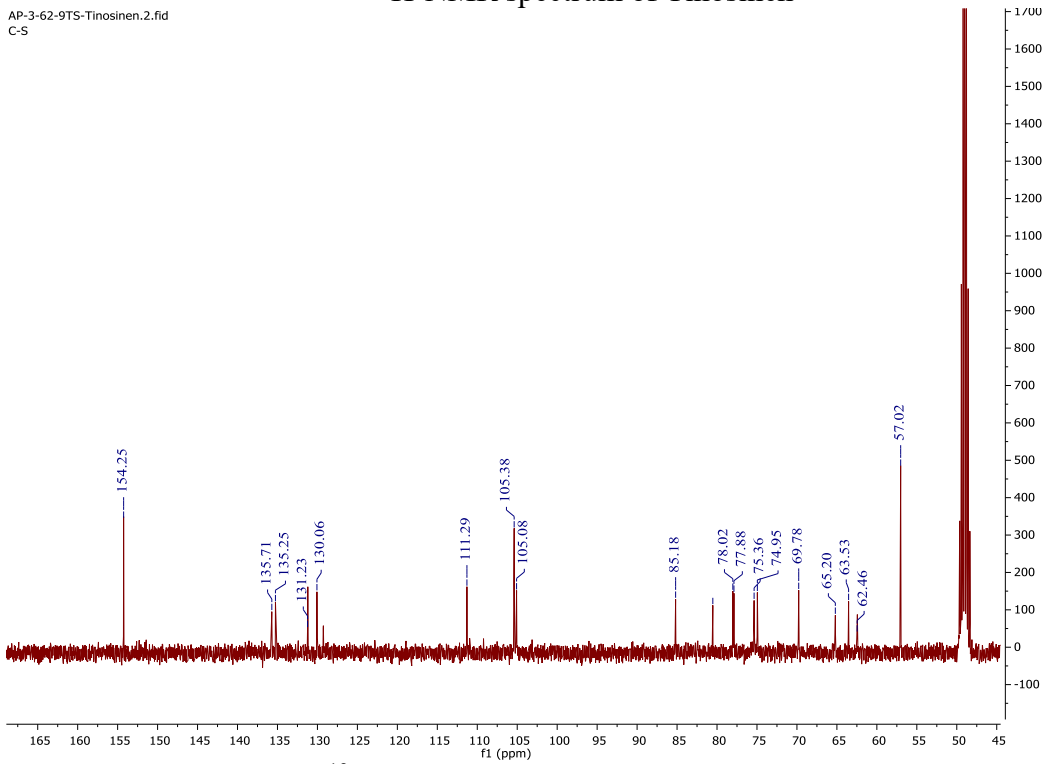


HMBC spectrum of Tinosineside A

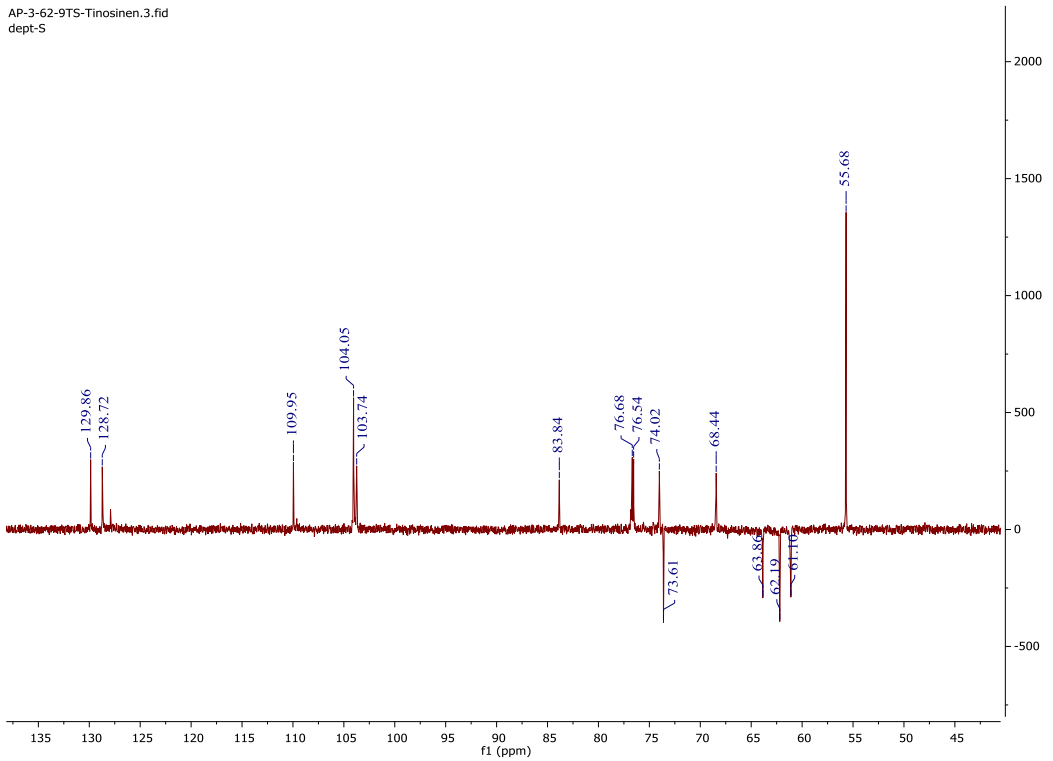
SI 18 1D and 2D NMR data of Tinosinen



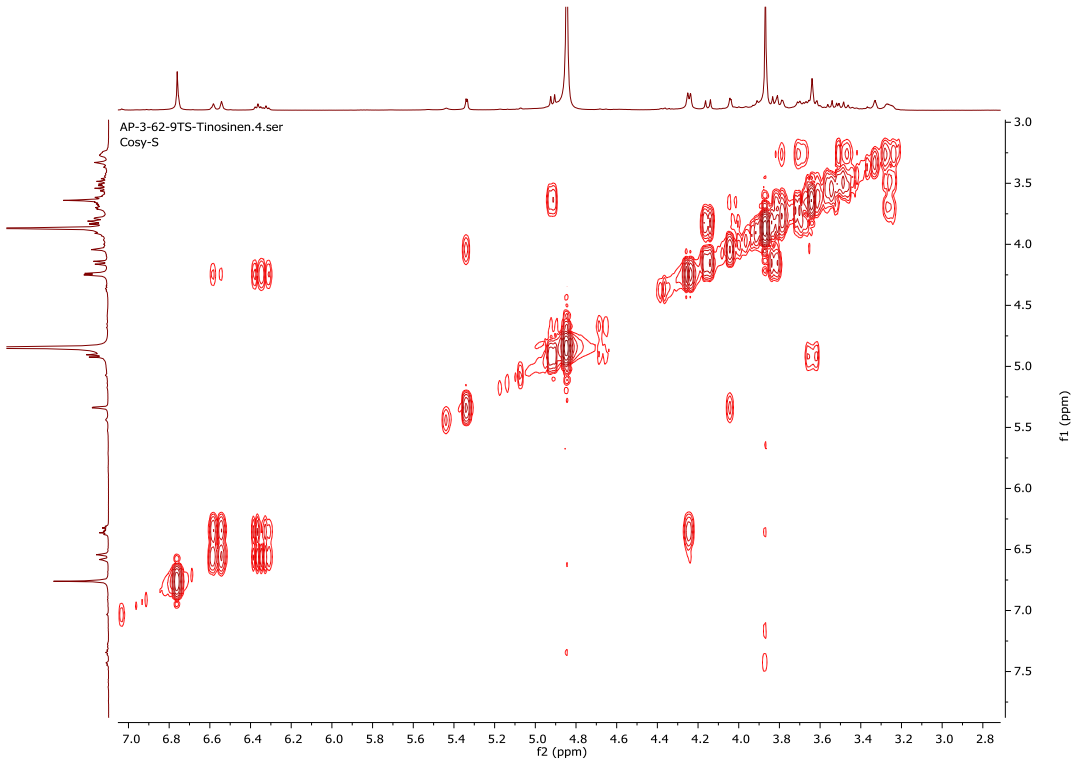
^{1}H-NMR spectrum of Tinosinen



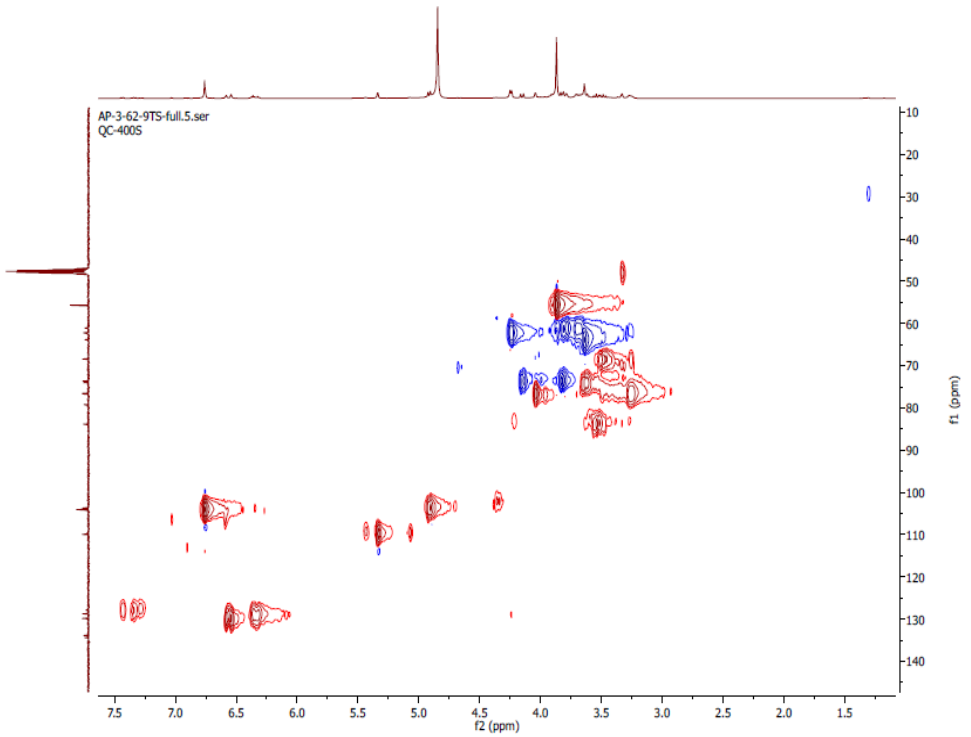
^{13}C-NMR spectrum of Tinosinen



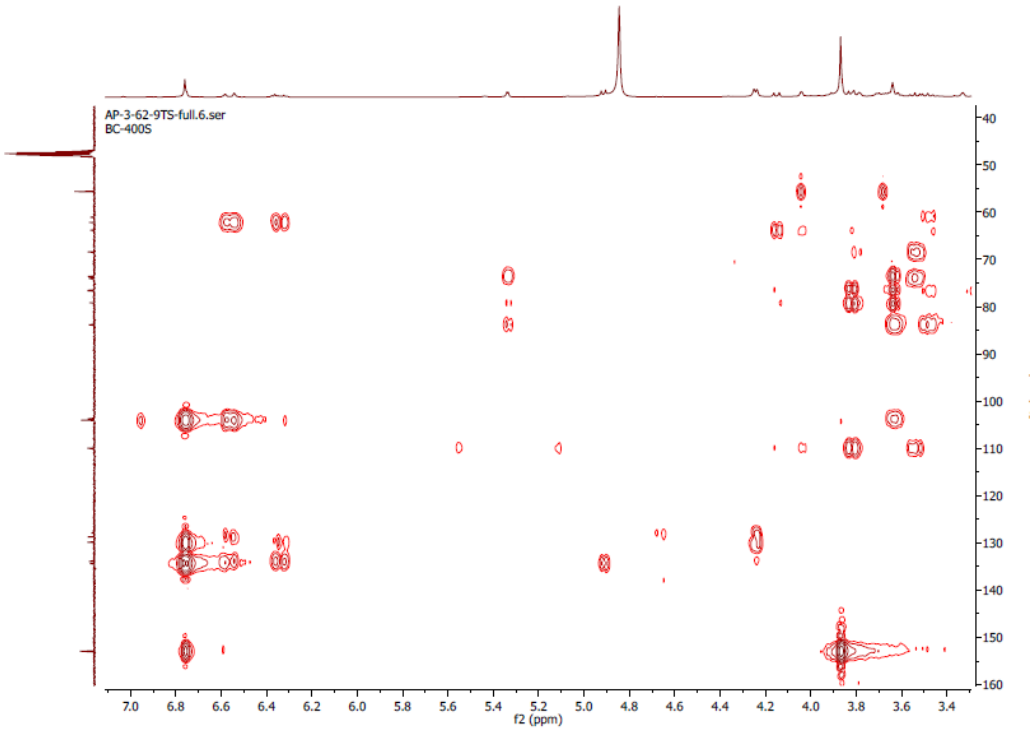
DEPT135 spectrum of Tinosinen



COSY spectrum of Tinosinen

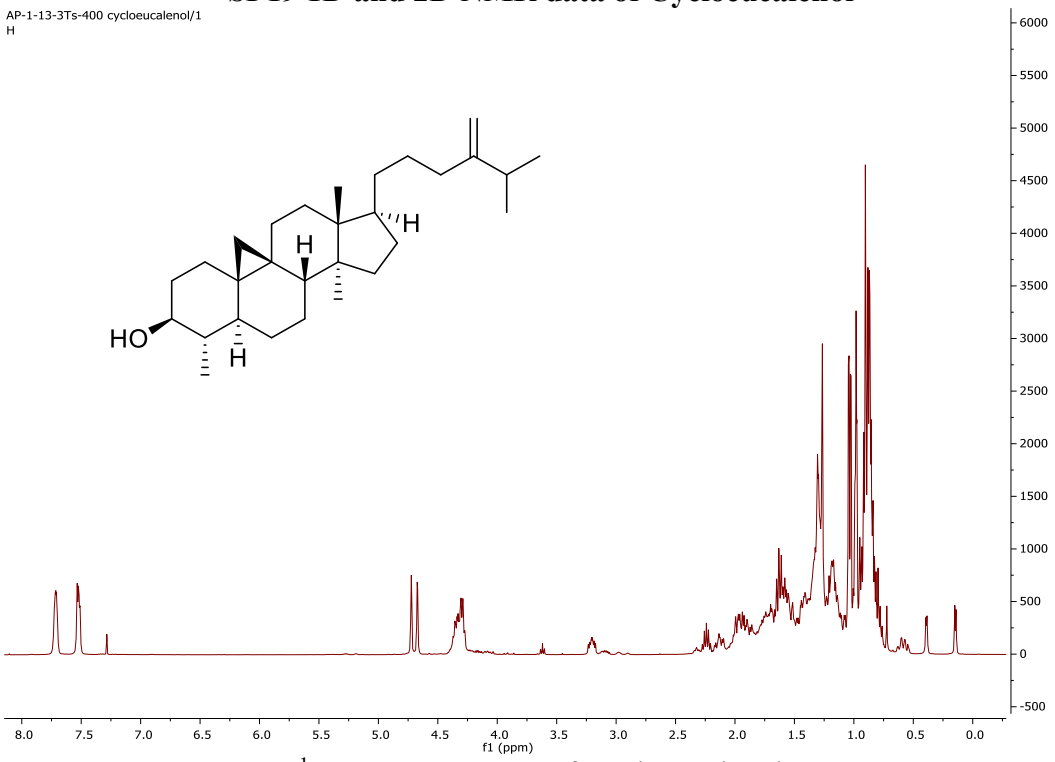


HSQC spectrum of Tinosinen

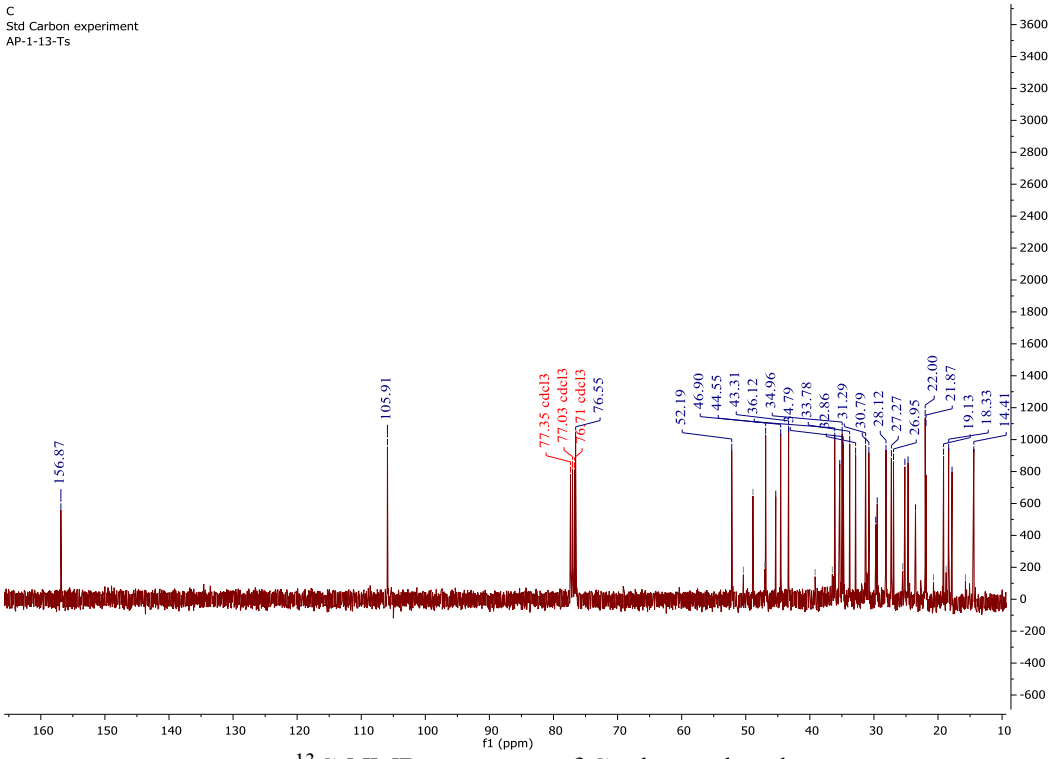


HMBC spectrum of Tinosinen

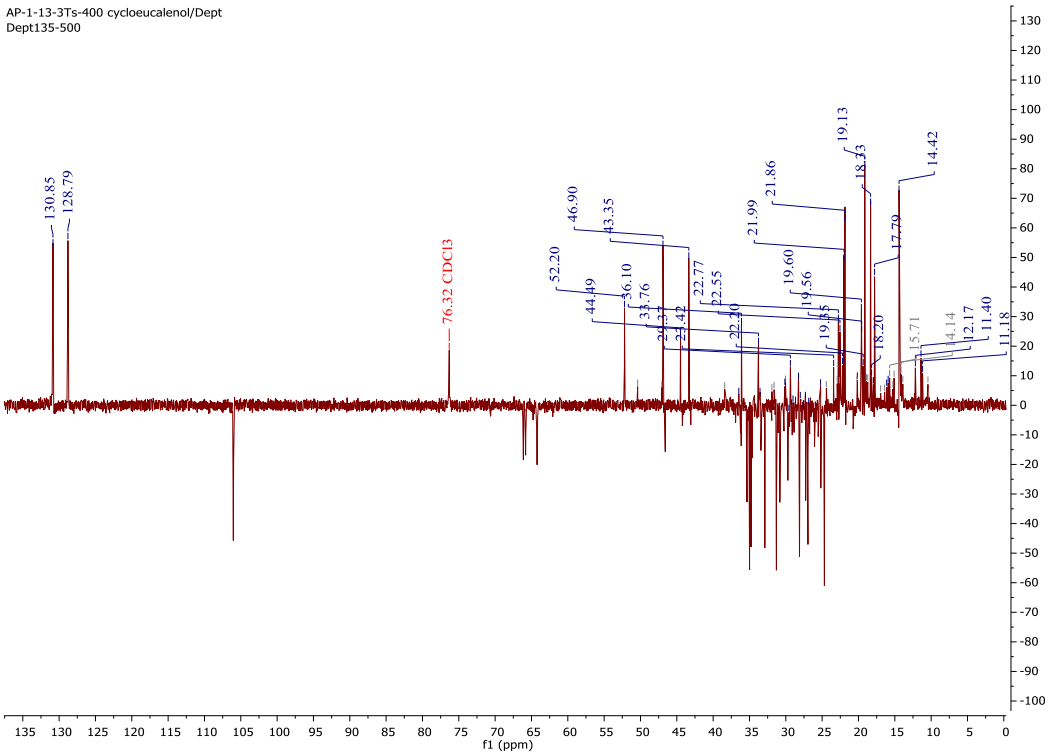
SI 19 1D and 2D NMR data of Cycloeucalenol



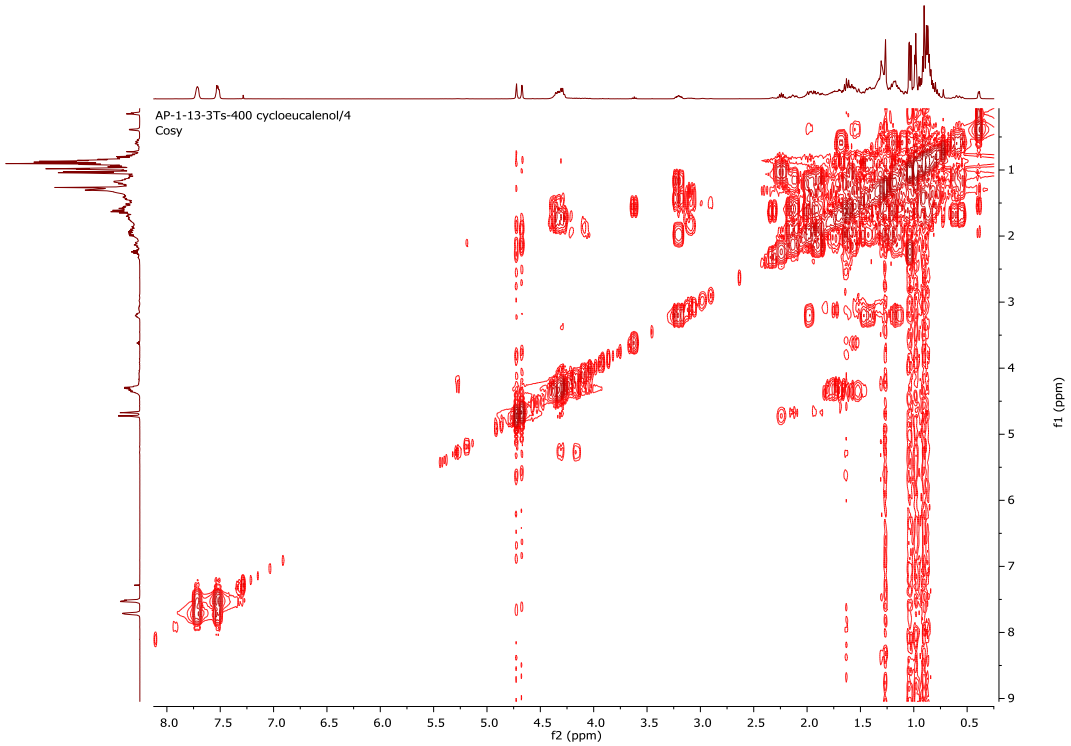
^{1}H-NMR spectrum of Cycloeucalenol



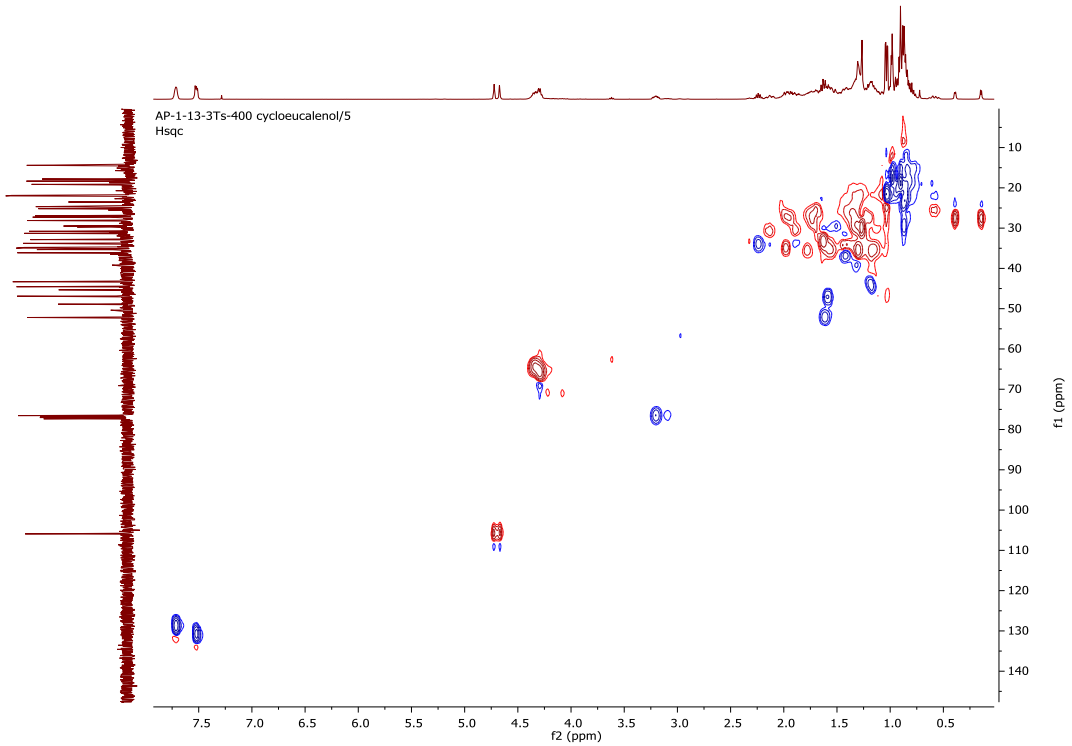
^{13}C-NMR spectrum of Cycloeucalenol



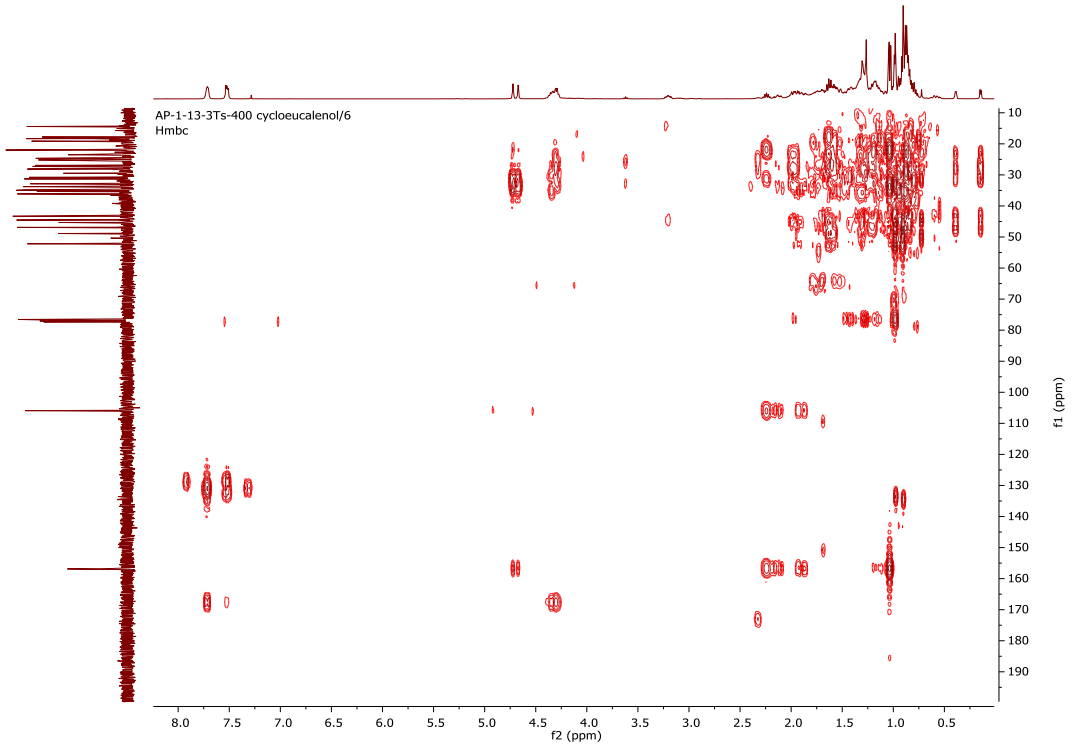
COSY spectrum of Cycloeucalenol



DEPT135 spectrum of Cycloeucalenol

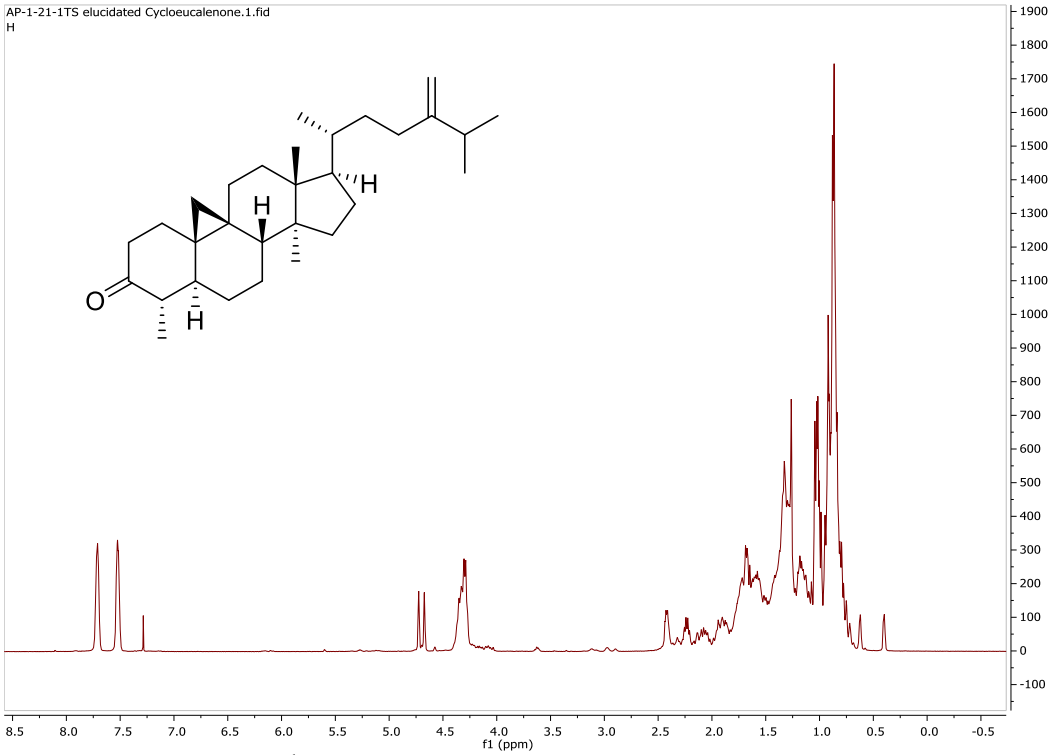


HSQC spectrum of Cycloeucalenol

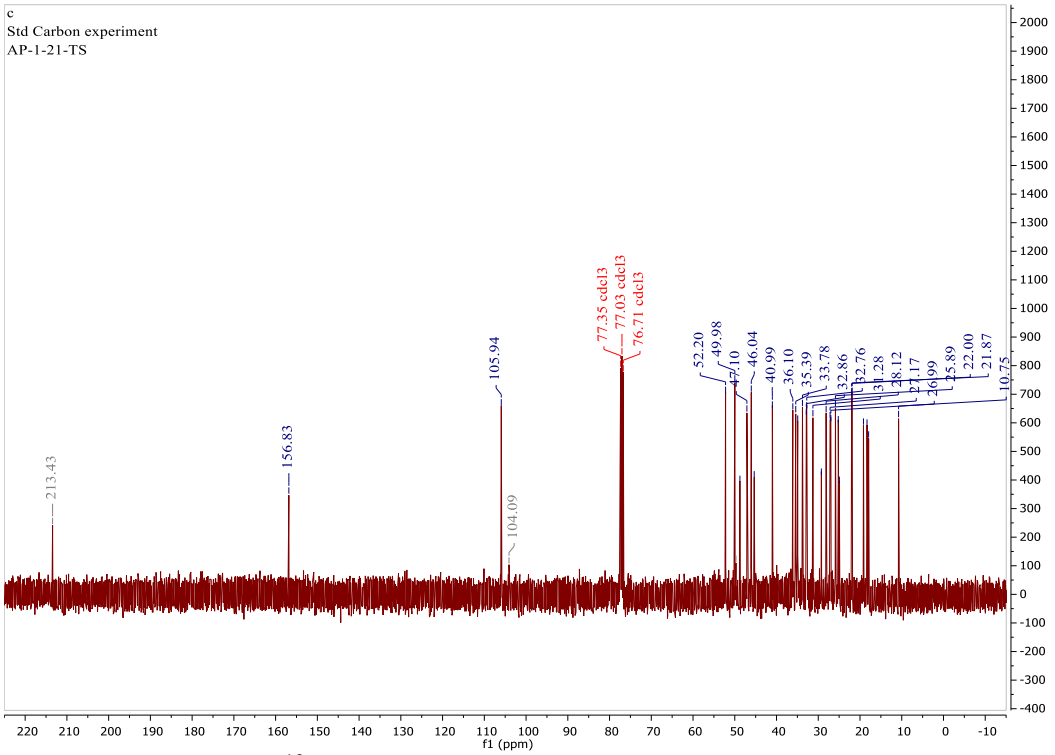


HMBC spectrum of Cycloeucalenol

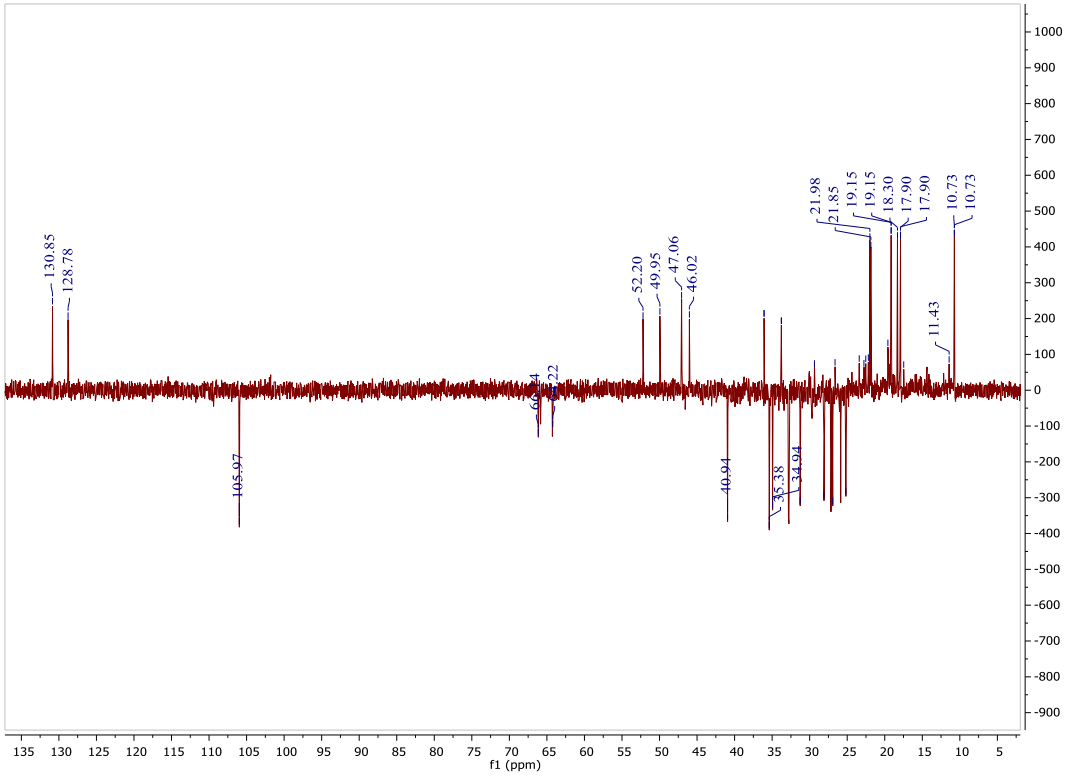
SI 20 1D and 2D NMR data of Cycloeucalenone



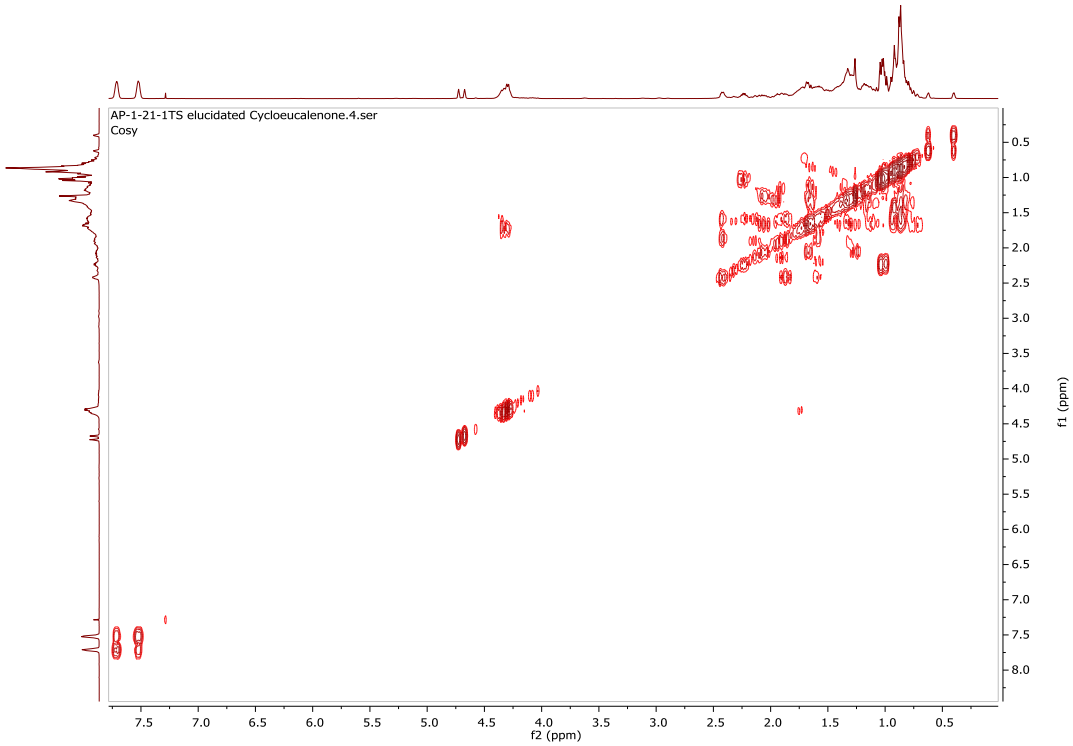
^{1}H-NMR spectrum of Cycloeucalenone



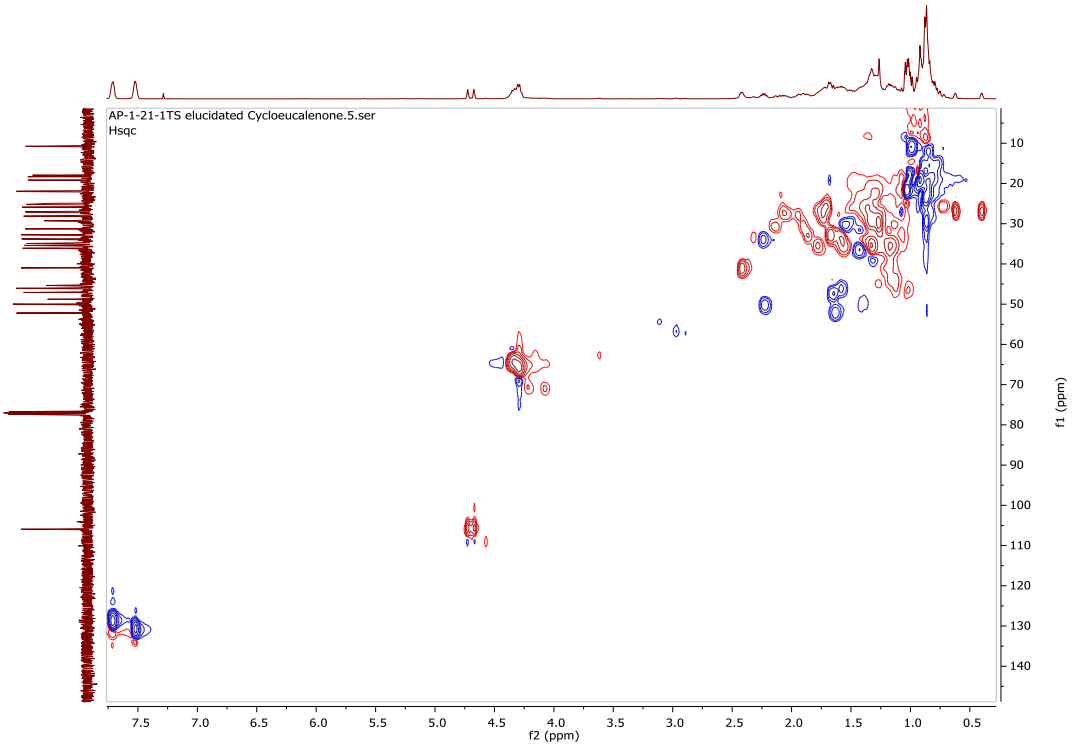
^{13}C-NMR spectrum of Cycloeucalenone



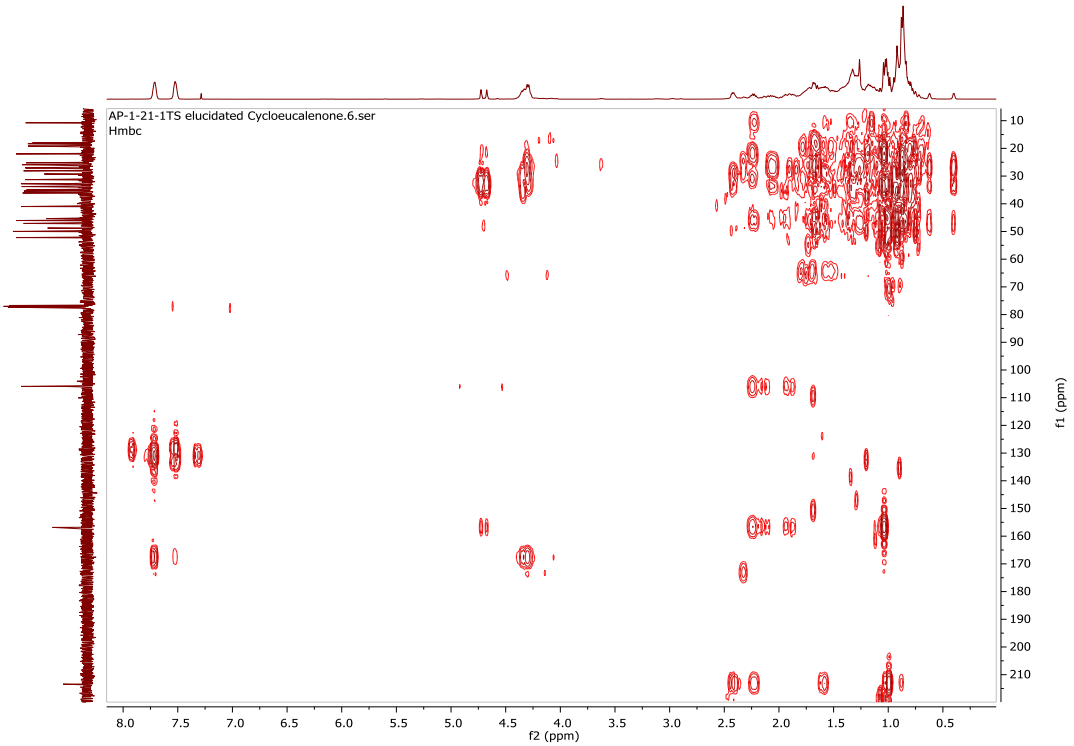
DEPT135 spectrum of Cycloeucalenone



COSY spectrum of Cycloeucalenone

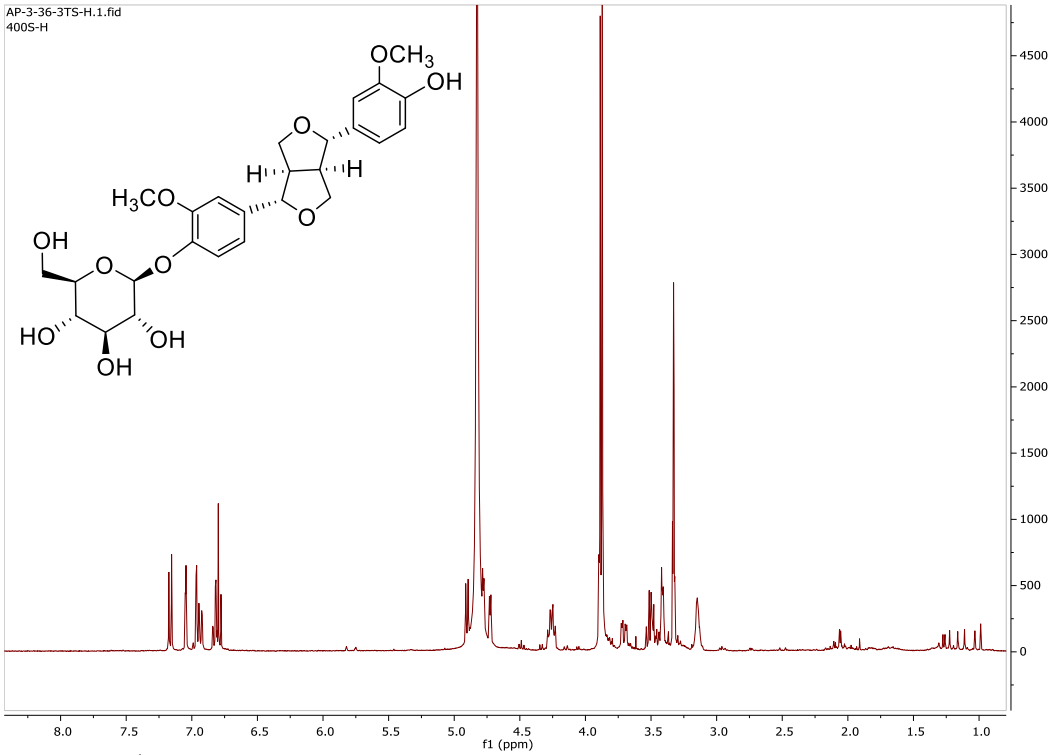


HSQC spectrum of Cycloeucalenone

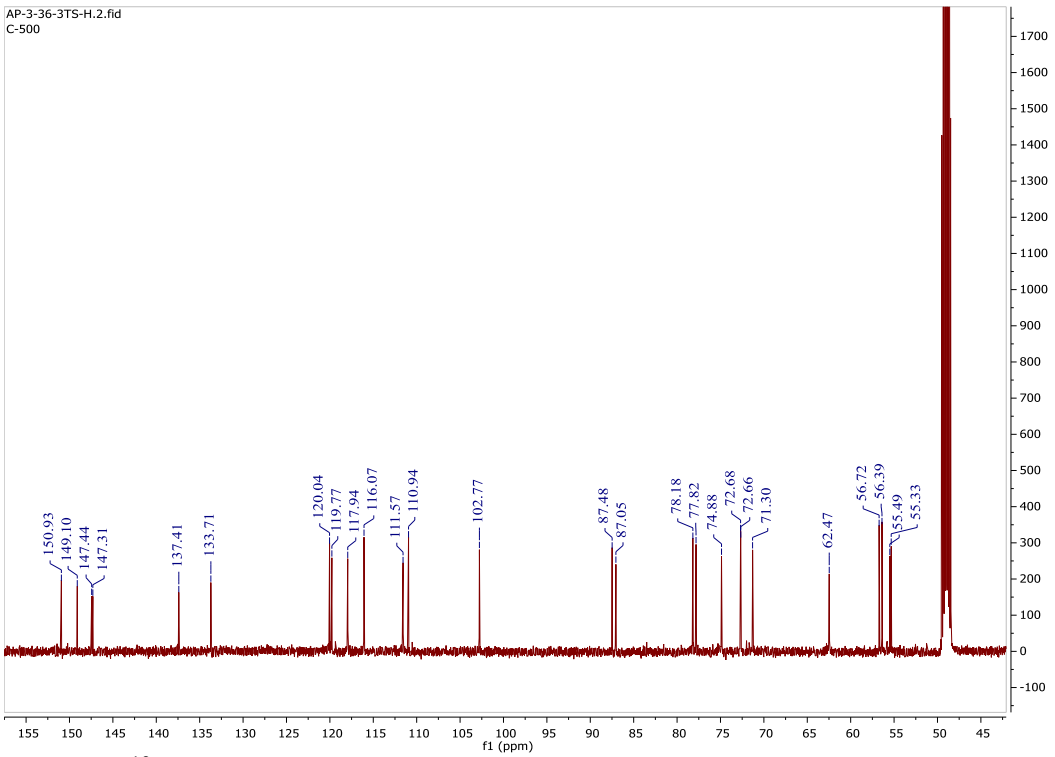


HMBC spectrum of Cycloeucalenone

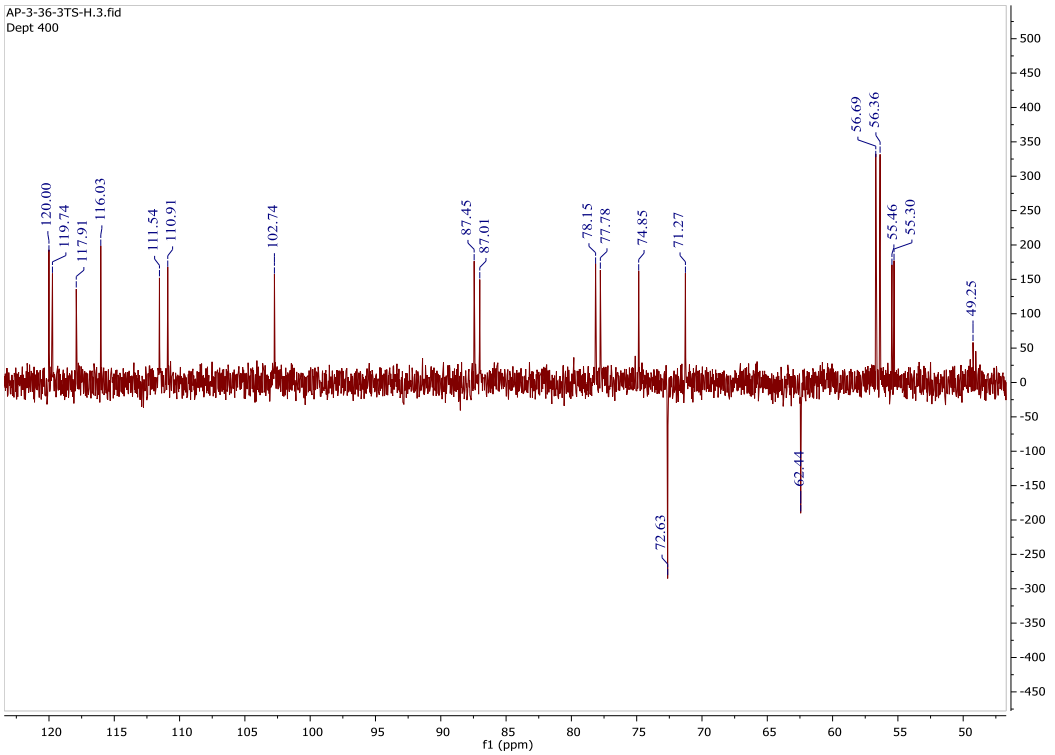
SI 21 1D and 2D NMR data of (-)-pinoresinol 4-O-β-D-glucopyranoside



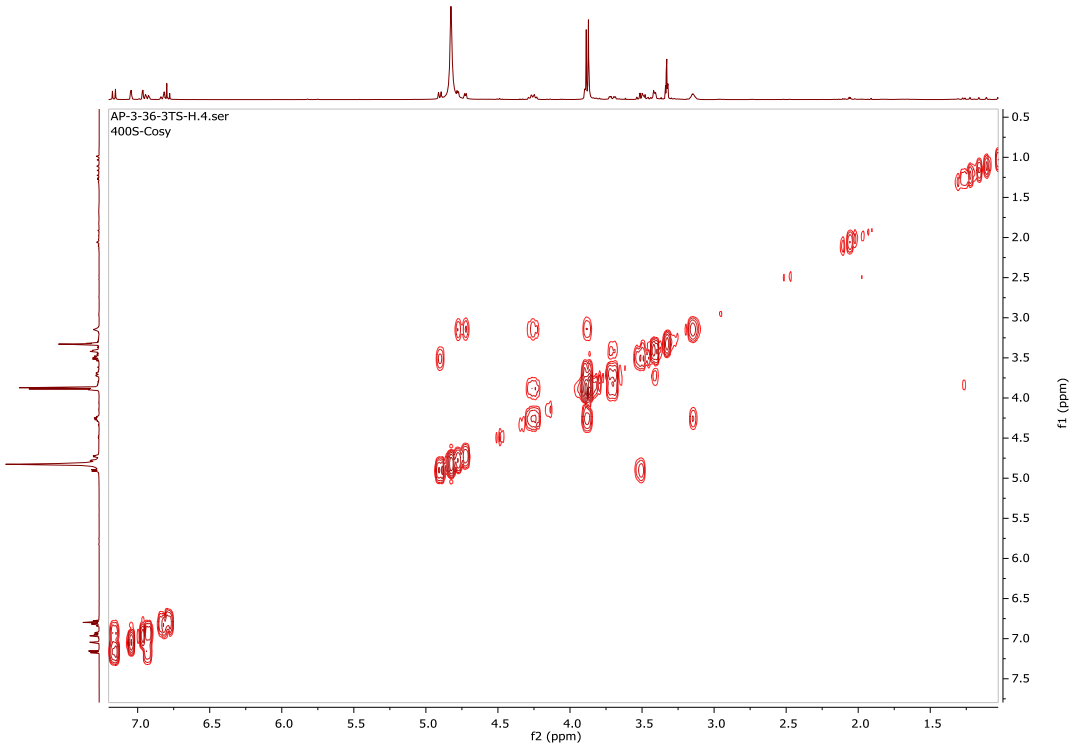
^{1}H-NMR spectrum of (-)-pinoresinol 4-O-β-D-glucopyranoside



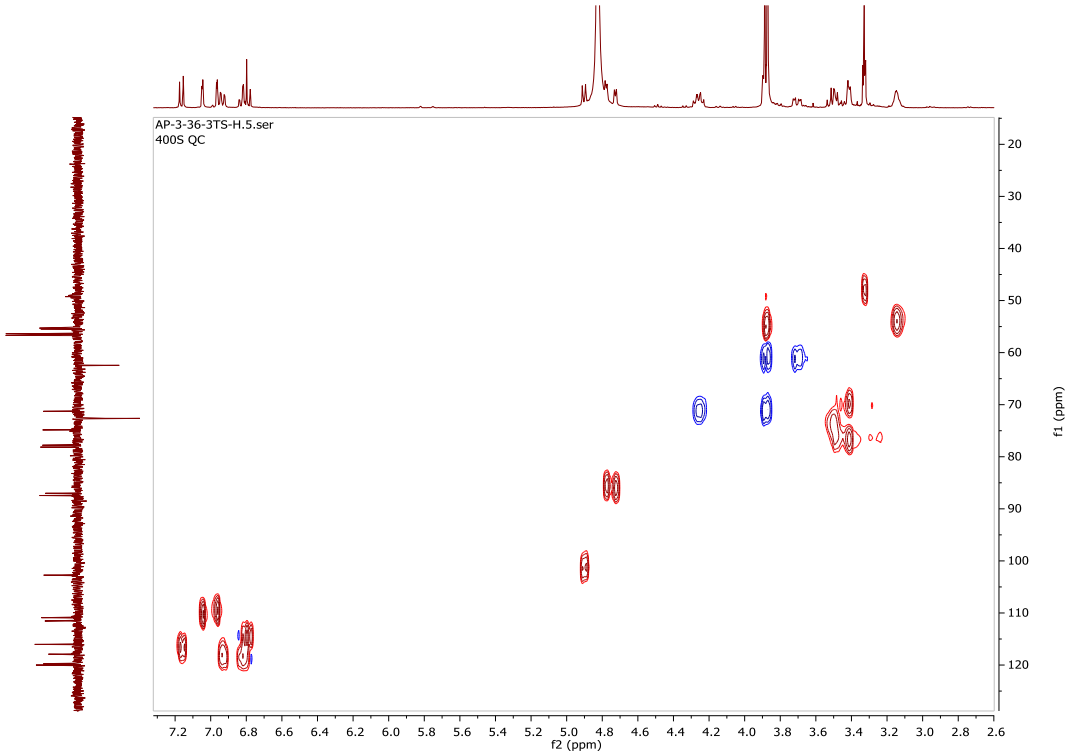
^{13}C-NMR spectrum of (-)-pinoresinol 4-O-β-D-glucopyranoside



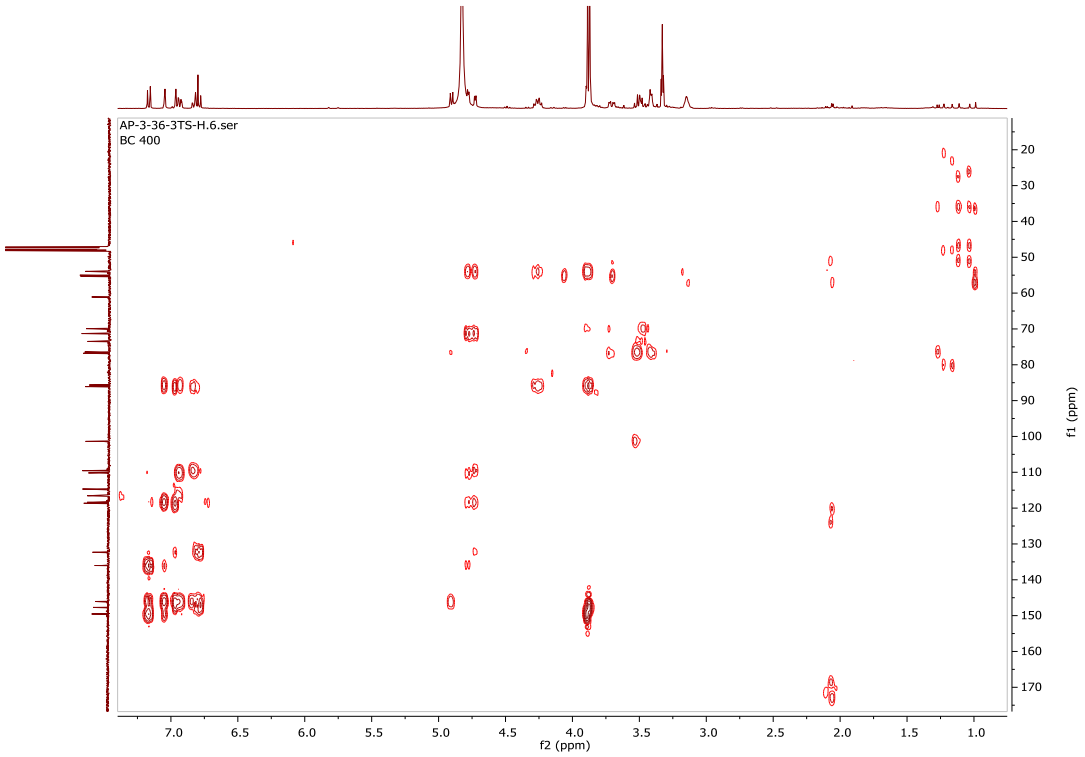
DEPT135 spectrum of (-)-pinoresinol 4-O-β-D-glucopyranoside



COSY spectrum of (-)-pinoresinol 4-O-β-D-glucopyranoside

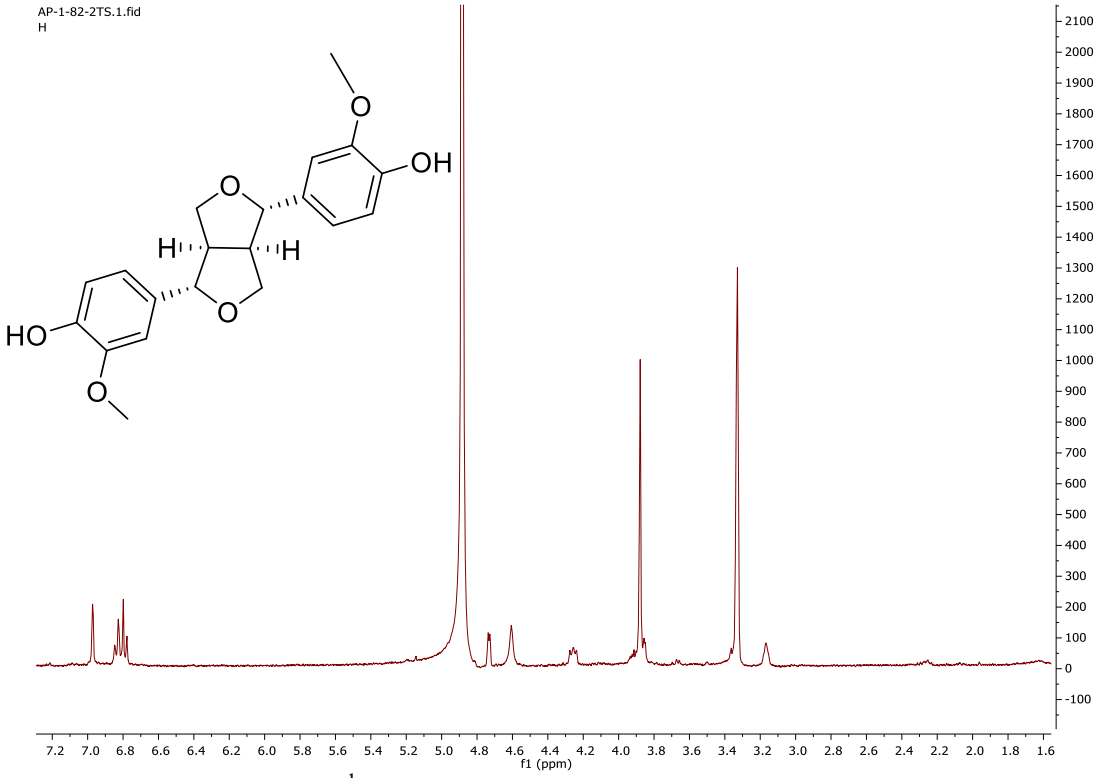


HSQC spectrum of (-)-pinoresinol 4-O-β-D-glucopyranoside

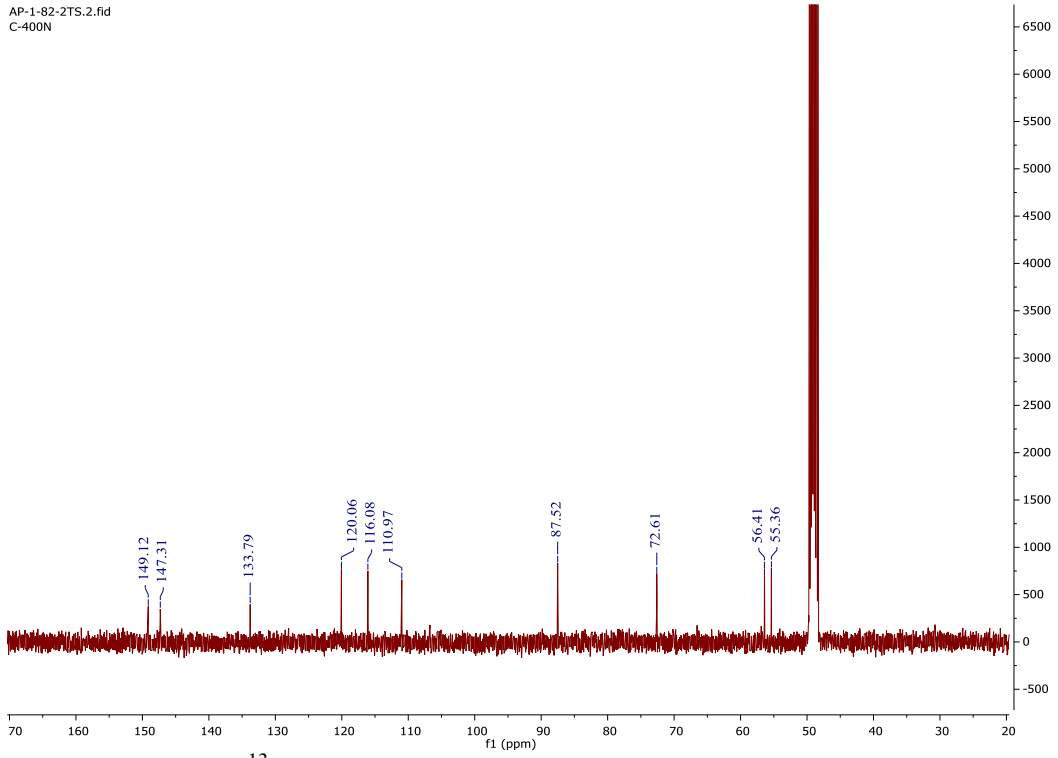


HMBC spectrum of. (-)-pinoresinol 4-O-β-D-glucopyranoside

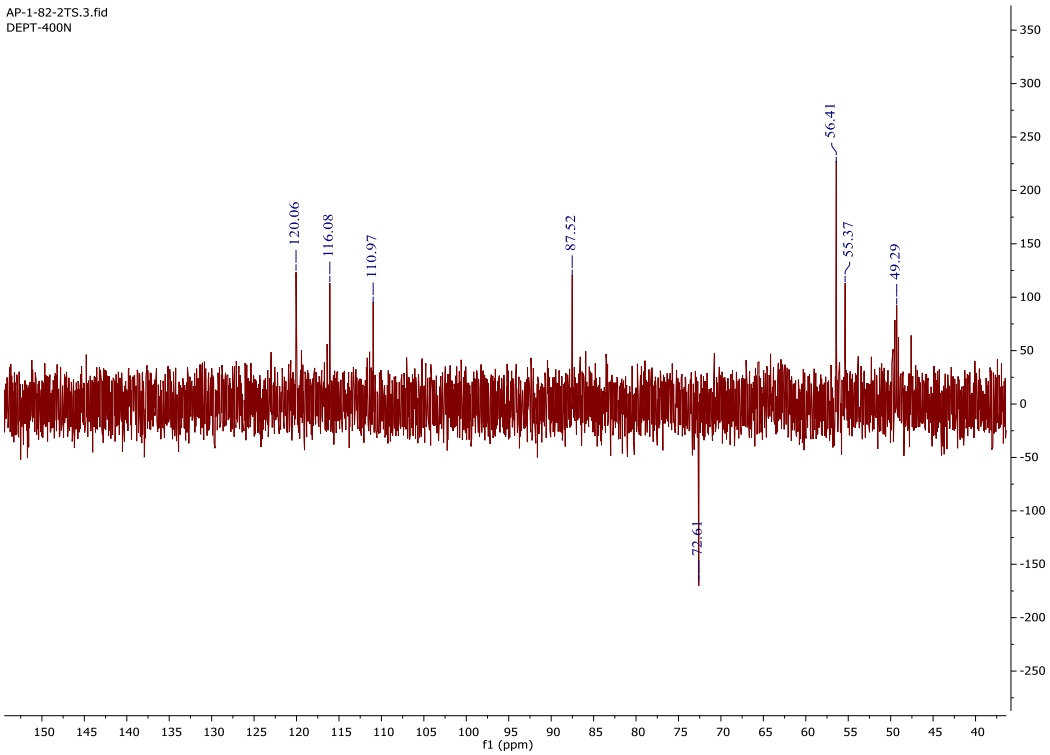
SI 22 1D and 2D NMR data of dl-Pinoresinol



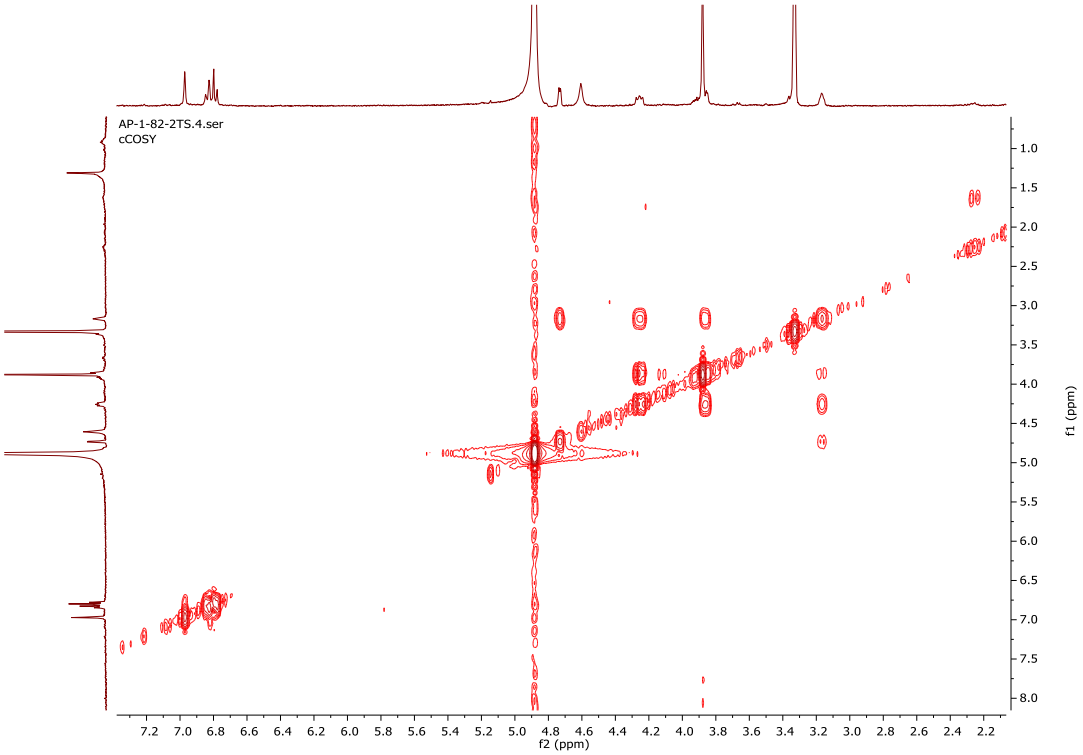
^{1}H-NMR spectrum of dl-Pinoresinol



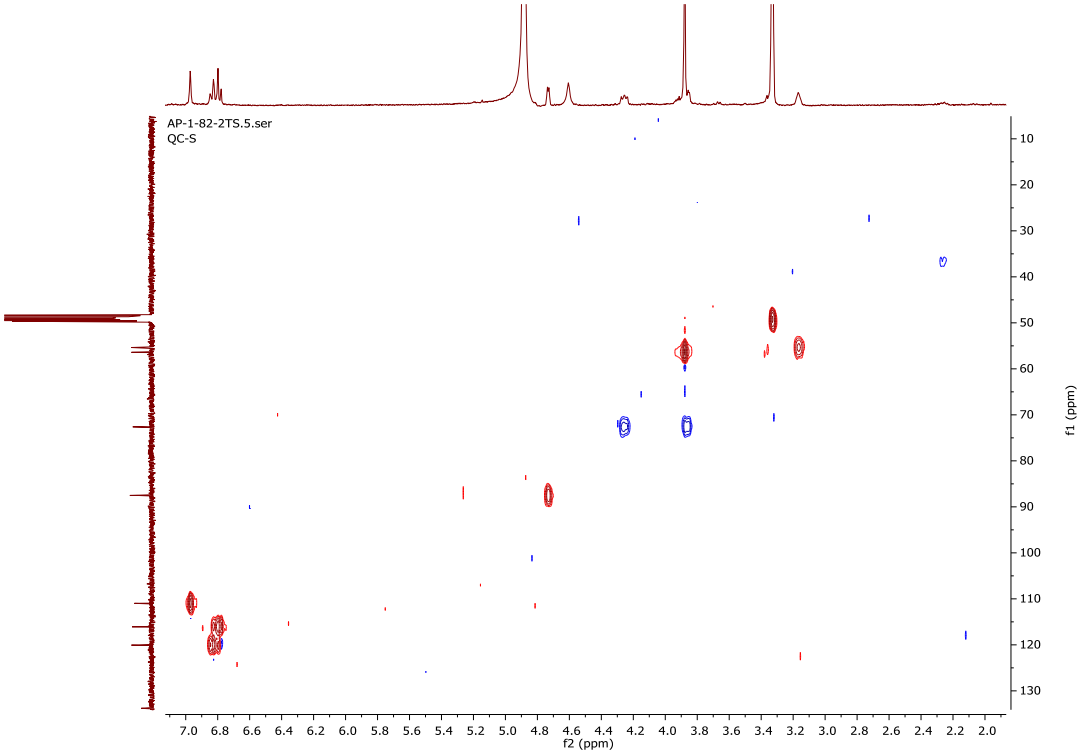
^{13}C-NMR spectrum of dl-Pinoresinol (3.3.11.)



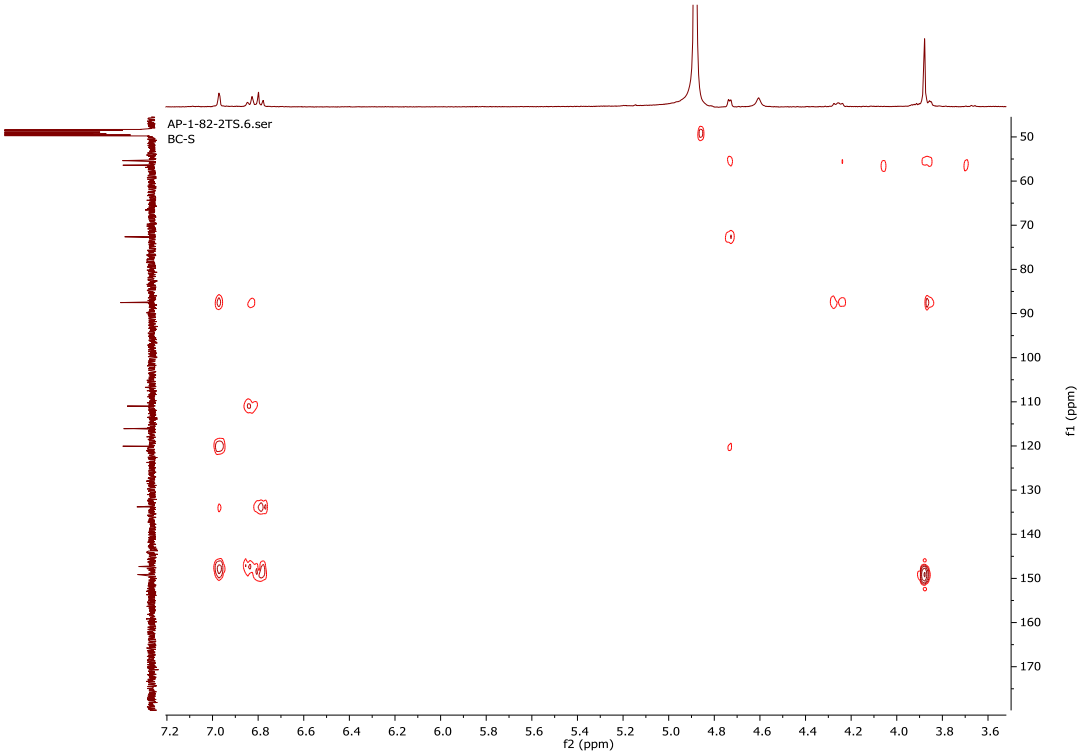
DEPT135 spectrum of dl-Pinoresinol



COSY spectrum of dl-Pinoresinol

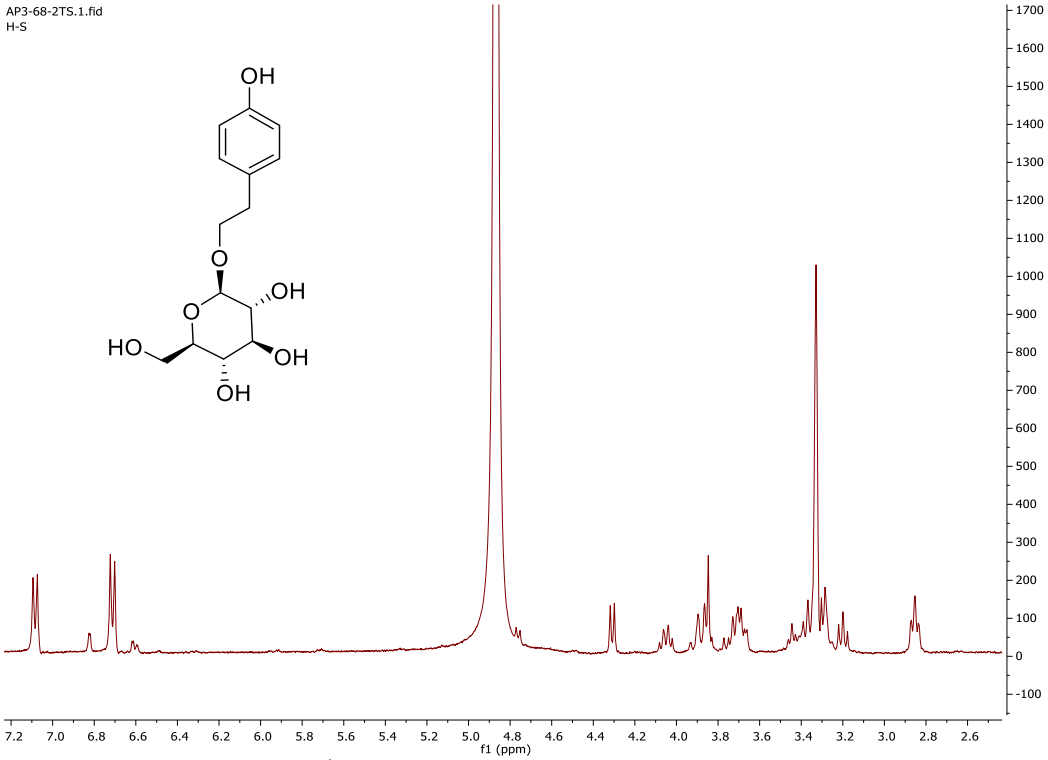


HSQC spectrum of dl-Pinoresinol

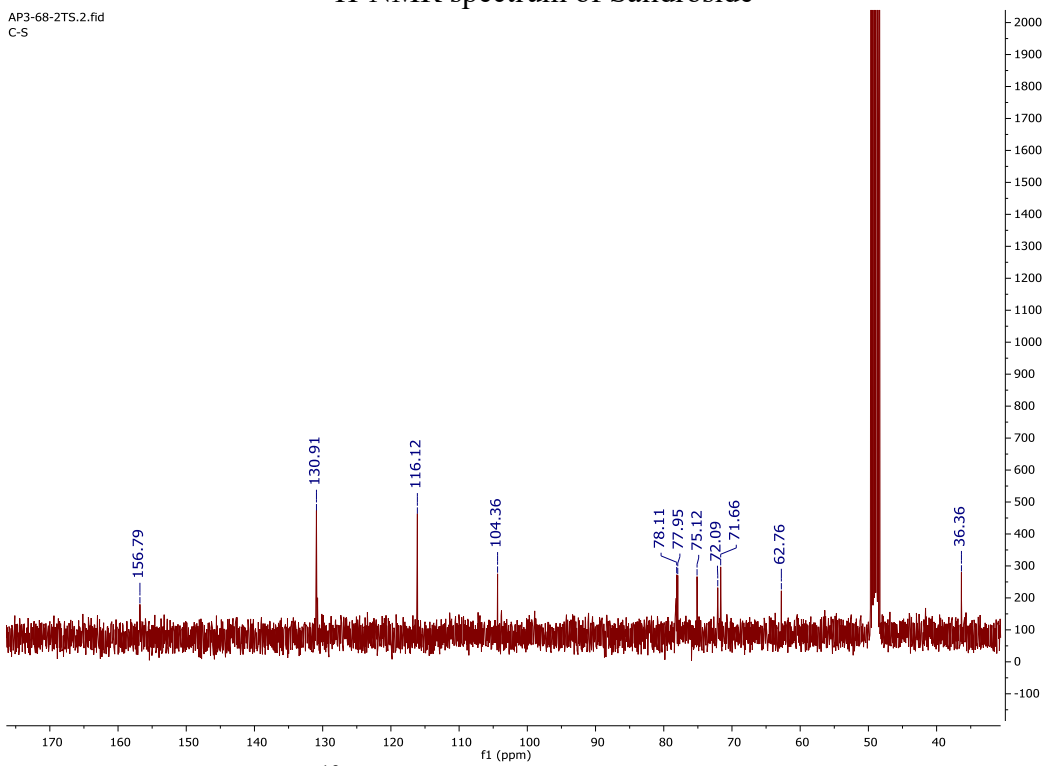


HMBC spectrum of dl-Pinoresinol

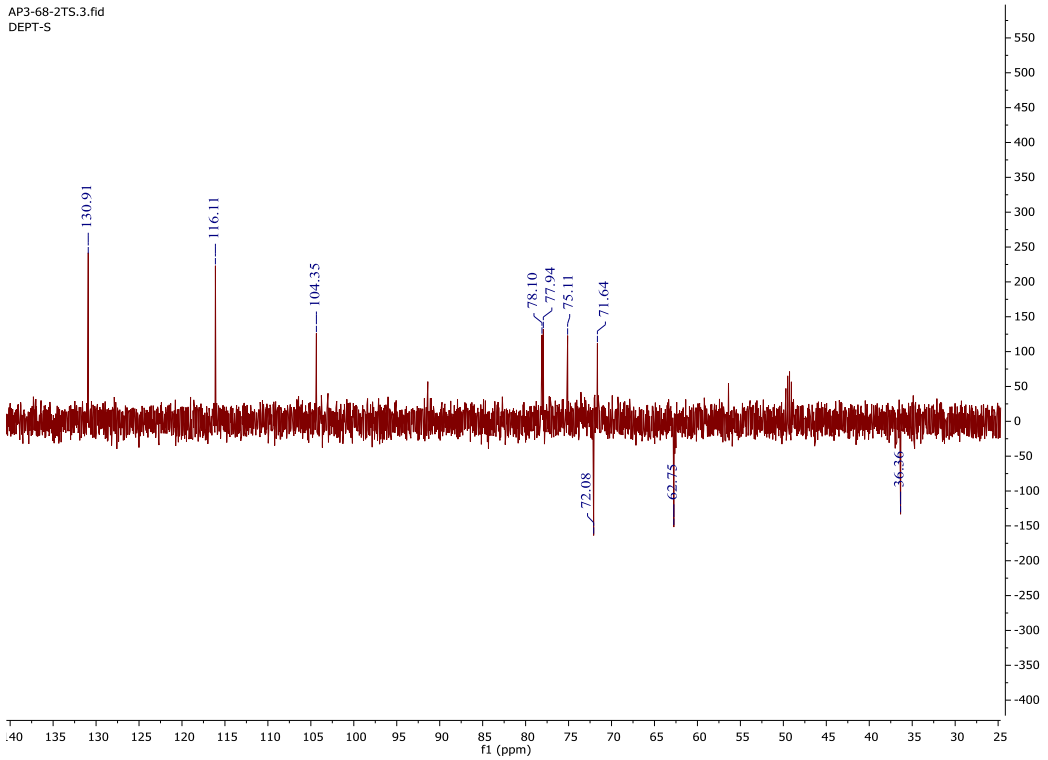
SI 23 1D and 2D NMR data Salidroside



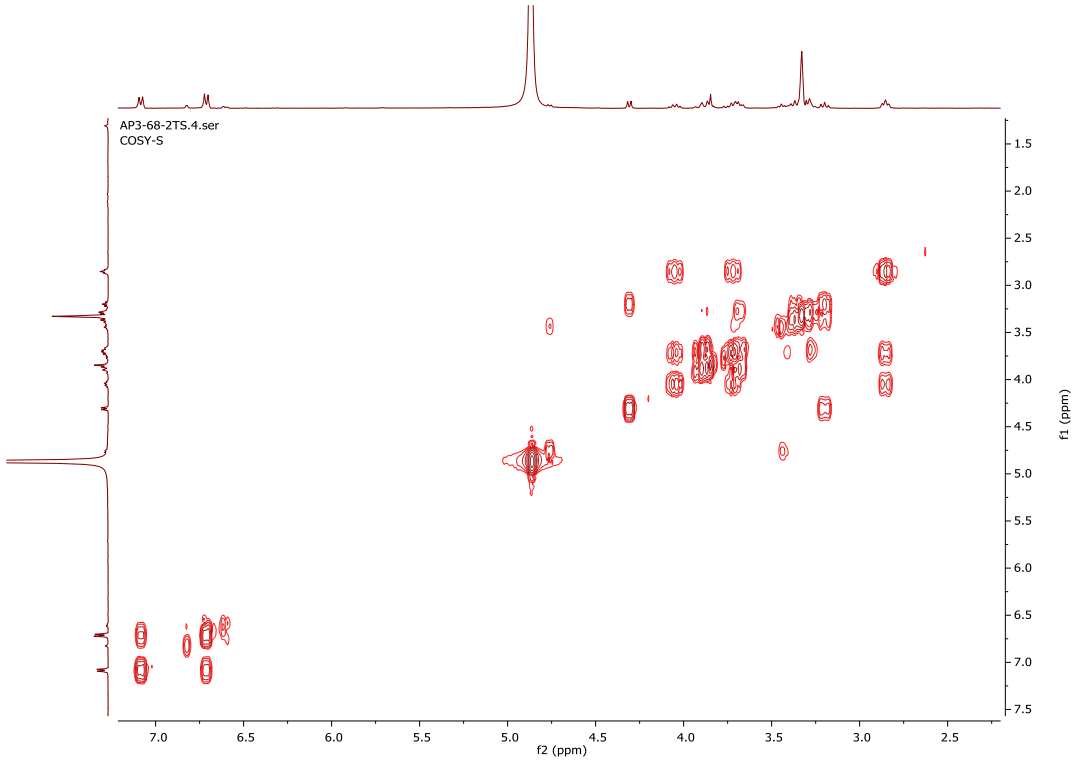
^{1}H-NMR spectrum of Salidroside



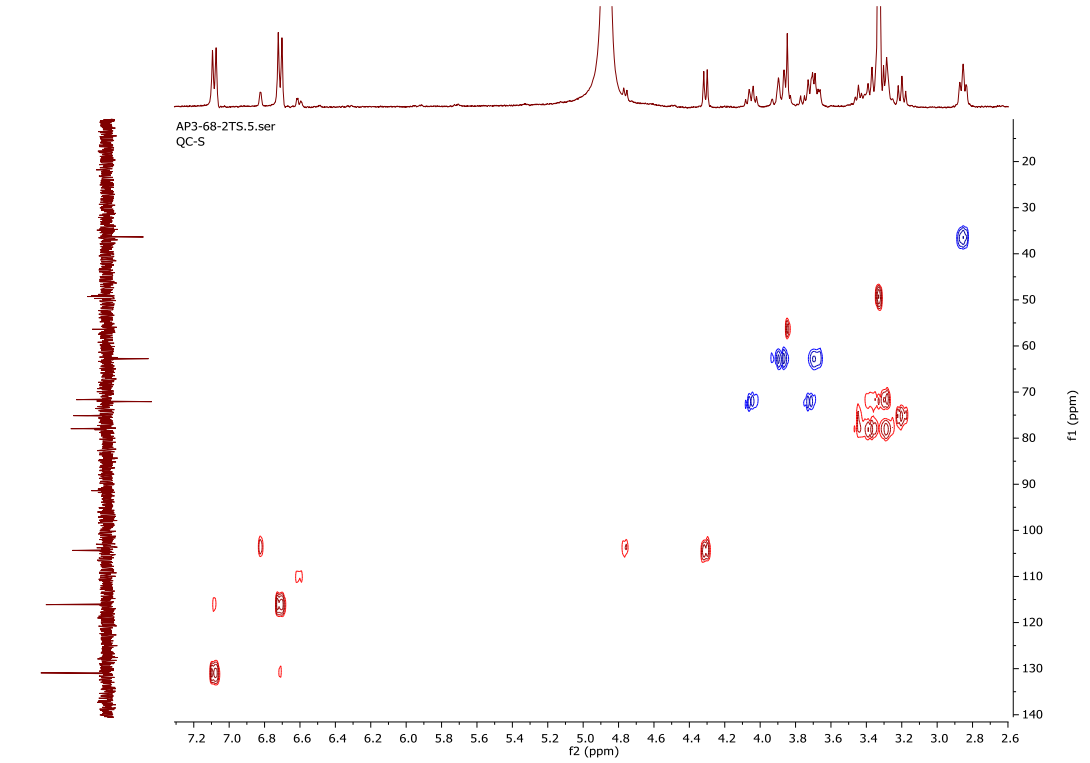
^{13}C-NMR spectrum of Salidroside



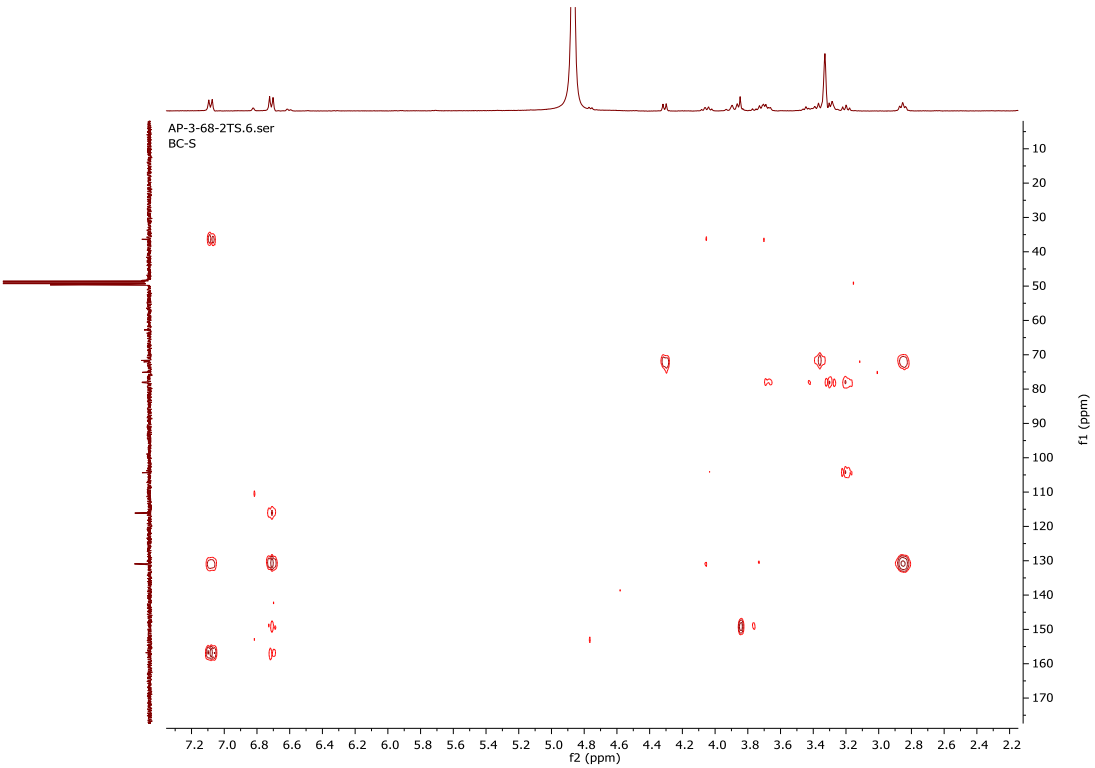
DEPT135 spectrum of Salidroside



COSY spectrum of Salidroside

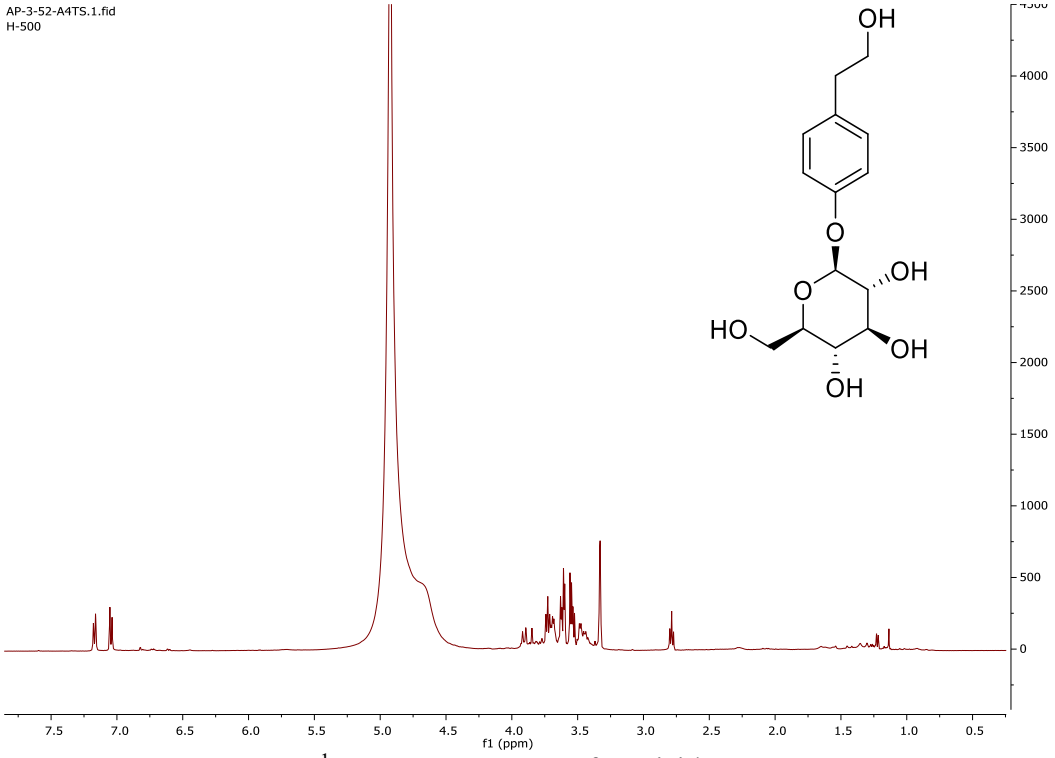


HSQC spectrum of Salidroside

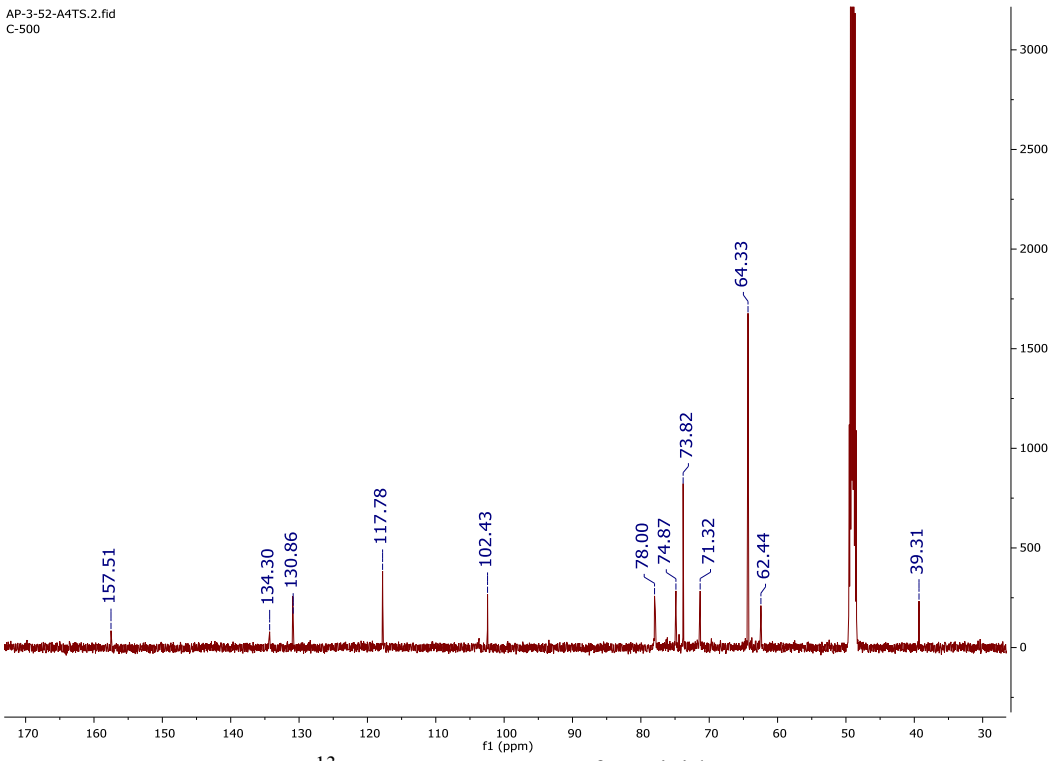


HMBC spectrum of Salidroside

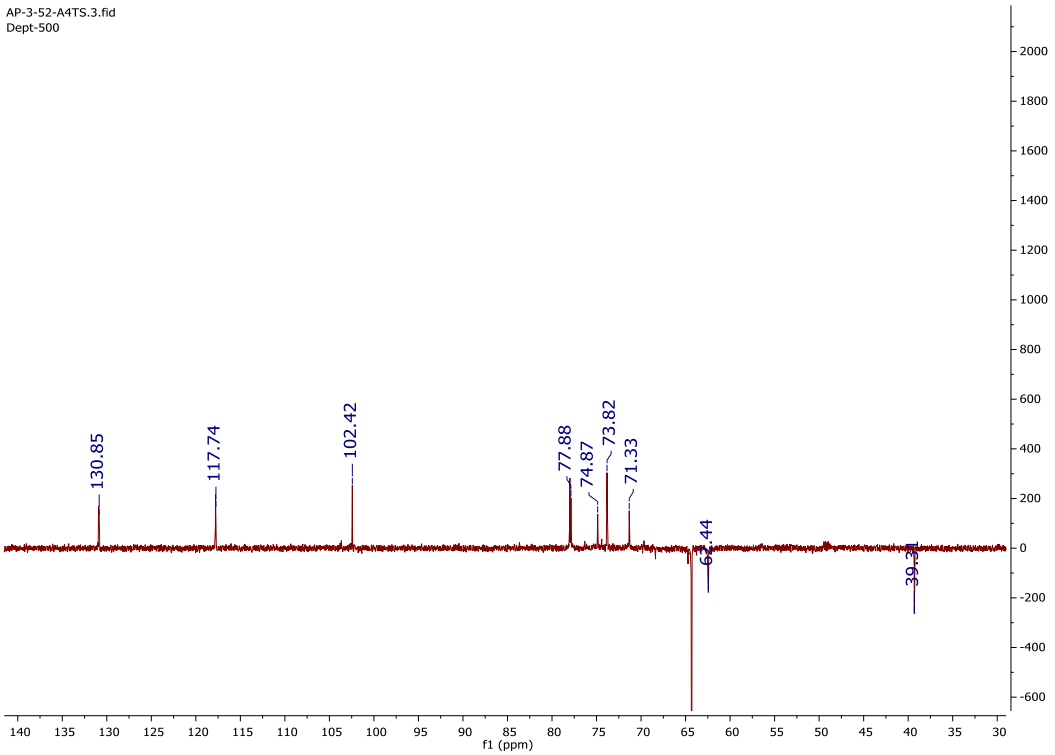
SI 24 1D and 2D NMR data Icariside D2



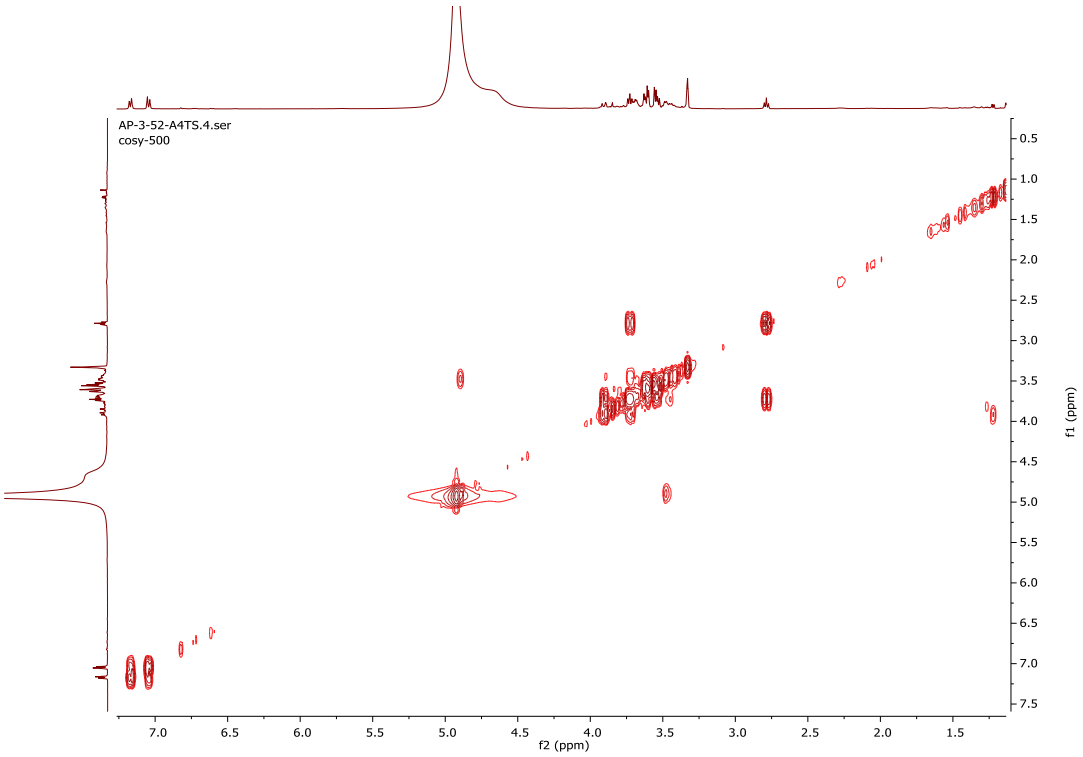
^{1}H-NMR spectrum of Icariside D2



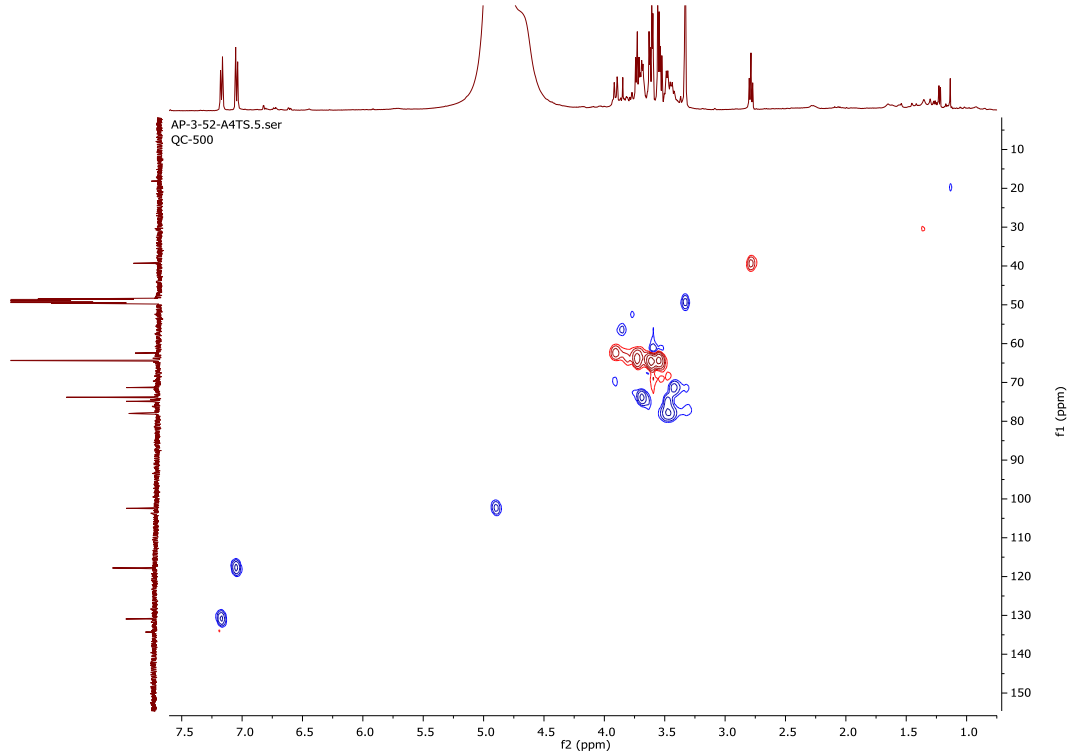
^{13}C-NMR spectrum of Icariside D2



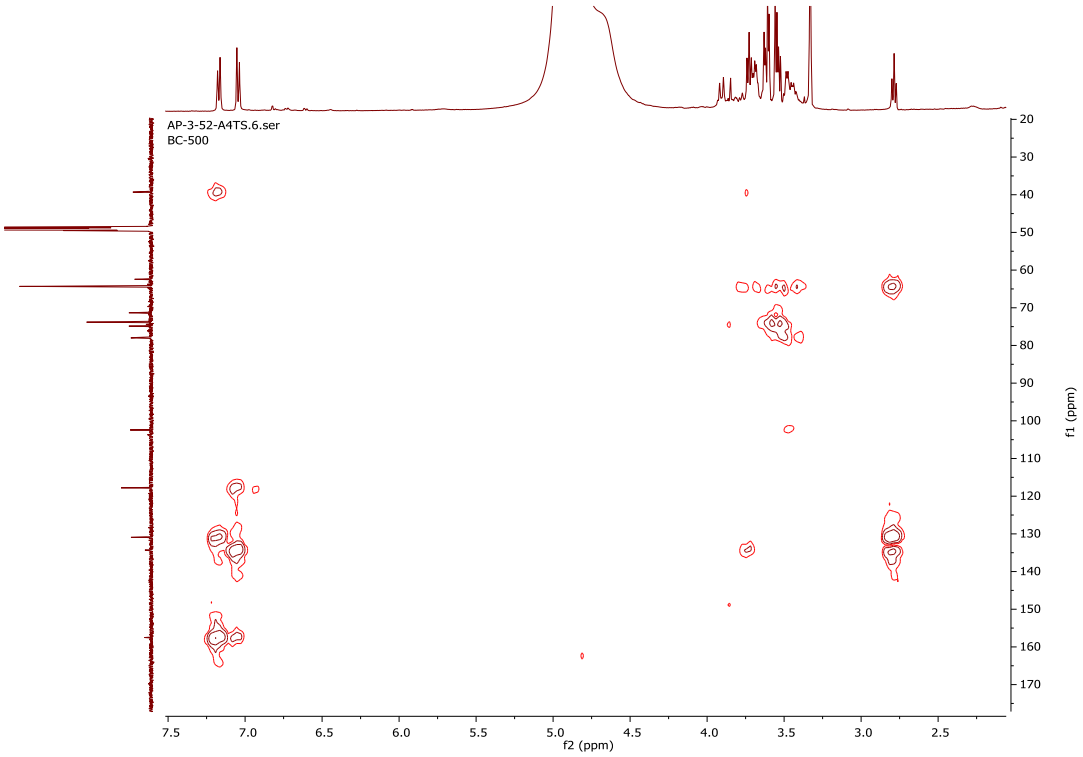
DEPT135 spectrum of Icariside D2



COSY spectrum of Icariside D2

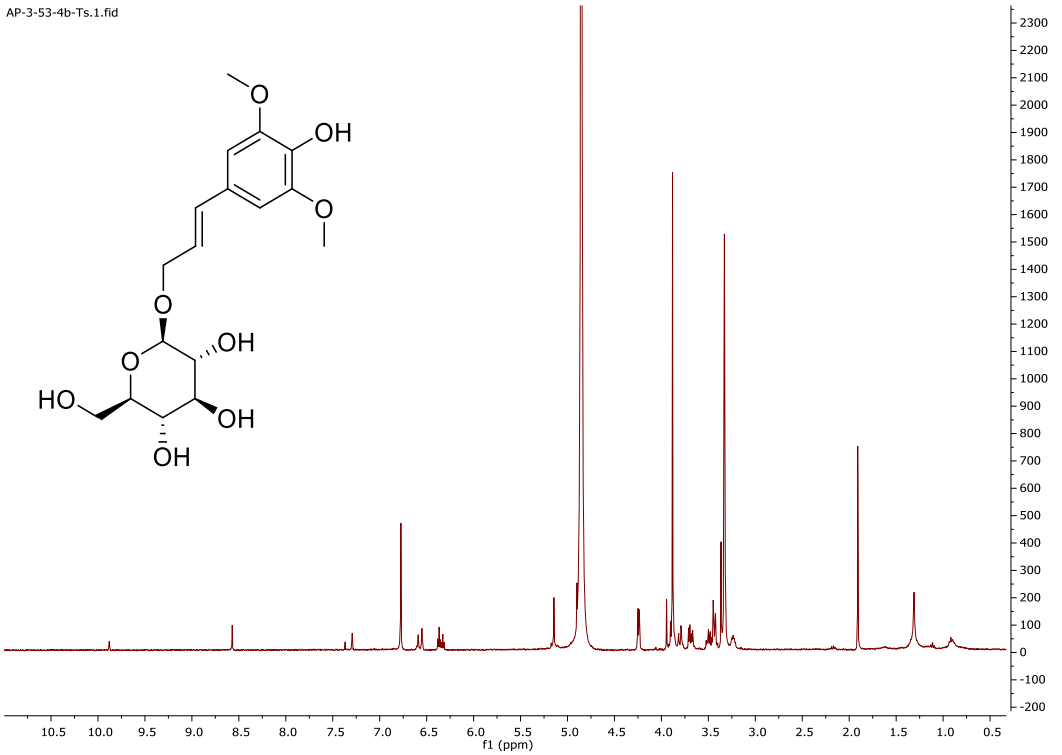


HSQC spectrum of Icariside D2

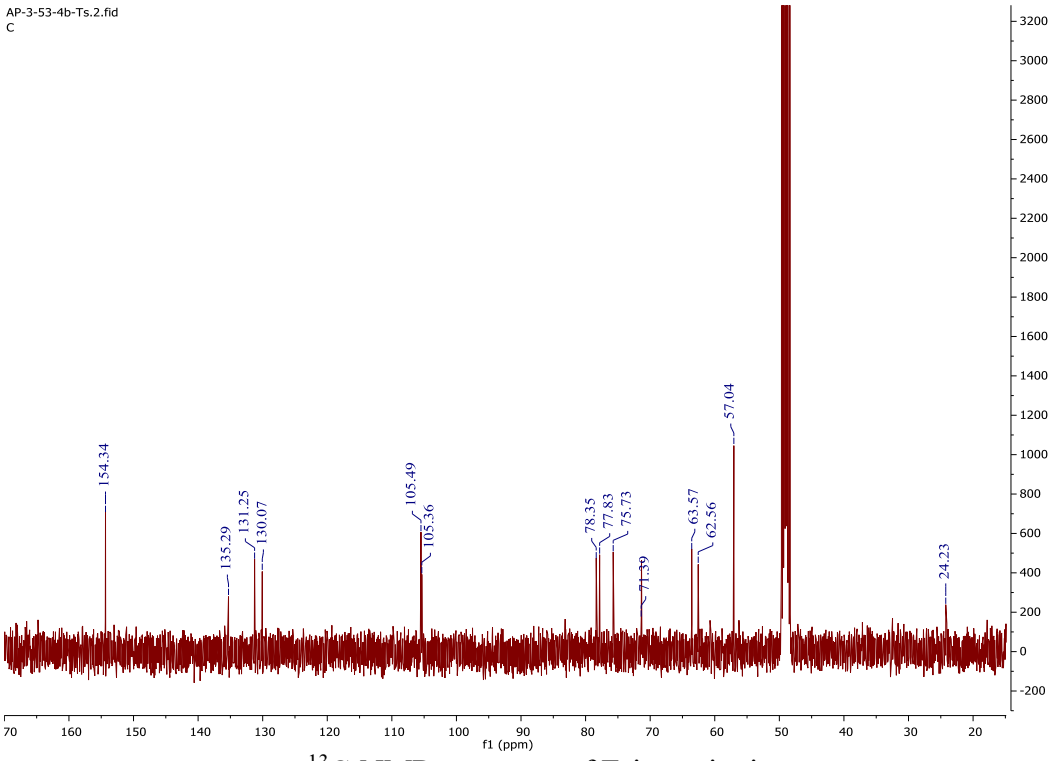


HMBC spectrum of Icariside D2

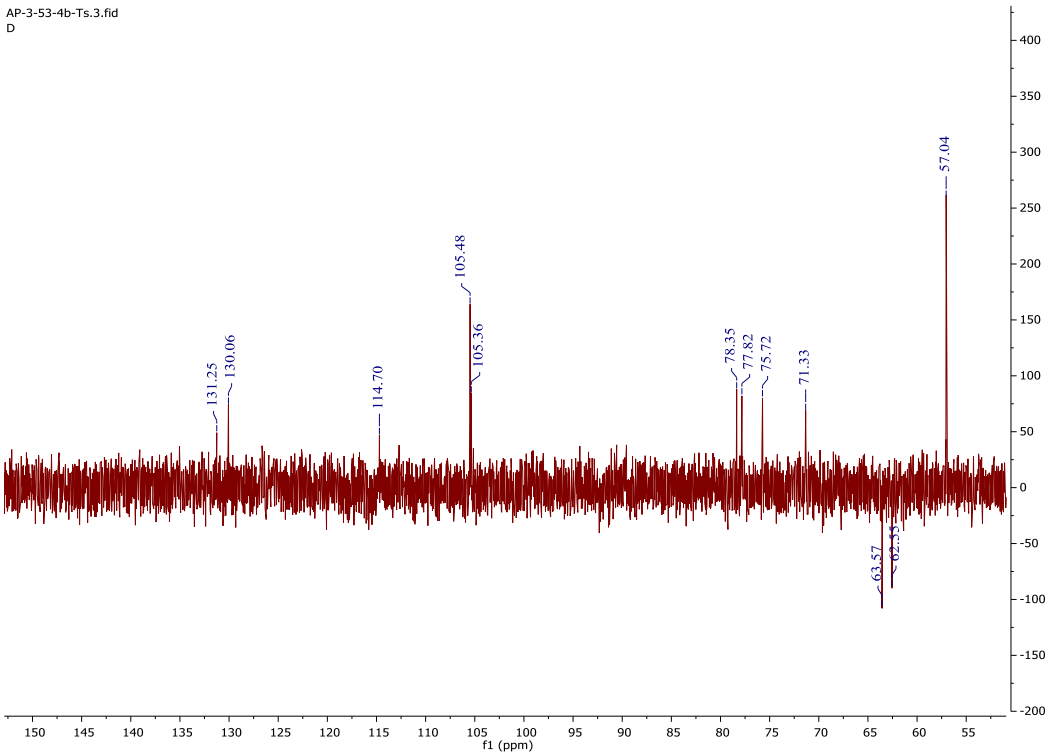
SI 25 1D and 2D NMR data E-isosyringin



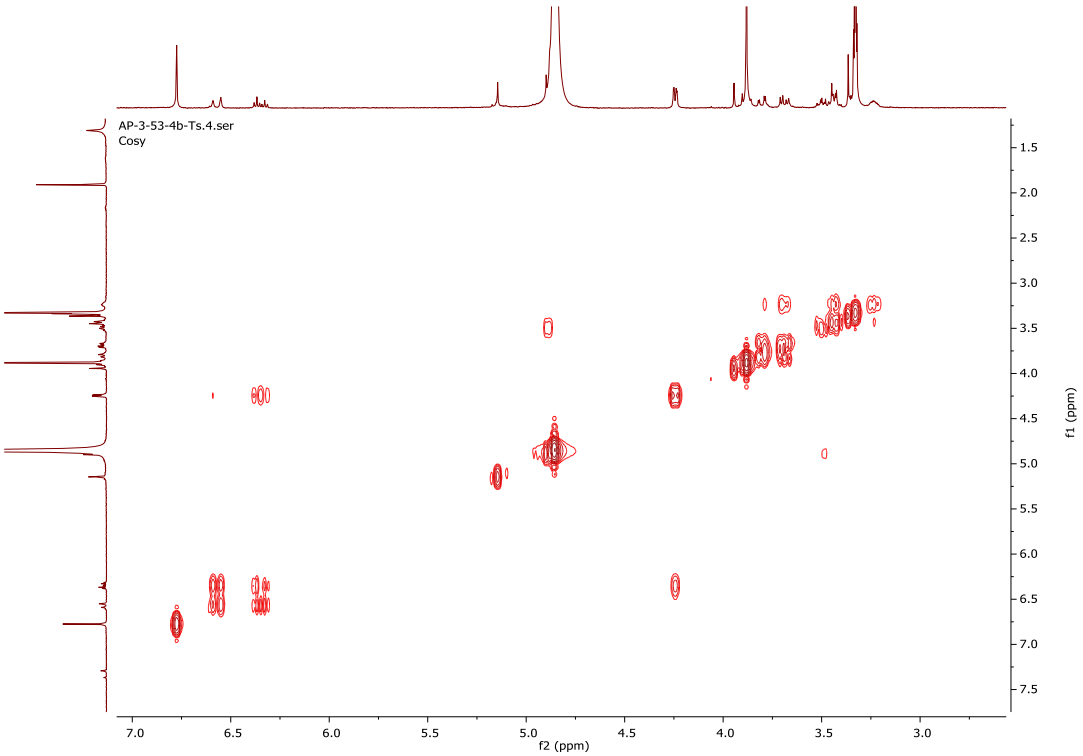
^{1}H-NMR spectrum of E-isosyringin



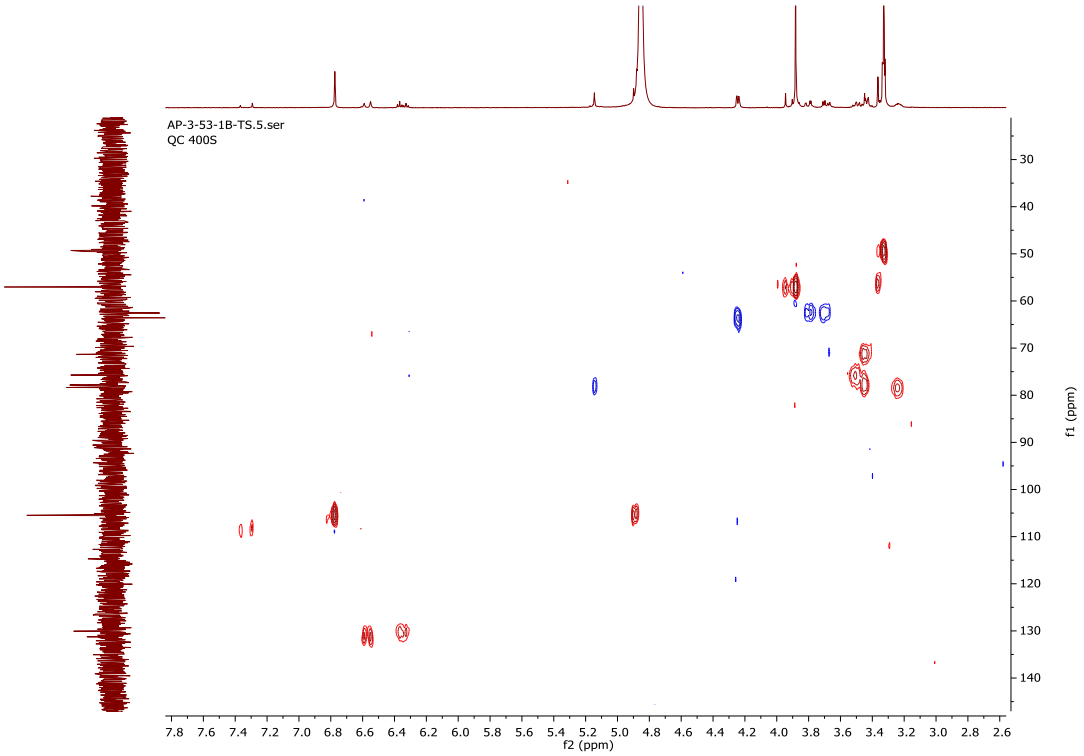
^{13}C-NMR spectrum of E-isosyringin



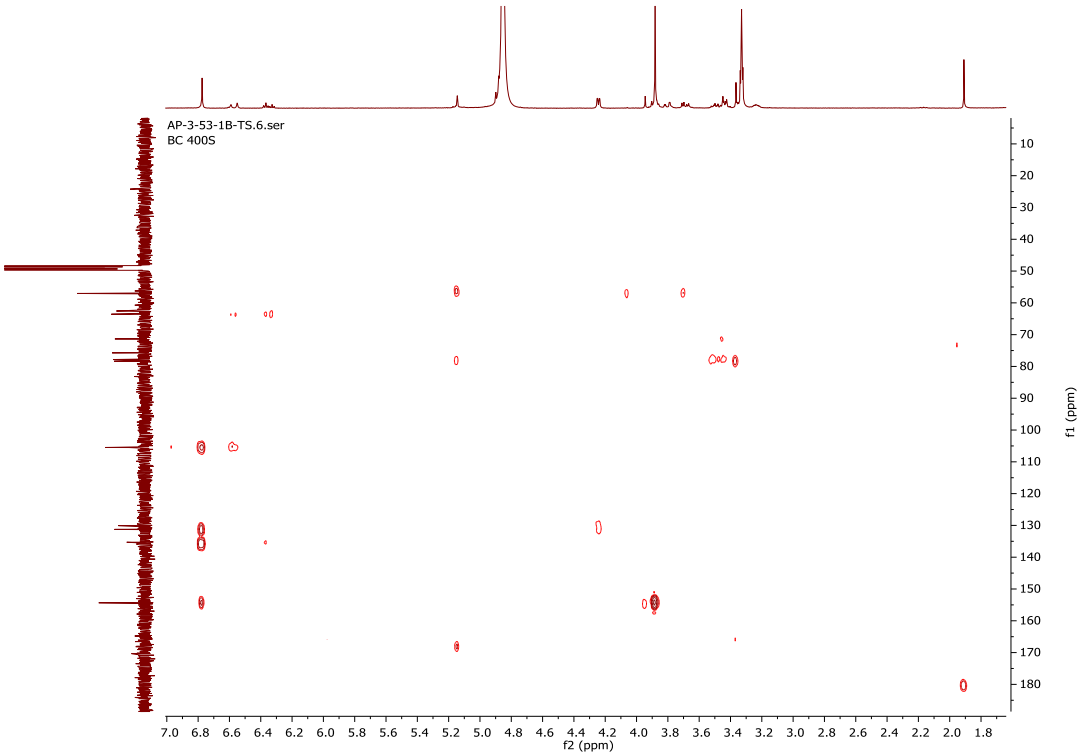
DEPT135 spectrum of E-isosyringin



COSY spectrum of E-isosyringin

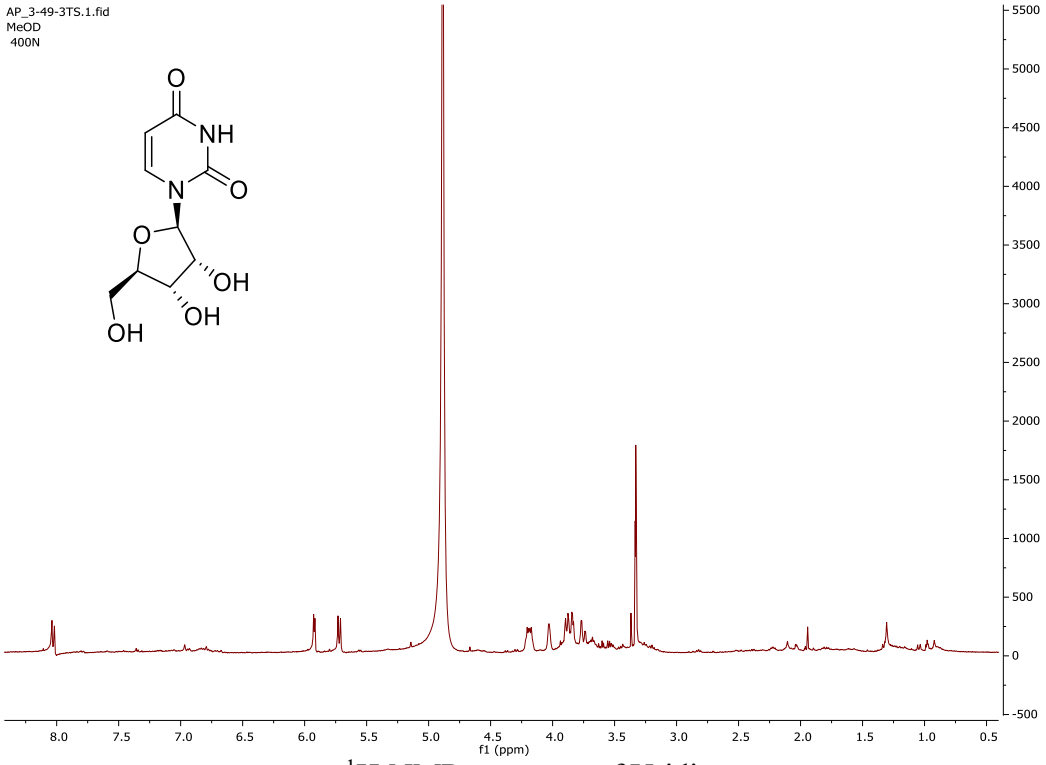


HSQC spectrum of E-isosyringin

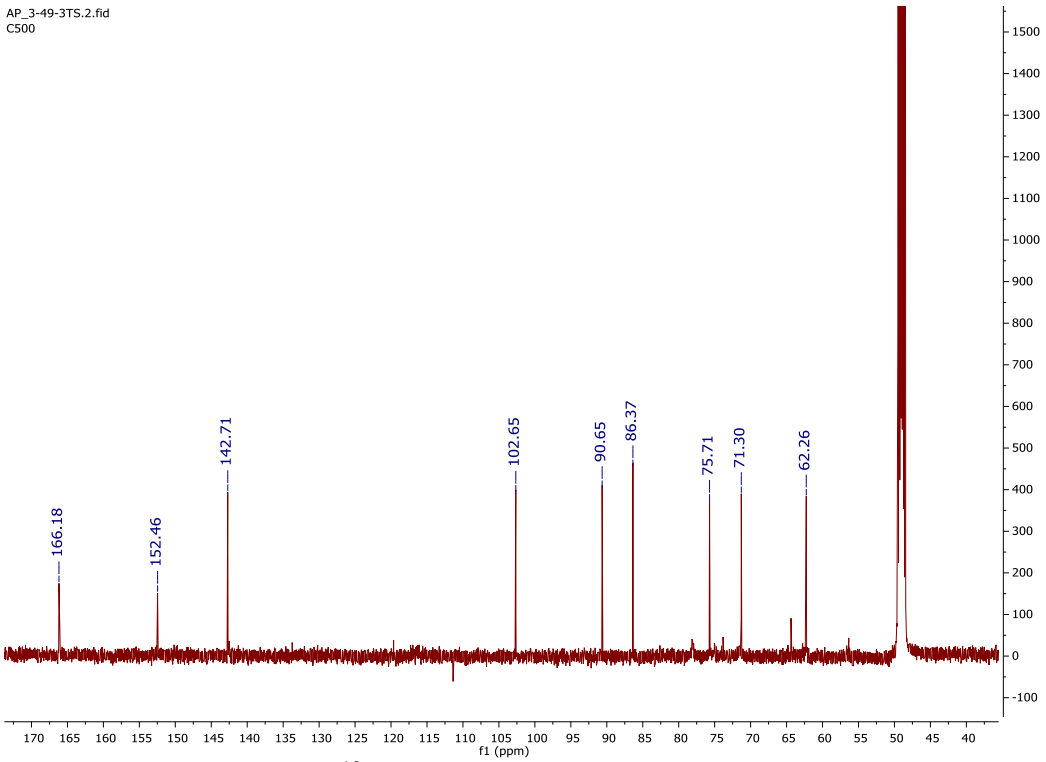


HMBC spectrum of E-isosyringin

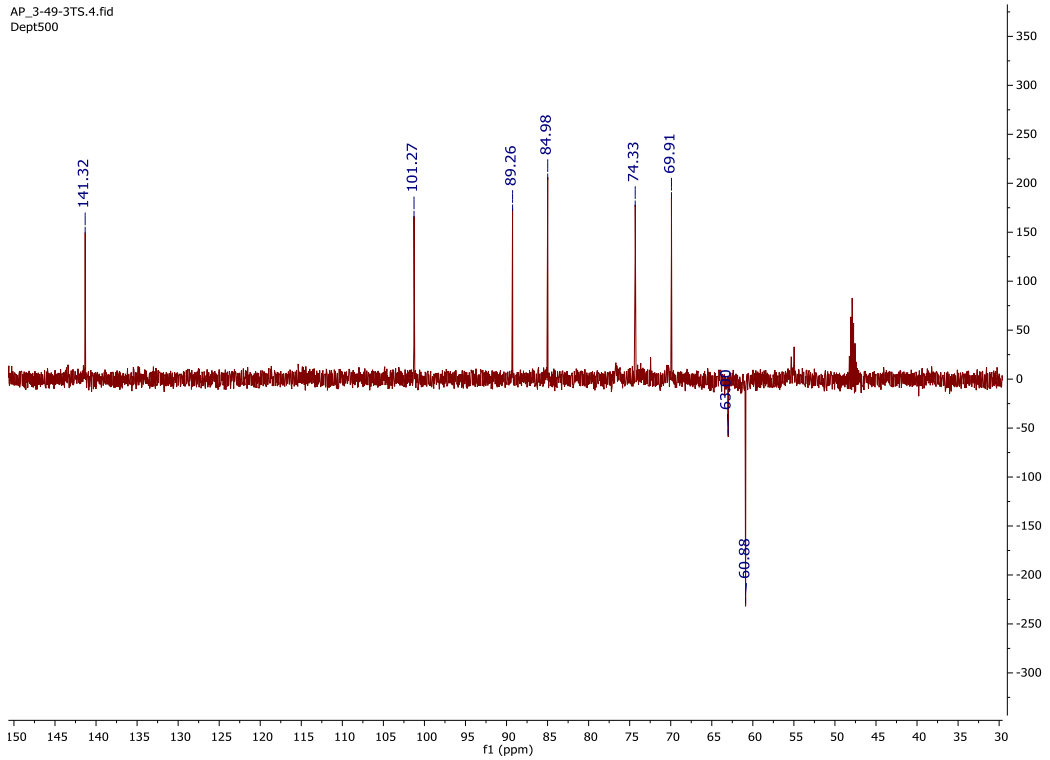
SI 26 1D and 2D NMR data Uridine



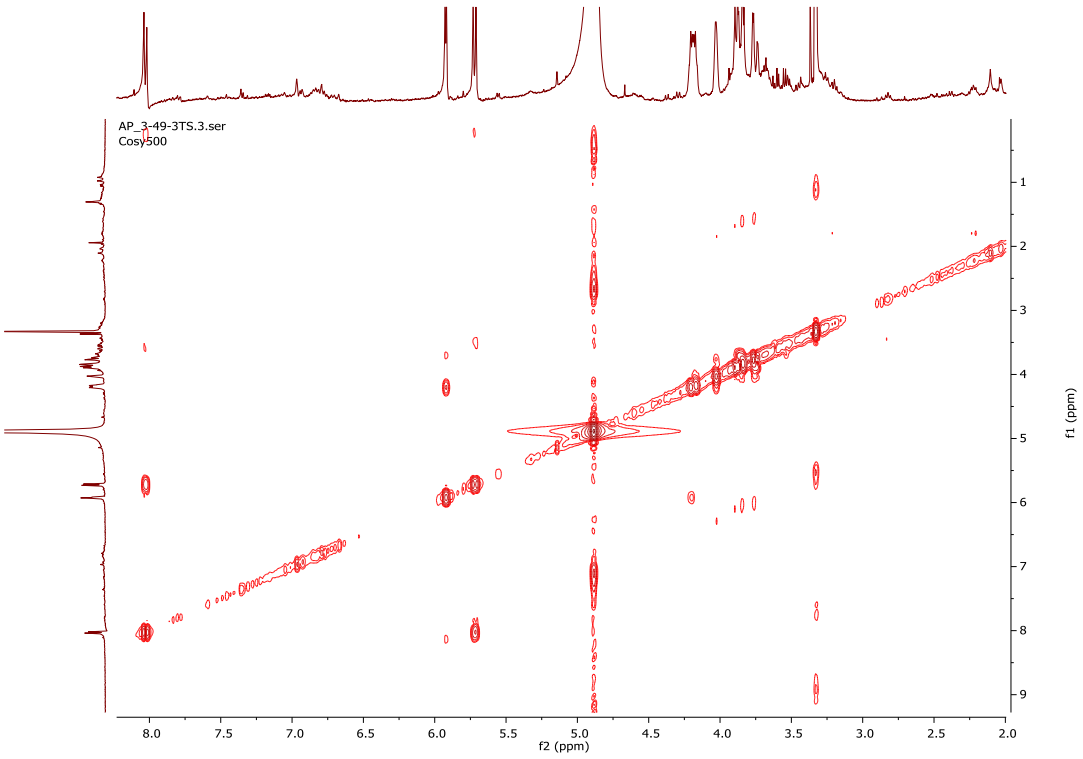
^{1}H-NMR spectrum of Uridine



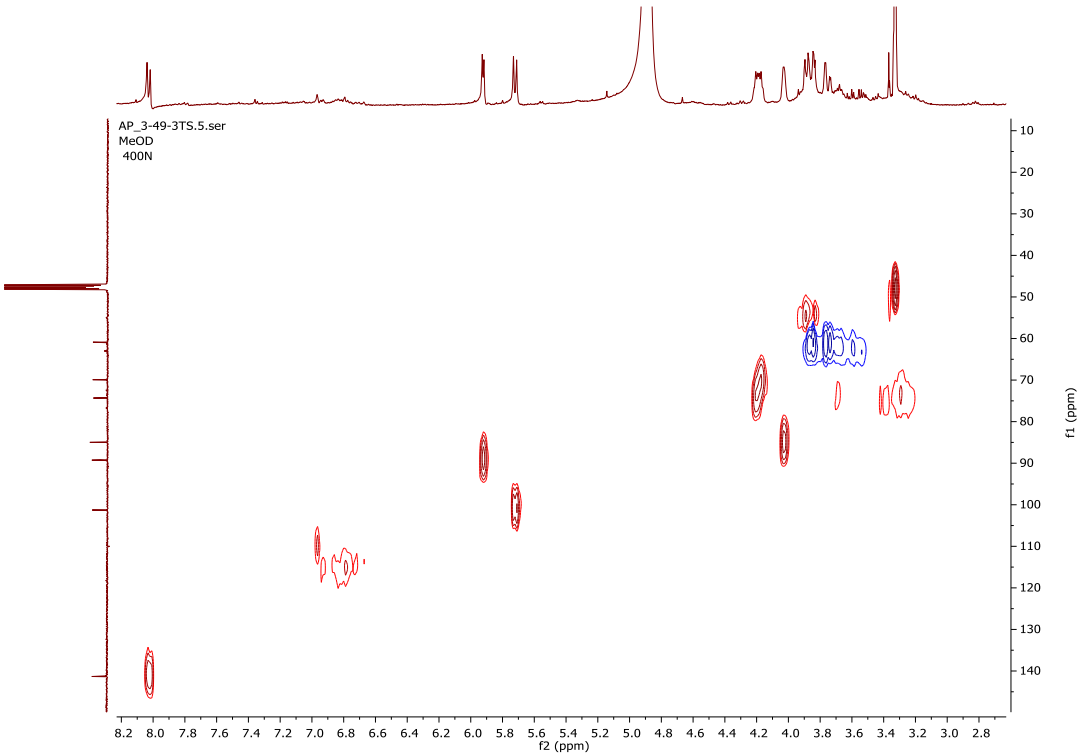
^{13}C-NMR spectrum of Uridine



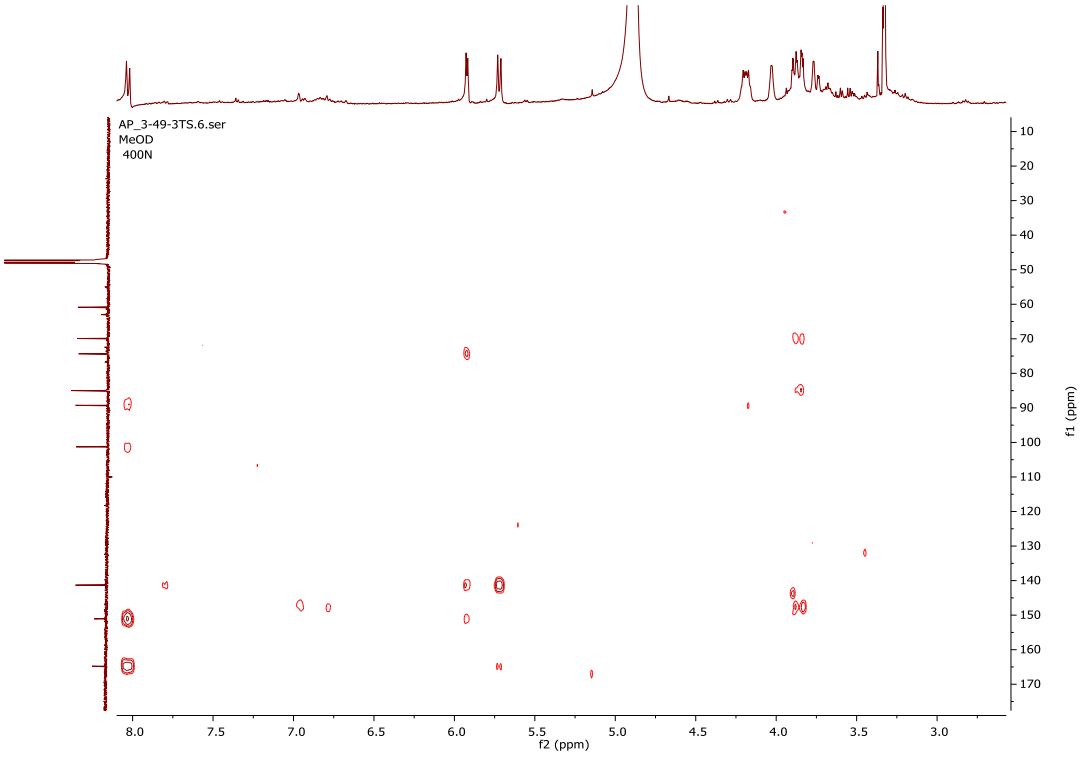
DEPT135 spectrum of Uridine



COSY spectrum of Uridine

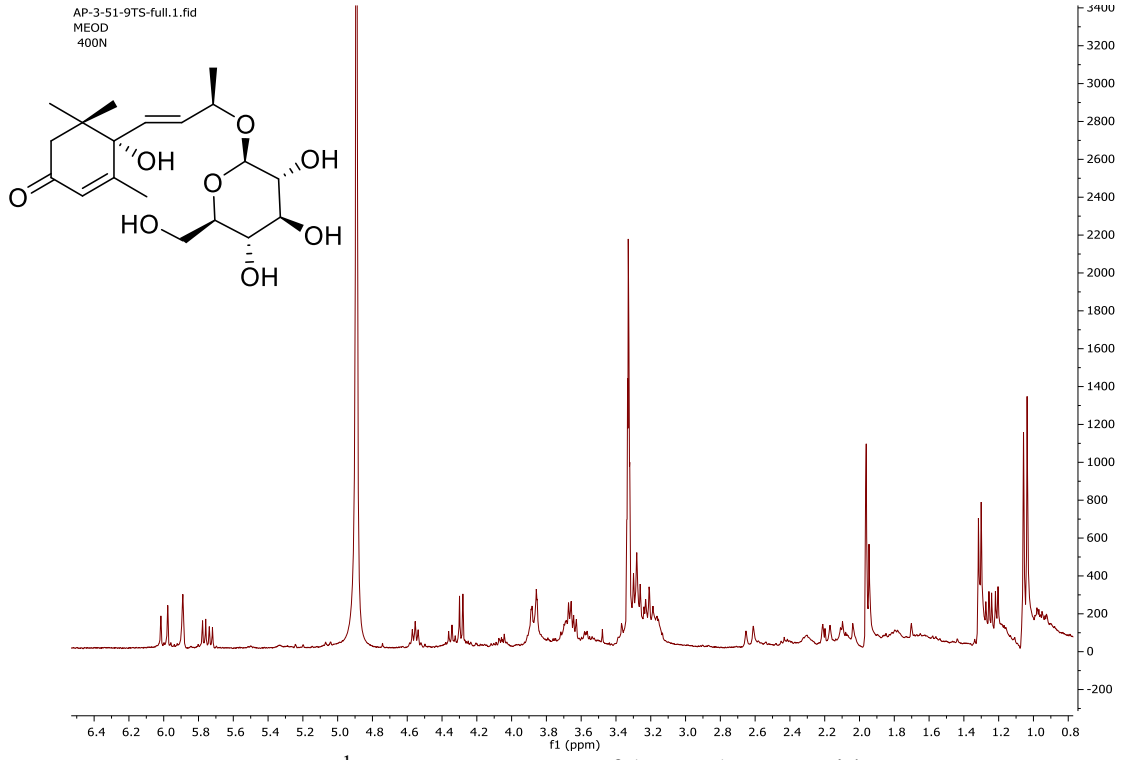


HSQC spectrum of Uridine

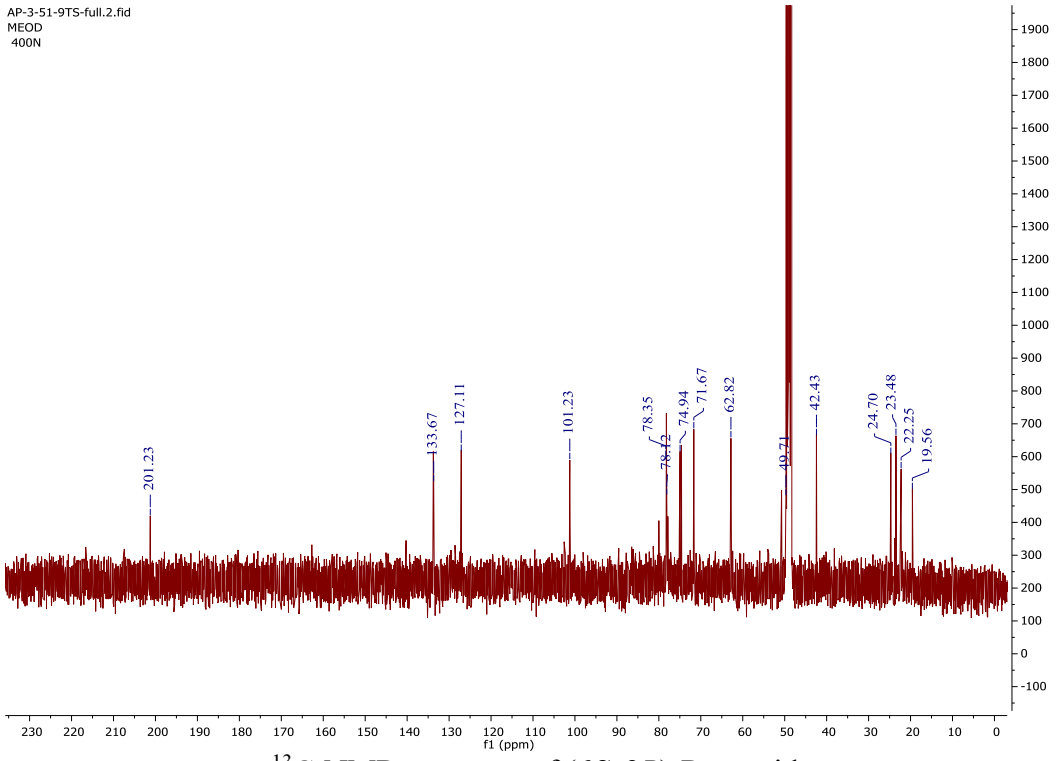


HMBC spectrum of Uridine

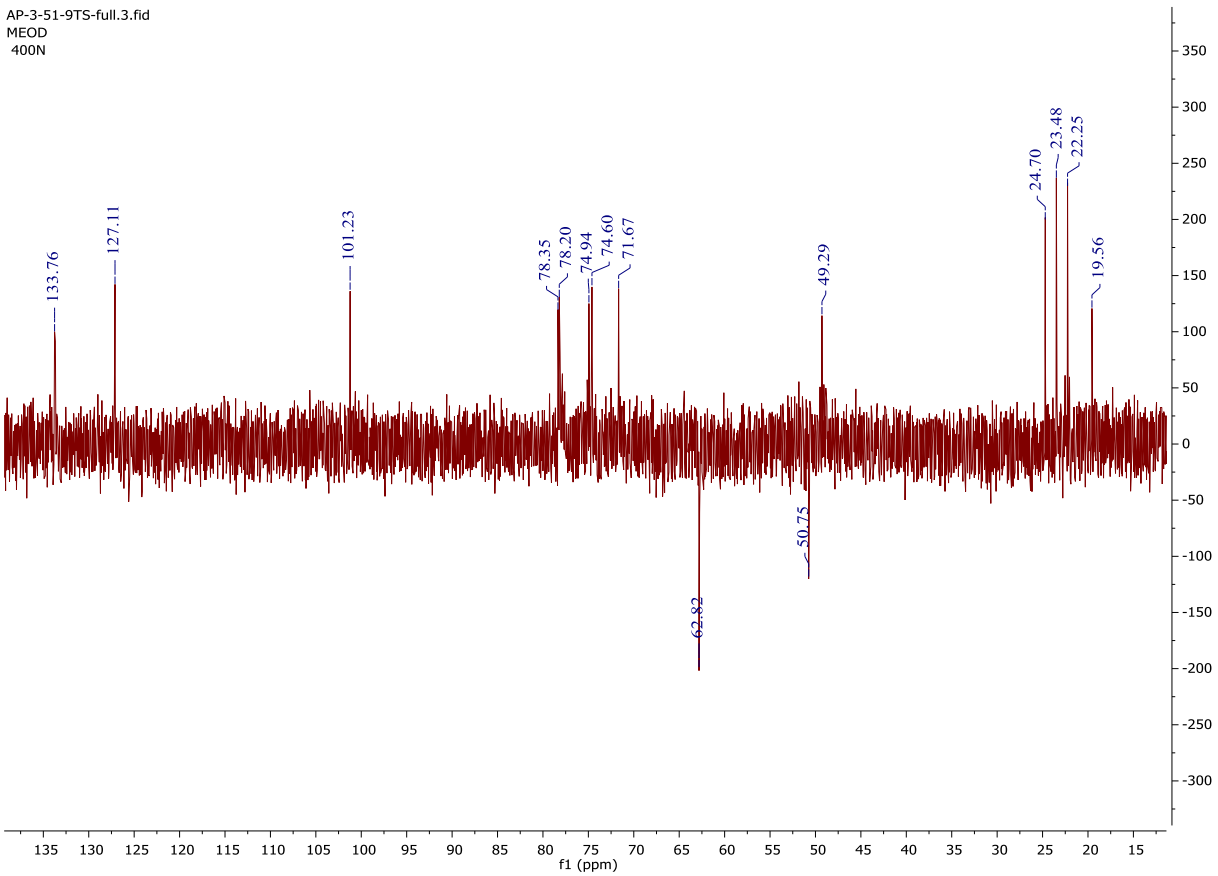
SI 27 1D and 2D NMR data (6*S*, 9*R*)-Roseoside



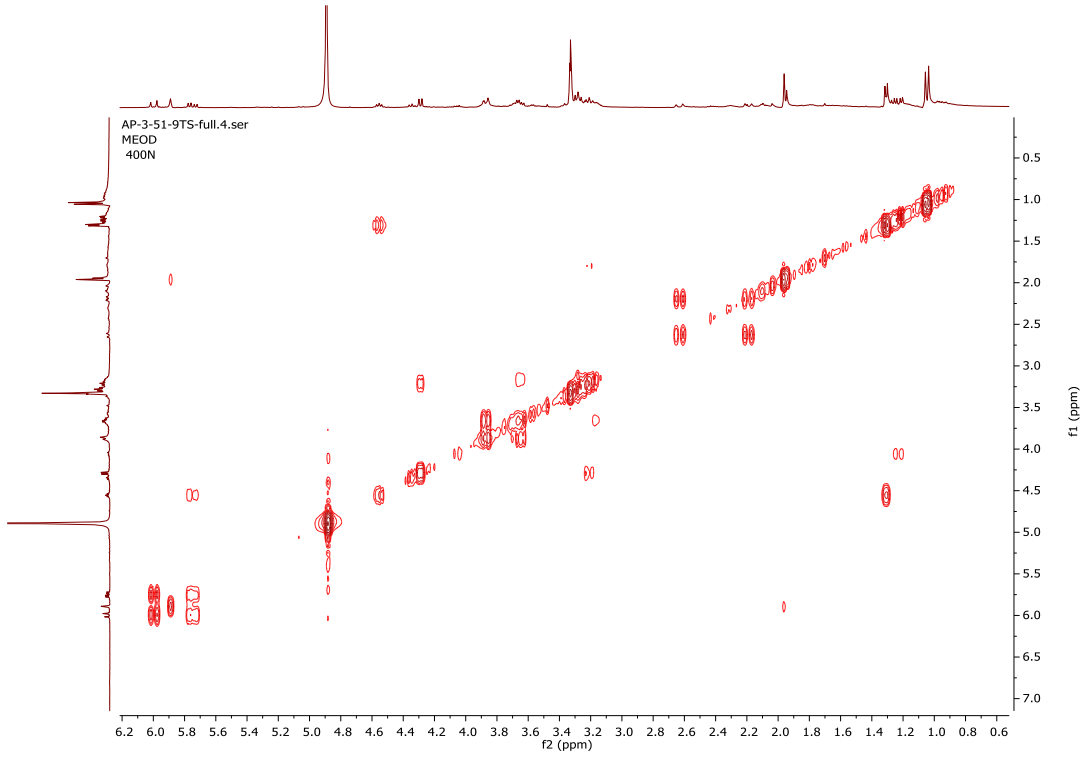
^{1}H-NMR spectrum of (6*S*, 9*R*)-Roseoside



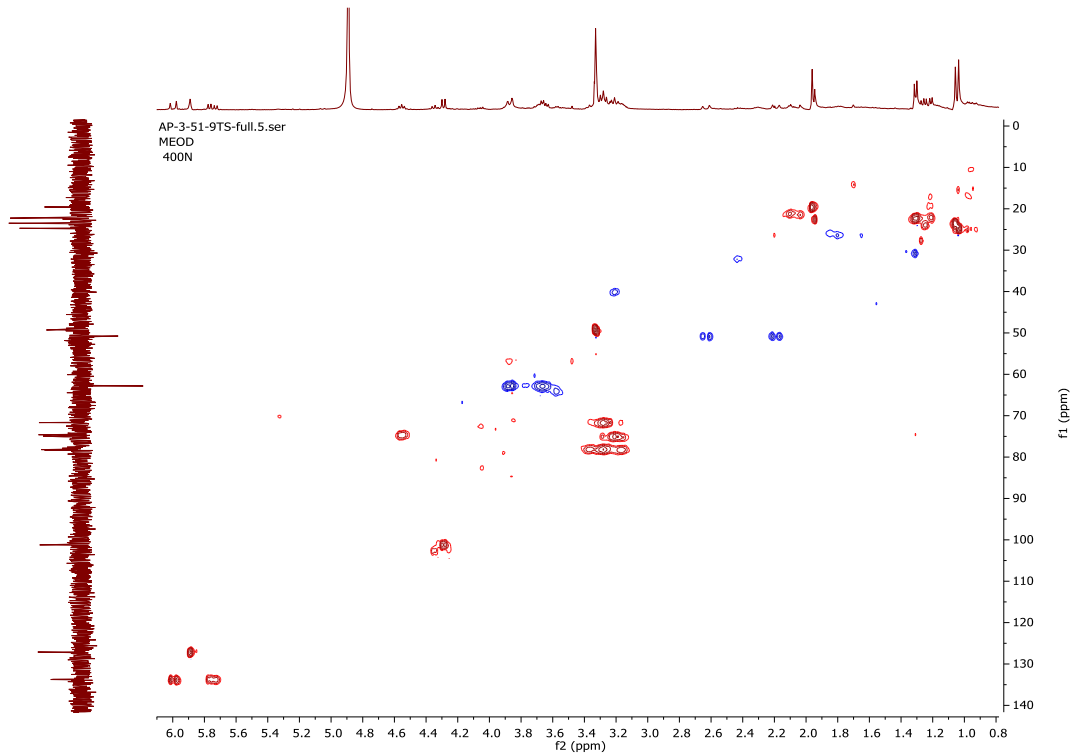
^{13}C-NMR spectrum of (6*S*, 9*R*)-Roseoside



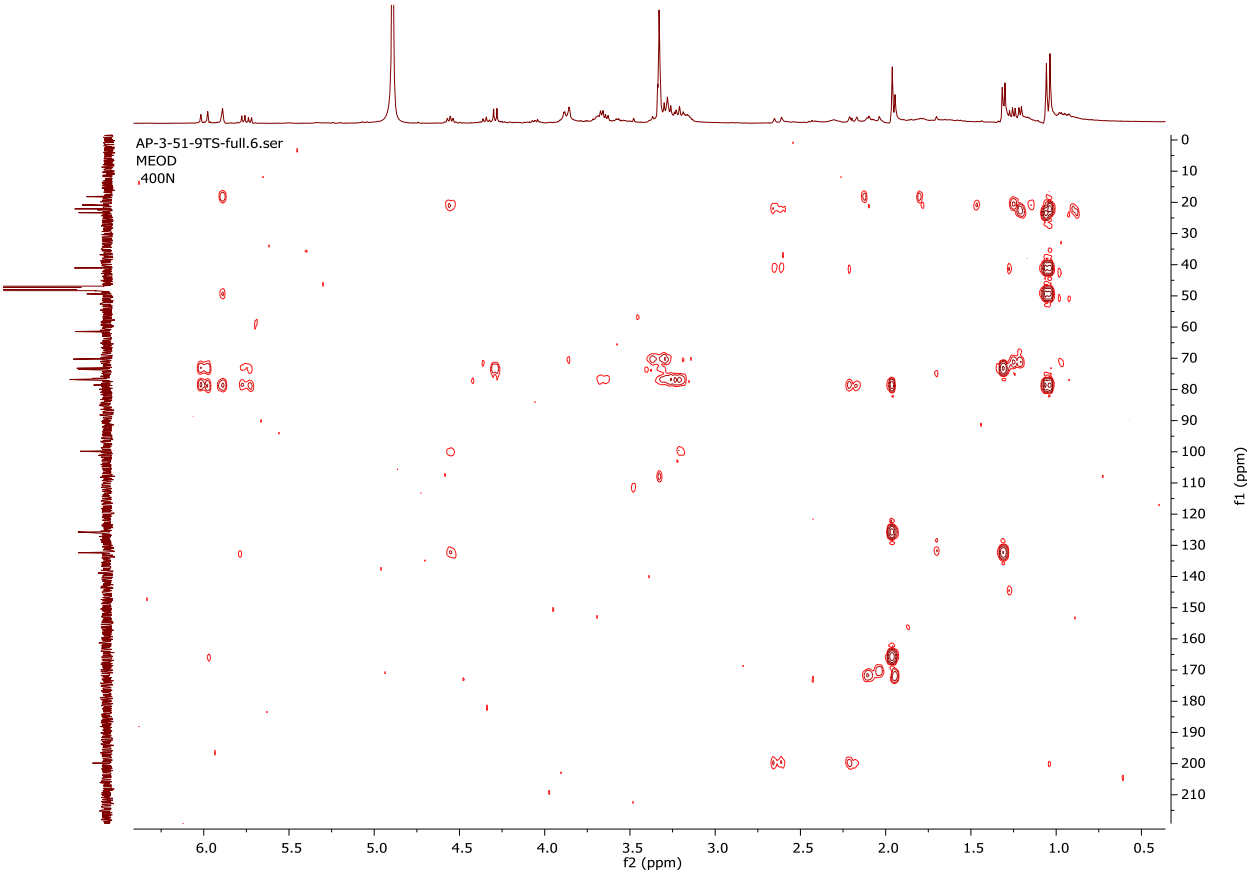
DEPT135 spectrum of (6*S,* 9*R*)-Roseoside



COSY spectrum of (6*S,* 9*R*)-Roseoside

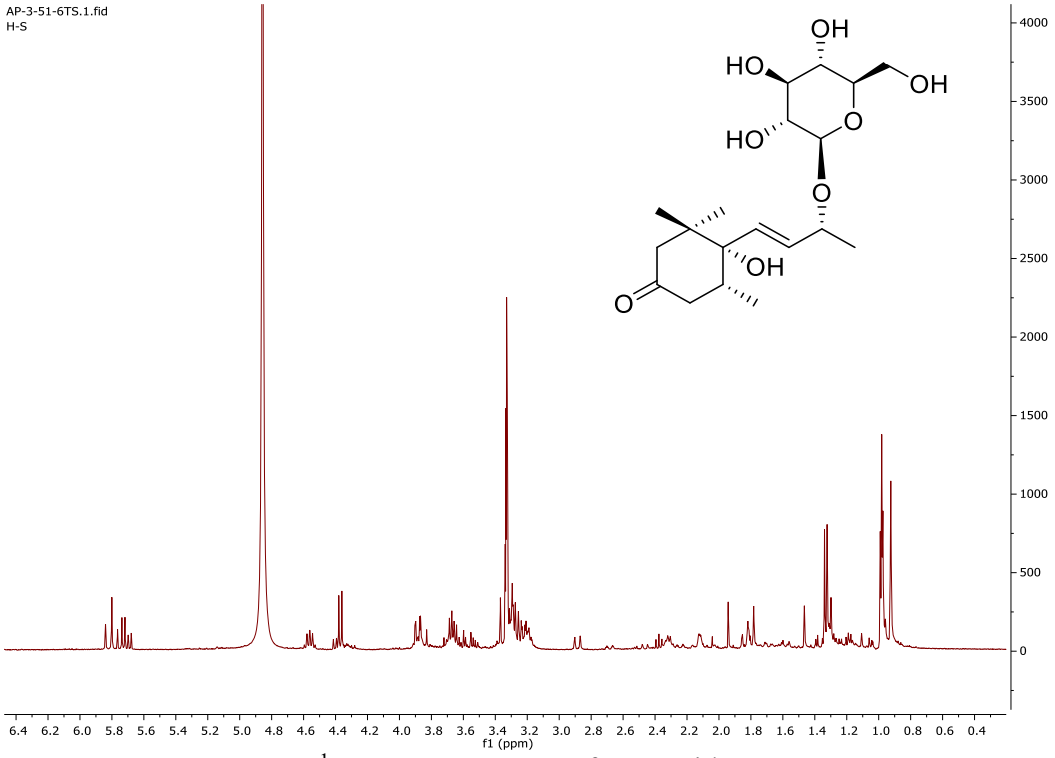


HSQC spectrum of (6*S,* 9*R*)-Roseoside

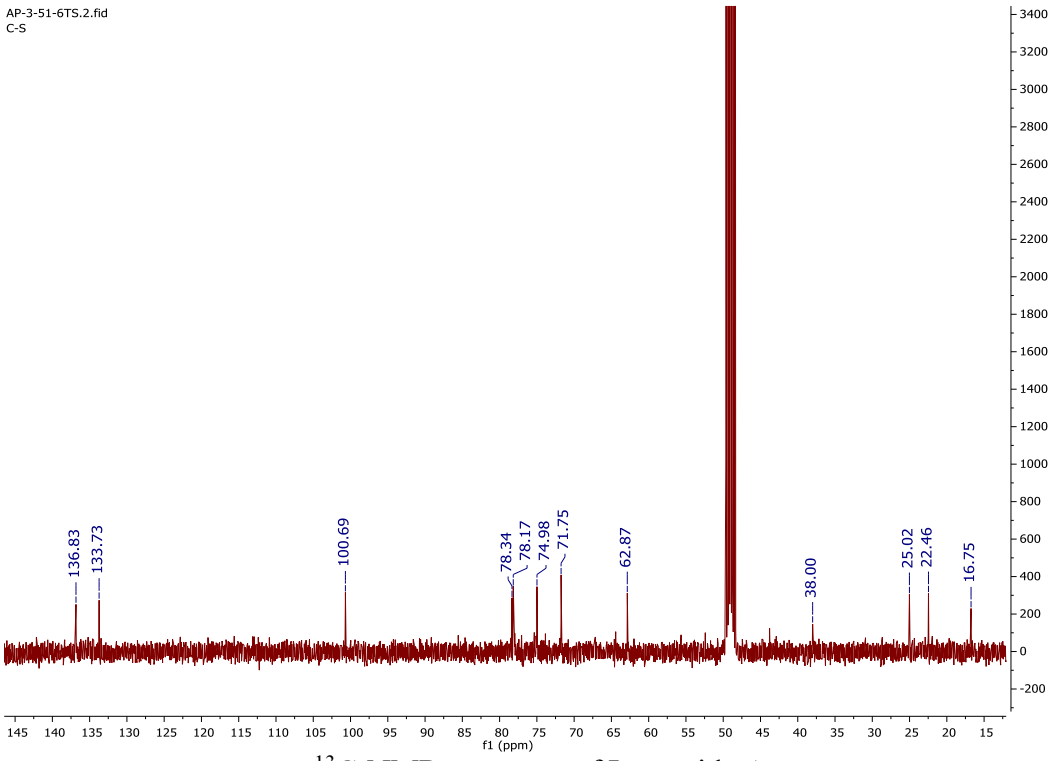


HMBC spectrum of (6*S,* 9*R*)-Roseoside

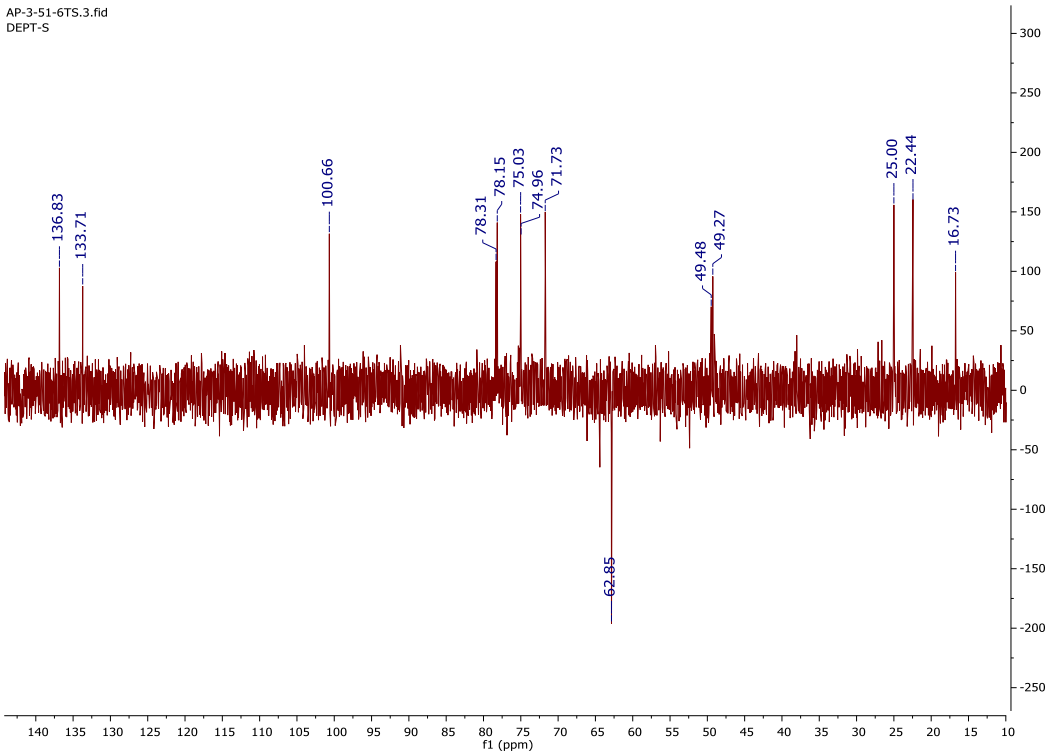
SI 28 1D and 2D NMR data Lauroside A



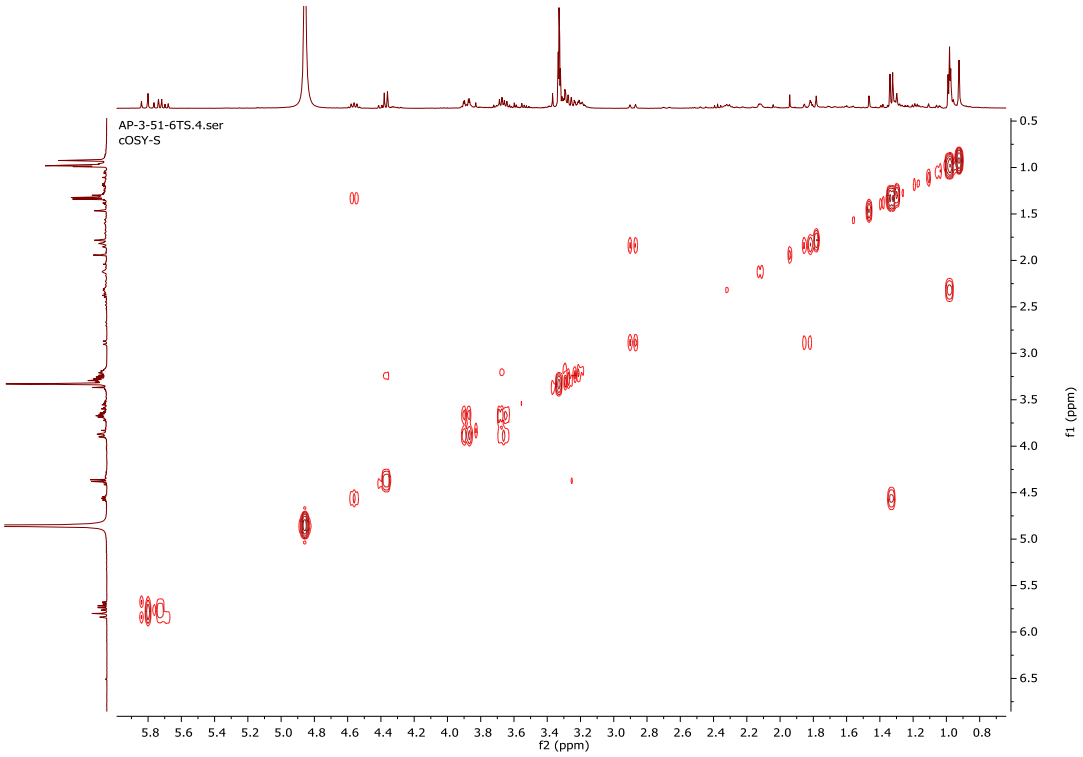
^{1}H-NMR spectrum of Lauroside A



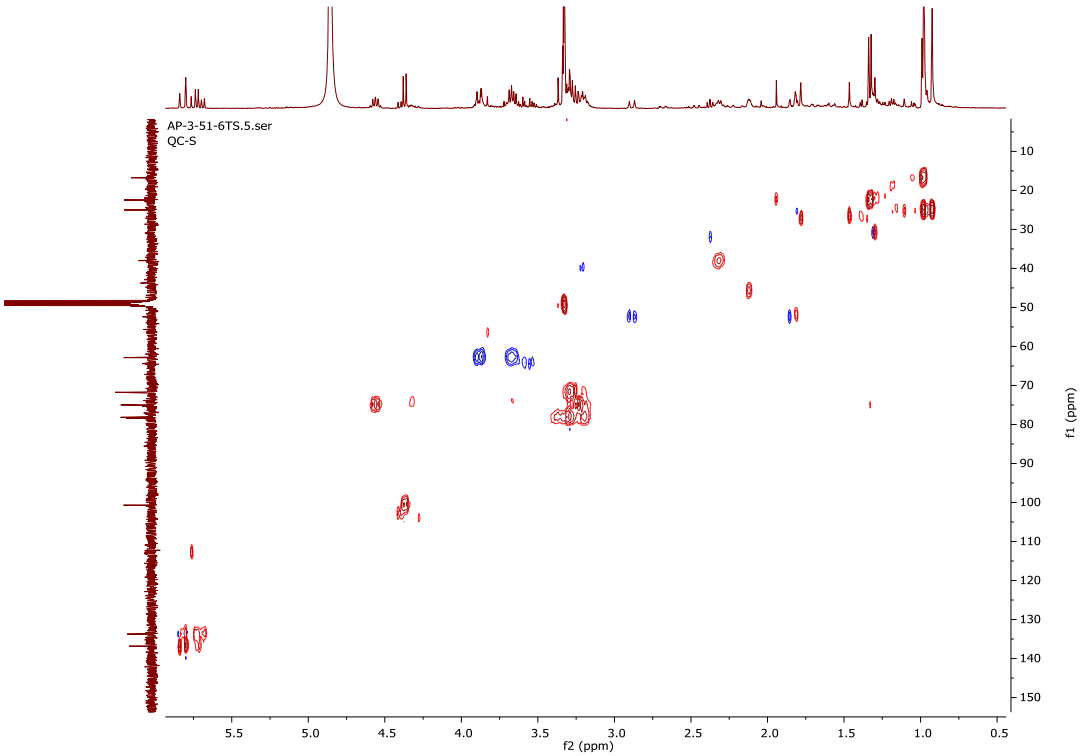
^{13}C-NMR spectrum of Lauroside A



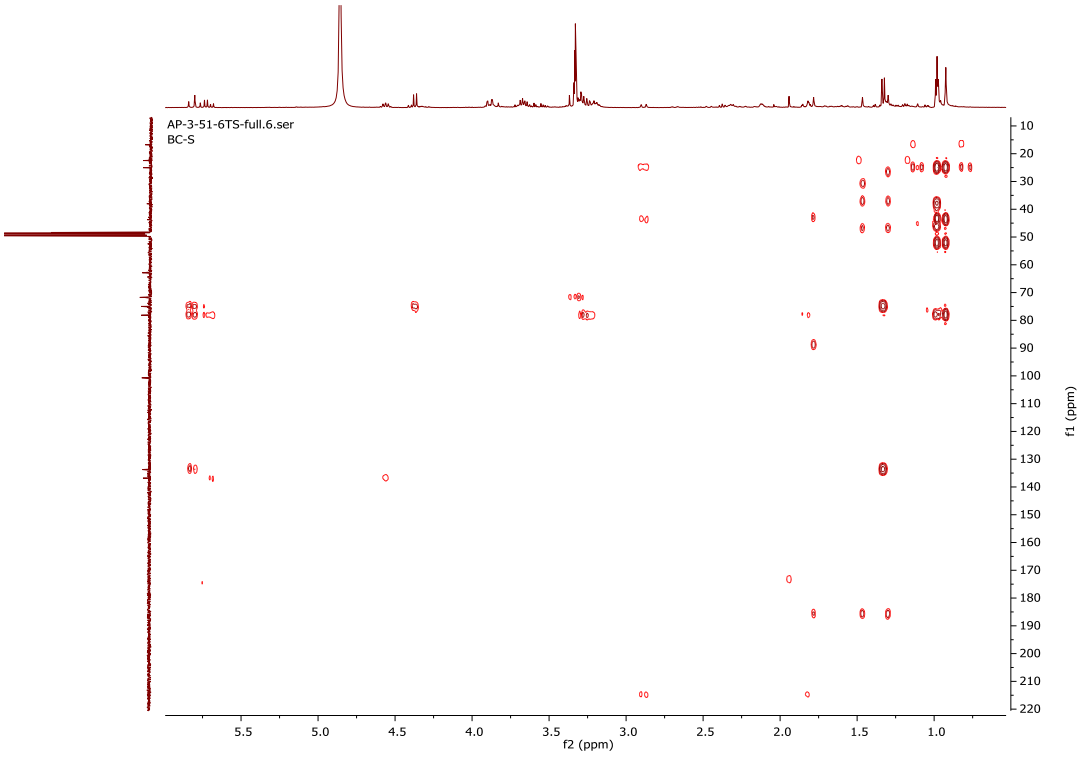
DEPT135 spectrum of Lauroside A



COSY spectrum of Lauroside A

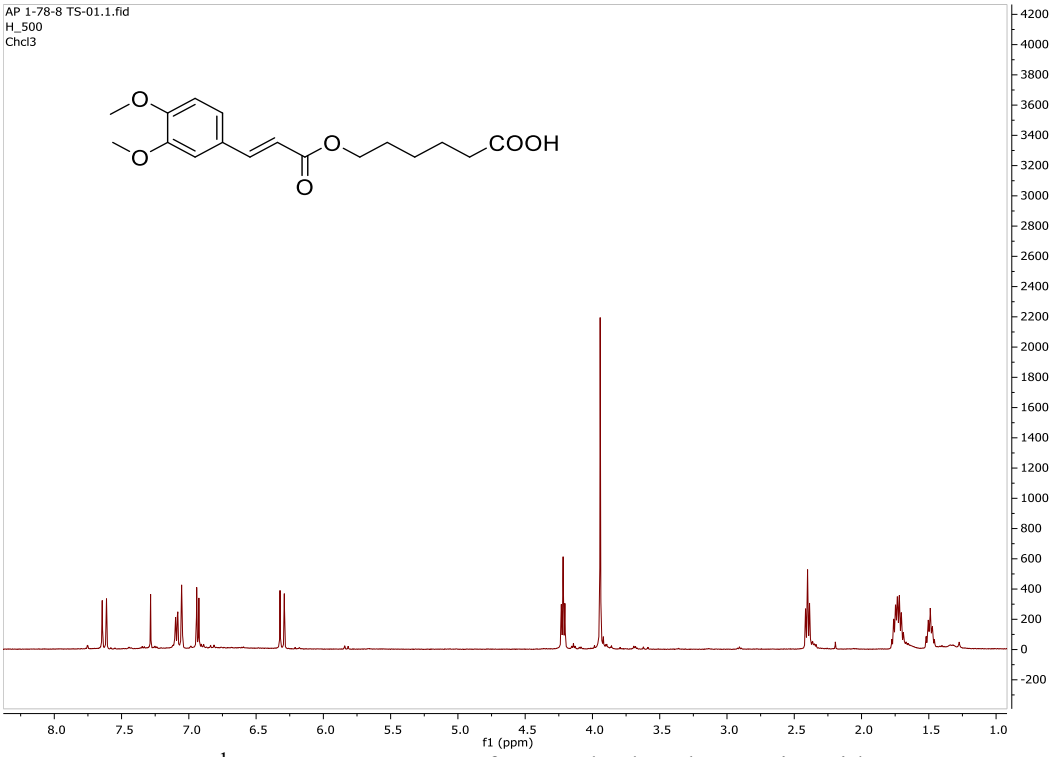


HSQC spectrum of Lauroside A

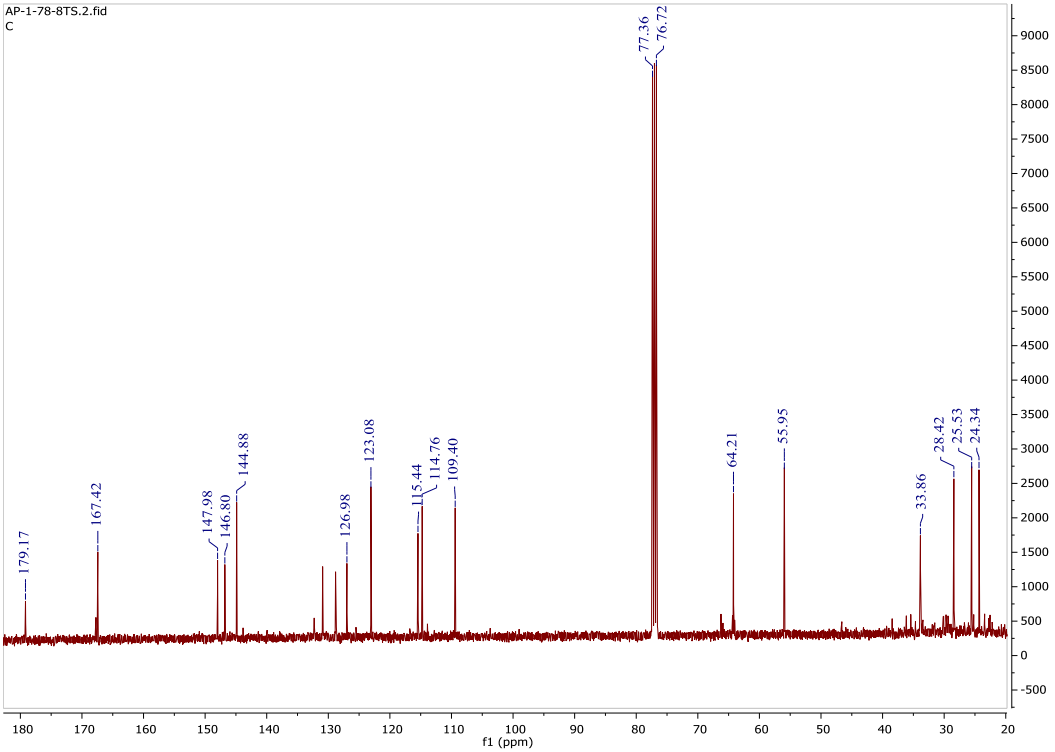


HMBC spectrum of Lauroside A

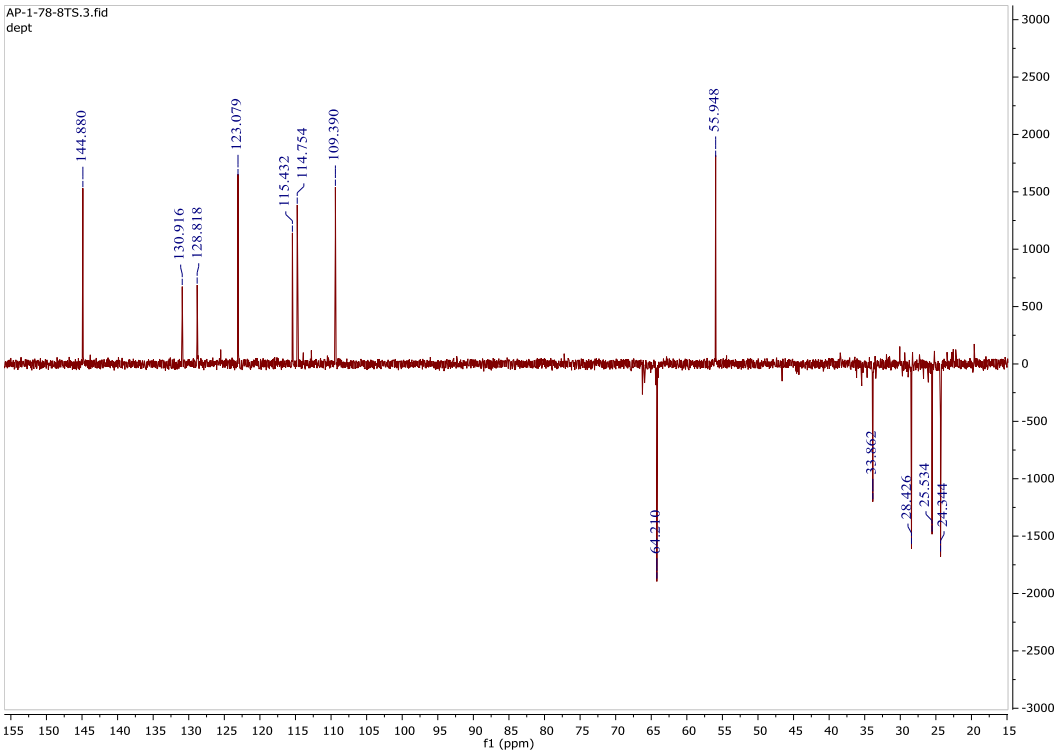
SI 29 1D and 2D NMR data 6-Feruloyloxyhexanoic acid



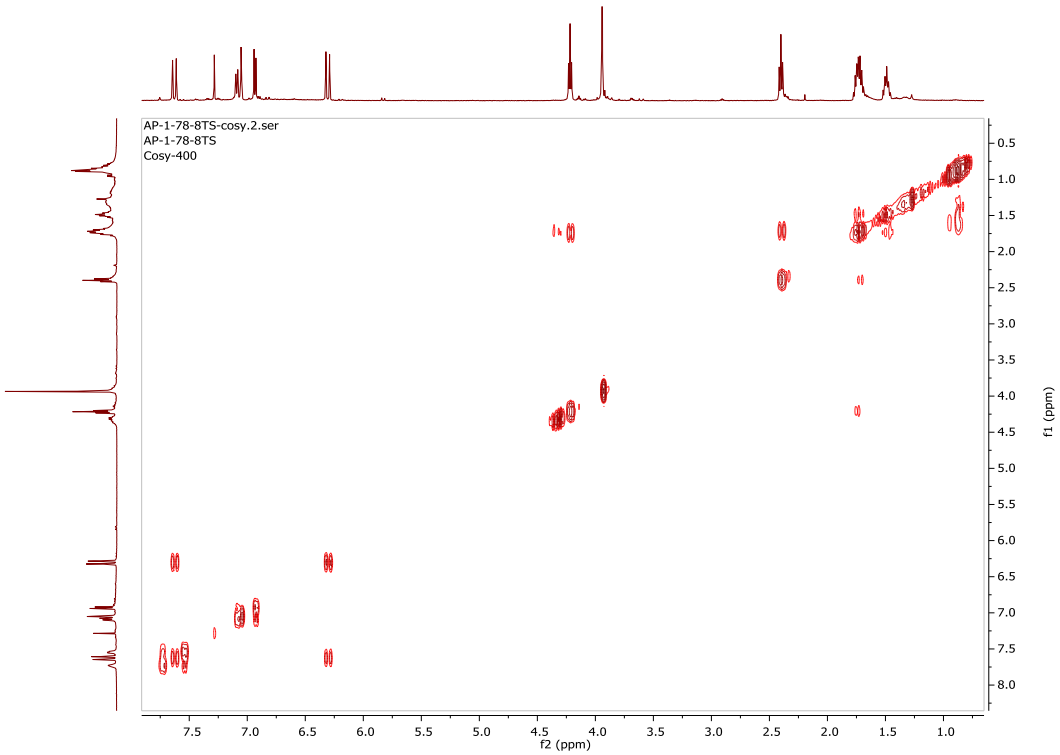
^{1}H-NMR spectrum of 6-Feruloyloxyhexanoic acid



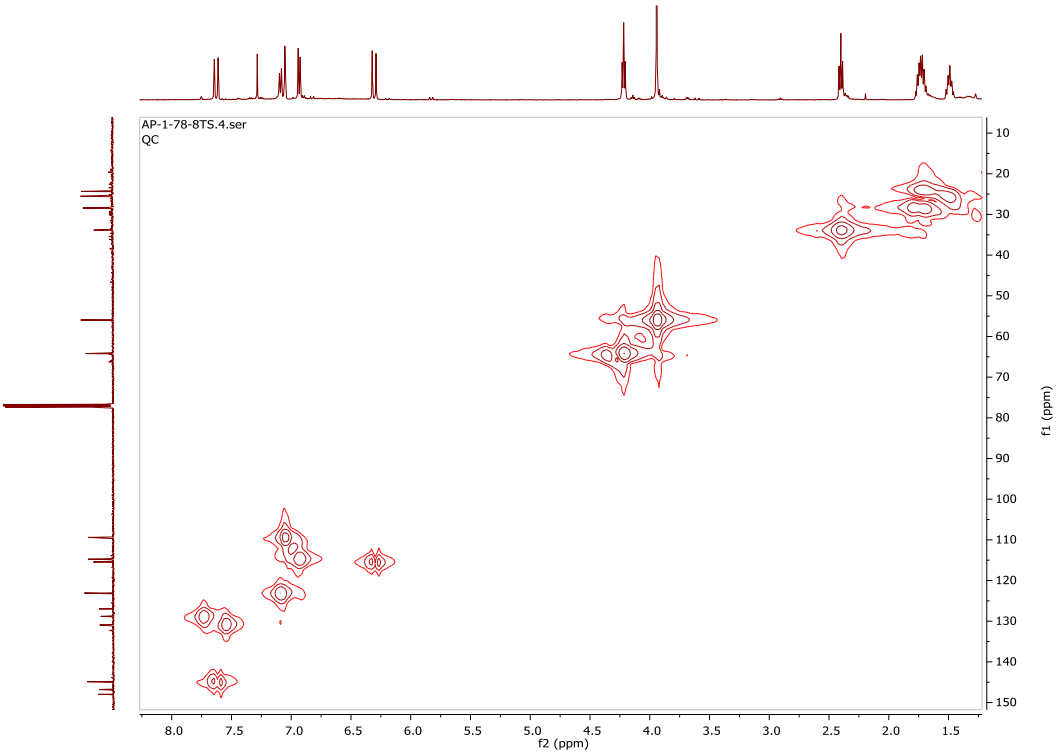
^{13}C-NMR spectrum of 6-Feruloyloxyhexanoic acid



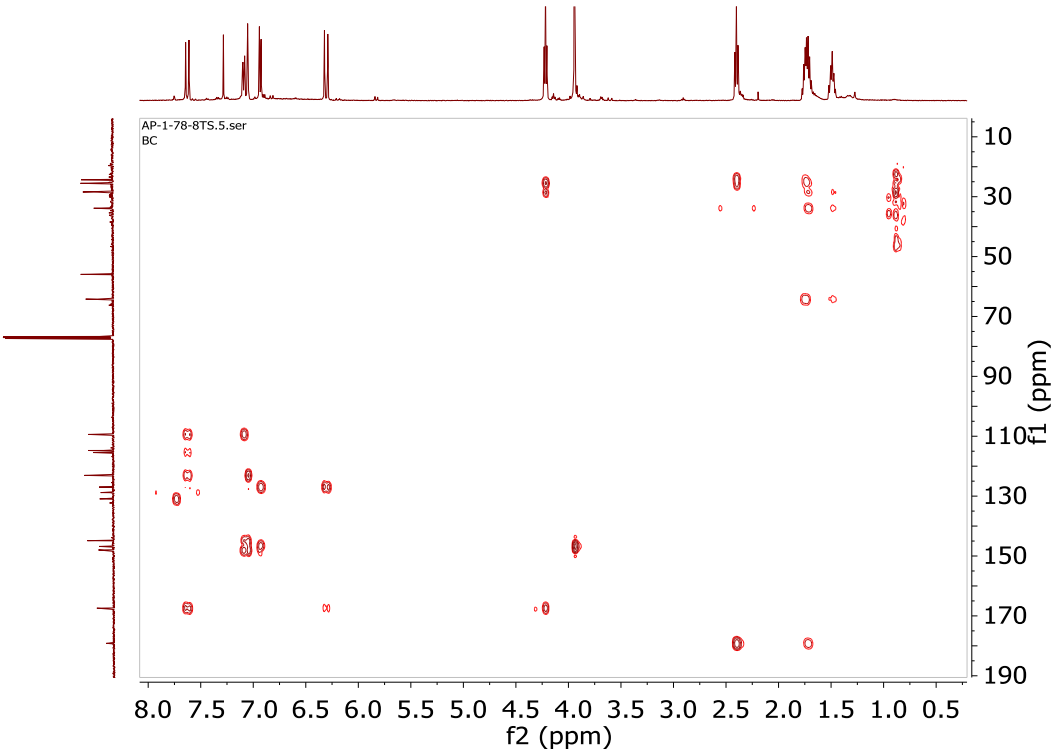
DEPT135 spectrum of 6-Feruloyloxyhexanoic acid



COSY spectrum of 6-Feruloyloxyhexanoic acid



HMQC spectrum of 6-Feruloyloxyhexanoic acid



HMBC spectrum of 6-Feruloyloxyhexanoic acid

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Vijayasankar Raman, Ph.D.
Research Scientist
National Center for Natural Products Research
The University of Mississippi
University, MS 38677, USA

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Abidah Parveen Ph.D.,
Department of Biomolecular Sciences,
School of Pharmacy, University of Mississippi

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Signature: 

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Date: 06/14/2019

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Sincerely,
Abidah Parveen
Phd, Department of Biomolecular Sciences,
School of Pharmacy, University of Mississippi

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Signature: -

VITA

Abidah Parveen
Department of Pharmaceutical Sciences
Abbottabad University of Science and Technology
Havelian, KPK, Pakistan
abidahp@gmail.com

Education

- **Doctor of Philosophy** in Pharmacognosy, 2019
  School of Pharmacy, University of Mississippi, United States.
- **Master of Philosophy** in Pharmacognosy, 2008
  Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan
- **Bachelor of Pharmacy**, 1999
  Faculty of Pharmacy, Gomal University, Dera Ismail Khan, Pakistan

Awards

- "**Poster Award First Place**", 19th ICSB, International Conference on the Science of Botanicals, Oxford, MS. April 8-11**,** 2019
- "**Poster Award 3rd Place**", Pharmacy Podium session, Graduate Student Council 9th Annual Research Symposium, University MS, USA. March 26, 2019
- "**Poster Award 2nd Place**", Pharmacy Poster, Graduate Student Council 8th Annual Research Symposium, University of Mississippi, USA, 2018
- **Honorable Mention Prize**, 17th ICSB, International Conference on the Science of Botanicals, Oxford MS, USA. 2017
- **Fulbright PhD** Scholarship Award. 2014
- **Gold Medal** by Gomal University for distinction in B. Pharmacy, 1999

Experience

- **Assistant Professor**, 2010 - present

  Department of Pharmaceutical Sciences, Abbottabad University of Science and Technology, Havelian, Pakistan (2015)

  Previously, Department of Pharmaceutical Sciences, Havelian Campus, Hazara University, Mansehra, Pakistan
- **Lecturer**, 2004-2010

  Faculty of Pharmacy, Gomal University, D. I. Khan, Pakistan
- **Visiting Lecturer**, April 2008-Sept 2008

  Department of Horticulture, Faculty of Agriculture, Gomal University,

  Dera Ismail Khan, Pakistan

Service

- Vice Chancellor Search Committee for Vice Chancellor of Research and Sponsored Programs, University of Mississippi, USA, 2018
- External Examiner and Paper setter in Pharmacognosy for Faculty of Pharmacy, Gomal University, D. I. Khan, Pakistan and Department of Pharmacy, Peshawar University, Peshawar, Pakistan, 2006-2010

Internship

- Global Pharmaceuticals, Islamabad, Oct – Dec,1999
- Pakistan Institute of Medical Sciences, Islamabad, June-Aug, 2009

Publications

1. **Abidah Parveen,** Ying Huang, Omer Fantoukh, Manal Alhusban, Vijayasankar Raman, Yan-Hong Wang, Wei Wang, Zulfiqar Ali, and Ikhlas A. Khan. (2019). Rearranged clerodane diterpenoid from Tinospora crispa. *Natural Product Research.* Manuscript accepted for publication by Taylor & Francis in Natural Product Research, available at http://dx.doi.org/10.1080/14786419.2019.1633648.

2. Fantoukh OI, Dale OR, **Parveen A**, Hawwal MF, Ali Z, Manda VK, Khan SI, Chittiboyina AG, Viljoen A and Khan IA. Safety Assessment of Phytochemicals Derived from the Globalized South African Rooibos Tea (Aspalathus linearis) through Interaction with CYP, PXR, and P-gp *J Agric Food Chem*. 2019; 67: 4967–4975.

3. Fantoukh OI, Abouelsaoud MA, **Parveen A**, Hawwal MF, Majrashi T, Ali Z, Khan SI, Chittiboyina AG and Khan IA. Isolation, Synthesis, and Drug Interaction Potential of Secondary Metabolites Derived from the leaves of Miracle Tree (Moringa oleifera) against CYP3A4 and CYP2D6 Isozymes (2019). Accepted for publication in *Phytomedicine*. https://doi.org/10.1016/j.phymed.2019.153010

4. Ul Abidin, S. Z., **Parveen, A**., Khan, R., Ahmad, M., Khan, I. A., & Ali, Z. (2018). Chemical constituents from Ferula oopoda (Boiss. & Buhse) Boiss. *Biochemical Systematics and Ecology*, *78*, 49–51. https://doi.org/10.1016/j.bse.2018.03.012

5. Jilani, M. S., Waseem, K., Ameer, K., Jilani, T. A., Kiran, M., Alizia, A. A., & **Parveen, A.** (2013). Evaluation of elite tomato cultivars under the agro-climatic conditions of Dera Ismail Khan. *Pakistan Journal of Agricultural Sciences*, *50*(1).

6. **Parveen, A**., Irfan, M., Jilani, M., Wasim, K., Kiran, M., & Muhammad, F. (2013). Lack of brine shrimp lethality and hemagglutination activity in Grewia asiatica Linn. *Journal of Pharmaceutical Negative Results*, *4*(1). https://doi.org/10.4103/0976-9234.116754

7. **Parveen, A**., Irfan, M., & Mohammad, F. (2012). Antihyperglycemic activity in grewia asiatica, a comparative investigation. *International Journal of Pharmacy and Pharmaceutical Sciences*, *4*(1), 210-213

Published Abstracts

1. **Parveen, A**., Ali, Z., Fantoukh, O., & Khan, I. A. (2016). Phytochemical constituents of Tinospora sinensis. *Planta Medica*, 82(05). doi:10.1055/s-0036-1578763
2. Fantoukh, O., Ali, Z., **Parveen, A**., Viljoen, A., & Khan, I. (2016). Flavonoid C- And O-Glycosides from Aspalathus Linearis. *Planta Medica,* 82(05). doi:10.1055/s-0036-1578730

Podium Presentation

- "Phytochemical investigation of *T. crispa* and *T. sinensis* to develop a validated method to analyze herbal dietary supplements of closely related *Tinospora* species", **46th Annual MALTO** Medicinal Chemistry and Pharmacognosy Meeting, School of Pharmacy, University of Tennessee Health Science Center, Memphis, TN, USA. May 20-22, **2019**.

- "Development of a chemical fingerprint as a tool to distinguish closely related *Tinospora* species and quantitation of major compounds". **19th ICSB,** International Conference on the Science of Botanicals, Oxford, MS, United States. 8-11 April, **2019**.
- **Graduate Student Council** 9th Annual Research Symposium, University MS, USA. March 26, **2019**
- "Assessment of Oral Contraceptive Pills as a Risk factor for Breast Cancer in Pakistan" at **17th IPCE**, International Pharmacy Conference & Exhibition organized by Pakistan Pharmacists Association, Pakistan at Marriott Hotel, Islamabad, Pakistan. **2012**
- "Antidiabetic activity of *Grewia asiatica*" at **16th IPCE,** International Pharmacy Conference & Exhibition organized by Pakistan Pharmacists Association, Pakistan at Pearl Continental, Lahore, Pakistan. **2011**

Posters Presentations

2014

- **18th School of Pharmacy** Annual Poster Sessions, University MS, USA

2015

- **19th School of Pharmacy** Annual Poster Sessions, University MS, USA
- **42nd Annual MALTO** Medicinal Chemistry and Pharmacognosy Meeting, University MS, USA - *May 17-19*
- **15th ICSB***,* International Conference on the Science of Botanicals, Oxford, MS, USA *April 13-16*

2016

- **20th School of Pharmacy** Annual Poster Sessions, University MS, USA
- **43rd Annual MALTO** Medicinal Chemistry and Pharmacognosy Meeting, USA, Houston, TX, USA-*May 22-24*
- **16th ICSB**, International Conference on the Science of Botanicals, Oxford, MS, USA *April 11-14*

2017

- **21st School of Pharmacy** Annual Poster Sessions, University MS, USA
- **44th Annual MALTO** Medicinal Chemistry and Pharmacognosy Meeting, Monroe School of Pharmacy in Monroe, Louisiana, USA *–May 21-23*

- **17th ICSB**, International Conference on the Science of Botanicals, Oxford, MS, USA *April 3-6*
- **ASP**, American Society of Pharmacognosy, Portland, Oregon, USA- *July 29-Aug 2*

2018

- **22nd School of Pharmacy** Annual Poster Sessions, University MS, USA
- **18th ICSB**, International Conference on the Science of Botanicals, Oxford, MS. USA. *April 9-12*
- **Graduate Student Council** 8th Annual Research Symposium, University MS, USA. *March 20*
- **ASP**, American Society of Pharmacognosy, Lexington, Kentucky, USA. *July 21-25*
- **GRC**, Gordon Seminar and Conference, Procter Academy, Andover, NH, USA. *July 28-Aug 3*

2019

- **19th ICSB**, International Conference on the Science of Botanicals, Oxford, MS. *April 8-11*
- **ASP**, American Society of Pharmacognosy, Madison, Wisconsin, USA. *July 13-17*

Professional Societies

2017 Rho Chi Society, United States

2006-2008 Board of studies, Faculty of Pharmacy, GU, D. I. Khan, Pakistan

2006-2008 Board of studies, Department of Pharmacognosy, GU, D. I. Khan, Pakistan

Elected Office

2009-Present Executive member, KPK, Central Executive Council, Pakistan Pharmacist Association

2013-2014 Lady Representative, Hazara University Academic Staff Association, HUASA

Registered Pharmacist:

Registration No. 534 – A / 2000 – PC, Pharmacy Council of N.W.F.P, Pakistan