In Vitro ANTITRYPANOSOMAL AND ANTIOXIDANT ACTIVITIES OF METHYL LINOLEATE FROM THE STEM BARK OF Strychnos spinosa LAM.

Garba, Ahmad[1*] and Aminu Muhammed[2]

[1*] Dept of Science Laboratory Technology, Nuhu Bamalli Polytechnic, Zaria, Nigeria
[2] Dept of Pure and Applied Chemistry, Bayero University, Kano, Nigeria
*Corresponding author Email: - garbaahmad4all@yahoo.com; GSM: +2348059786730

ABSTRACT

The urgent needs in search of an alternative compound for chemotherapy of trypanosomiasis was the priority in this study. The column chromatography of the methanol extract of the stem bark of Strychnos spinosa leads to the isolation of (Z,Z) – 9,12 – octadecadienoic acid methyl ester (otherwise known as methyl linoleate) which was found to be active against trypanosomes brucei brucei at a low concentration of 12.5mg/ml. The Total Phenolic contents of the methanol extract was found to be 132mg GAE/g, which is higher in comparison to petroleum ether and ethyl acetate extracts. Thus, the results obtained suggest the ethno- pharmacological usefulness of Strychnos spinosa plant.

Keywords: Antitrypanosomal, Antioxidant, Methyl Linoleate, Chemotherapy, Strychnos Spinosa Lam.

INTRODUCTION

African trypanosomiasis is a wasting disease of animals and man caused by haemoprotozoan, trypanosomes. It occurs across more than a third of Africa affecting almost all animals' species except poultry (Ooijen, 1983) causing sleeping sickness. The current chemotherapy of the trypanosomiasis relies on only six drugs (Saramin, Pentamidine, Melarsoprol, Eflorinthine, Arsobal and Mal B). Five of which were developed for more than 30 years; hence these drugs display undesirable toxic side effect (Steverding, 2005) also studies have shown the emergence of drug resistant trypanosomes (Peroz –morga, 2007). Therefore, there is the need for the development of a cost-effective new drug for the treatment and control of the disease.

Plant has an almost limitless ability to synthesize secondary metabolite. Which are substances that serve as a molecule of plant defense against predation by micro-organism, insects and herbivorous, some of which may evolve in plant odor (Terpenoids), pigmentation (Tanins and quinines) and flavanoid (capsacin) however, several of these molecules possess medicinal properties (Cowan, 1999). The genus *strychnos* (loganiaceae) consist of about 200 species that are used in folk medicine and in arrow dart poison in many parts of the world (Sitrit *et al*, 2003). *Strychnos spinosa* is a deciduous shrub commonly known as monkey orange, kokiya (Hausa), Atako (Yoruba), and has been utilized in African traditional medicines for many diseases such as skin bite, dropsy, ear ache, fever, epilepsy and rheumatism (Tor – anyli *et al*., 2015). The pharmacological properties of these plant include antiplasmodial (Bero *et al*., 2009), anti helmitic (Waterman *et al.*, 2010) antimicrobial activity (Nwodo *et al*., 2010) and anti-oxidant activity (Nhukarue *et al*., 2010) while the secondary metabolite isolated from these plant are tritepenoid and

sterol (Hoet *et al*., 2007), Secoiridoids (Msonthi *et al*., 1985), Alkaloid (Ohiri *et al.*, 1984) and monoterpenes (Adesogan *et al.*, 1981)

The isolation and structural elucidation of methyl linoleate from the stem bark of *Strychno spinosa* and its antitrypanosomal activity against *trypanosome brucei brucei* are hereby reported.

MATERIALS AND METHOD

Collection of plant materials

The stem bark of plant was collected at Kufena village, Zaria Local Government Area of Kaduna State, Nigeria in February, 2016.

The voucher specimen was identified and authenticated by the Taxonomist of Plant Biology Department, Bayero University, Kano, Nigeria with an accession number BUKHAN 0127.

Extraction and Isolation

The stem bark of *Strychnos spinosa* was washed, shade dried and made into powdered. The powdered stem bark (1 kg) was macerated for 2 weeks using 1 liter each of petroleum ether, ethyl acetate and methanol sequentially. The extracts were evaporated to dryness under vacuum at 40°C using rotary evaporator. Methanol extracts (2.4g) was subjected to column chromatography in a gradient elution of petroleum ether, ethyl acetate and methanol to afford 168 fractions (SSME 1 – 168). A number of fractions were pooled based on their TLC profile SSME 82 gave a single spot based on TLC analysis (petroleum ether – ethyl acetate; 1:1)

Characterization of isolated compound

^{1}H – NMR (400MHz), ^{13}C – NMR (100MHz) and 2D – NMR experiment were carried out using Agilent NMR spectrometer in $CDCl_3$. The GC – MS were performed on Agilent GC-MS system (GC: 5890 series II: MSD 5972). The fused-silica HP-5 capillary column (30 m × 0.25 mm, ID, film thickness of 0.25µm) was directly coupled to the MS. The carrier gas was helium with a flow rate of 1.2 ml min^{-1}. The IR spectrum was measured on Shimadzu FT – IR instrument.

Determination of Total Phenolic Content

The total phenolic content in the plant extracts was determined using method of singleton *et al* (1999) with a slight modification. The plant extract (1 mg/ml, 20µl) was mixed with of Folin – ciocalteau reagent (20µl) in a 96 well microtitter plate. After 5 minutes, sodium carbonate (0.01M, 20µl) was added to each sample and allowed to stand for 5 minutes before adding 125µl of distilled water. The mixture was measured at the absorbance at 765nm using Thermo -- scientific multiskan GO spectrophotometer (Thermofisher Scientific, Vartaa, Finland). The blank is the same reaction mixture with water instead of the extracts or standard. The total phenolic content was expressed as mg gallic acid equivalent per gram.

In vitro antitrypanosomal assay

The stabilate of *trypanosomes brucei brucei* were obtained from Nigerian Institute for trypanosomiasis Research, Vom, Plateau state, Nigeria and passage into mice. The *in vitro* antitrypanosomal activity was performed by blood incubation infectivity test (BIIT) by using 96 well microtitre plates as described by mergia *et al*, 2014 with a slight modification, 0.32g of the plant extracts and the isolates were dissolved in 2% tween 80 in phosphate buffered saline glucose (PBSG) to make an effective test concentration between 100mg/ml – 16.5mg/ml. Negative and positive controls were also prepared by

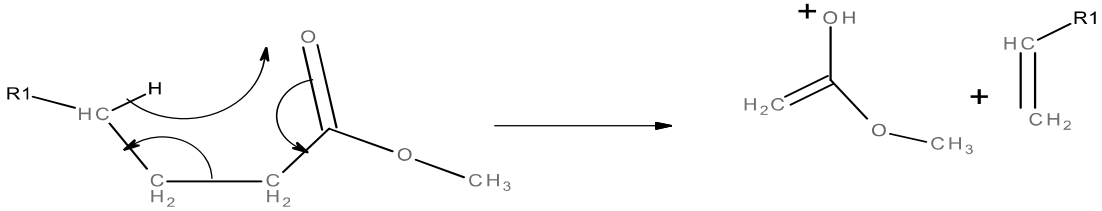
dissolving 200µl of infected blood in tween 80 in PBSG and similar effective test concentration of diminazene aceturate respectively. The blood was checked at 10 minutes interval for 2 hours for the inactivity of the parasite in the blood when mixed with the extracts/isolates using microscope at ×400 magnification. The shorter the time of cessation of motility of the parasite, the more active the extract was considered to be, under this in vitro system, the parasite survived for 4 hours when no extracts or isolates were present. The validation of these *in vitro* antitrypanosomal activity was done by injecting intraperitoneally the parasite suspension of the in vitro test from microtitre plate into five healthy mice and the level of parasitaemia was assessed everyday by collecting blood from the tail of each mouse and check for the presence of trypanosomes using the wet blood film by microhaematocrit buffy coat technique (Woo, 1970). The loss of infectivity of the trypanosomes to mice was concluded if no trypanosomes were detectable within 21 days (Maikai, 2011). The effect of the extracts and isolate in prolongation of establishment of infection was monitored by comparing with the negative control.

Statistical Analysis

MINITAB version 14 was used as a statistical tool, the p-value of less than 0.05 was considered to be statistically significant

RESULTS AND DISCUSSIONS

The methanol extract was subjected to gravity column chromatographic separation using petroleum ether – ethyl acetate – methanol gradient over silica gel. The elution afforded 168 fractions which were subsequently pooled to 20 fractions. The fraction SSME 82 was isolated as single spot with R_f value of 0.9 (Petroleum Ether; Ethyl acetate 1:1); the isolated compound appeared as a brown oil (37mg, 15.47%)**.** The MS analysis (Figure 2) revealed a molecular ion at m/z 294 which corresponds to the formula $C_{19}H_{34}O_2$. The base peak appears at m/z 55 due to allylic bond rupture ($C_4H_7^+$). A peak appeared at m/z 73 which is a characteristic for the methyl ester and additional reinforced entity appeared at m/z 59 and 74 which was due to McLafferty rearrangement (β- cleavage).



m/z 74

The ion that appeared at m/z 95.5 was due to cleavage at C9 – C10 to form an aliphatic chain [$C_7H_{13}^+$] and the m/z at 70 was for [$C_5H_{10}^+$]. All these are in lined with the work of Saeed (2010).

The FT-IR spectral data analysis (Figure 3) showed absorption band for the carbonyl and carbon- carbon double bond at 1730.73$cm^{-1}$and 1683cm^{-1} respectively. 1127cm^{-1} appeared for C—O stretching vibration. Normally methyl esters of long

chain fatty acid are characterized by three band patterns near 1250, 1205 and 1175cm^{-1} (Erno, 2008), but in this study, it appears at 1279.74cm^{-1} and 1127cm^{-1}. The C—H stretching vibration of aliphatic saturated chain appeared at 2929.67cm^{-1} and 2858.80cm^{-1} and the bending vibration appeared at 1465cm^{-1} and 1582cm^{-1}.

The ^{1}H - NMR spectral of **SSME 82** in $CDCl_3$ (figure 4) showed a multiplet of overlapping peaks that appeared in the region of δ = 0.49 – 2.0ppm which was highly suggestive of the nature and richness of an aliphatic skeleton in the compound. The methyl protons at δ = 0.89ppm was assigned to H—18, while the methylene proton was resonating between 1.00 to 2.13ppm. The methoxy group resonated at δ = 3.85ppm (3H, s) while the olefinic proton appeared at δ = 7.32 ppm (1H,dd,11Hz) which is the characteristic of vinylic conjugated dienes. The absence of peaks above δ values 7.5ppm is an evidence for the total absence of any aromatic rings (Saeed, 2010). The J-value of 11Hz proved that the compound is a cis--isomer

The ^{13}C - NMR spectral (figure 5 and table 1) of **SSME 82** shows the appearance of the methyl carbon at δ = 11.29ppm for C-18, the carbonyl group for ester appeared at δ = 168.11ppm for C- 1, while the carbinol (C – O) appeared δ = 77.7ppm. The methine carbon appeared at 132.75ppm as they are de-shielded due to the existence of a double bond at C—9 and C—12, the methylene carbons resonate between δ = 23.03 to 29.25ppm. The DEPT spectrum (Figure 6) classified the carbon atoms into five methane, twelve methylene, two methyl carbon atoms and one quaternary carbon atom. Furthermore, the COSY, HSQC and HMBC spectral data (figure 7, 8 and 9) indicates ^{1}H—^{1}H correlation as well as ^{1}H – ^{13}C relations. The COSY spectral confirmed the connectivity between the various protons

Thus from the above aforementioned evidences and comparison with literature of fatty acid methyl ester (FAME), (Saeed and Bashier, 2010) the structure of **SSME 82** was confirmed to be (Z,Z) -9,12-octadecadienoic acid methyl ester.

Table 1; ^{1}H – NMR data of SSME 82 in $CDCl_3$

Position	**δ_H (mult, J Hz)**	**Reference (Shinge, 2010)**	**δ_C**	**Reference (Ajoku *et al*., 2015)**
1	----------	----------	168.11	174.65
2	2.12(2H,t,7.6Hz)	2.30	23.0	22
3	1.31 (m)	1.26—1.63	23.0	-------
4	1.32 (m)	1.26—1.63	23.0	-------
5	1.39 (m)	1.26—1.63	24.0	-------
6	1.65 (m)	1.26—1.63	28.0	29.25
7	1.73 (m)	1.26—1.63	29.2	29.36
8	1.80 (m)	1.26—1.63	29.6	29.46
9	7.12 (1H,15Hz)	2.10	129.1	-------
10	7.13	5.62	129.9	--------

	(1H,15Hz)	(1H,dt,15Hz)		
11	7.12 (1H,dd,11.6Hz)	6.29 (1H,dd,11Hz)	131.1	--------
12	7.30 (1H,dd,11Hz)	5.30 (1H,10.8Hz)	131.2	--------
13	7.12 (1H,dd,111Hz)	5.30	132.7	--------
14	2.12 (2H,d,6.3Hz)	2.15 (2H,d,6.3Hz)	68.4	--------
15	1.92	-------	68.4	------
16	1.90 (m)	1.26—1.63	72.13	-------
17	1.12	----------	72.13	-----
18	0.89(3H,t,7.0Hz)	0.89(3H,t)	11.29	-----
OCH_3	3.85 (3H,S)	3.67	77.7	-----



SSME 82

Hexadecanoic acid methyl ester has previously been isolated from the calyx of *Hibiscus sabdariffa* (Ajoku *et al*., 2015). Although most unsaturated fatty acids in seed oils occurs in the cis forms but there are exceptions (Chisholm & Hopkin, 1963).

Total Phenolic Content

The total phenolic content in the petroleum ether, ethyl acetate and methanol extracts of *Strychnos spinosa* stem bark were found to be 49.14, 59.32 and 132.57 mg/GAE/g respectively (figure 1). This indicates that methanol extracts have the highest amount of phenolic content (132.57mg/GAE) while the least amount is found in the petroleum ether (47.14mg/GAE). Hence it can be seen that the total phenolic content in plant extracts depends on the polarity of the solvent used, thus high polar solvent has high concentration of phenolic compounds (Mohsen and Ammar, 2008). Phenolic compounds are present in food, beverages and medicinal plant and are reported to have useful pharmacological properties, they are known to inhibit inflammatory responses via many routes, and possess good antioxidant activity due to their scavenging ability on free radical as a result of their hydroxyl groups (Tosun *et al.*, 2009). Therefore, the methanol extracts (SSME in comparison with the ethyl acetate (SSEA) and petroleum ether (SSPE) of the plant would have higher antioxidant properties.

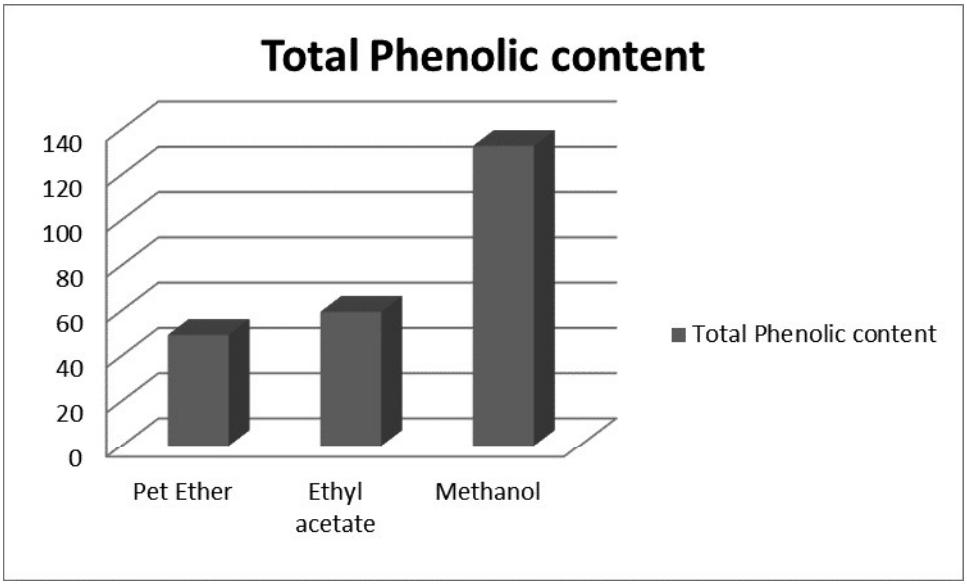


Figure 1; Total phenolic content of the extracts

In vitro antitrypanosomal activity

Table 2; *In vitro* effect of Petroleum ether, Ethyl acetate, Methanol extracts and Isolate compound (SSME 82) from stem bark of *Strychnos spinosa* on *Trypanosome brucei brucei*

Extracts/Isolate	**Time taken to immobilize parasite(minutes)**	**IC_{50} (mg/ml)**	**Activity on *Trypanosome brucei brucei***
Petroleum Ether	Motile	DE	-
Ethyl acetate	Motile	75.63	-
Methanol	Motile	112.50	-
Isolate (SSME 82)	120	DE	+
Diminazene (Positive control)	40	200	+
2% tween 80 PBSG (Negative control)	Parasite very active even after 4 hours	-	-

+ = Active against *Trypanosome brucei brucei,* - = Not active against *Trypanosome brucei brucei,* DE = Not estimated.

It can be seen that the isolate SSME 82 ceased motility of the trypanosomes within 120 minutes at concentration of 12.5mg/ml while the positive control diminazine aceturate immobilized the motility of the trypanosomes within 40 minutes and the negative control does not immobilized the motility even after 4 hours.

Parasites motility can serve as a reliable indicator of viability of most trypanosomes and complete ceased or reduced in motility of trypanosomes when compared to the control could be taken as an index of trypanocidal activity as reported by Wurochekke and Nok (2004). Thus, the isolated SSME 82 showed a considerable trypanocidal activity and this confirmed the work of Hopp *et al* (1976) and Sepulveda – Boza (1996) where natural products with trypanocidal activity belonging to a variety of photochemical classes have been identified. The mechanism by which the isolate SSME 82 eliminate or immobilize

the parasite is not known at this stage of study however many reports has shown that many natural products exhibits their trypanocidal activity by virtue of their interference with the redox balance of the parasite acting either on the respiratory chain or on the cellular defense against oxidative stress (Joseph *et al*., 2011). Also, fatty acid methyl ester (FAME) has been reported to cause autolysis of membranous structures, induces significant aortic dilution, inhibit phagocytic activity and nitric oxide productions of certain cells, reduce levels of tumor necrosils factor-alpha without affecting ATP levels (Wang *et al*., 2010). Trypanosomiasis inhibitors induce apoptosis and inhibit several enzymes activities such as human topoisomerases. Compounds with carboxylic functionalities are essential for the significant inhibitory activity of those DNA manipulating enzymes (Mizushina, 2003). It has also been reported that compounds with oxygenated side chain or oxygenated function seems to be important for anti-trypanosomal activity (Quilez, 2003) and this is in agreement with this study as the isolated compound SSME 82 was an oxygenated compound, a fatty acid methyl ester.

The IC_{50} values of the isolated compound SSME 82 could not be determine as there is no much responses of the trypanosomes to the various concentration of the isolate.

CONCLUSION AND RECOMMENDATIONS

The secondary metabolite (Z,Z)-9,12-octadecadienoic acid methyl ester has been isolated from the stem bark of *Strychnos spinosa* and it shows a potent anti - trypanosomal activity against *Trypanosome brucei brucei.* The methanol extracts have a high total phenolic content of 132mg GAE/g as such it can be used in the treatment and control of African animal trypanosomiasis.

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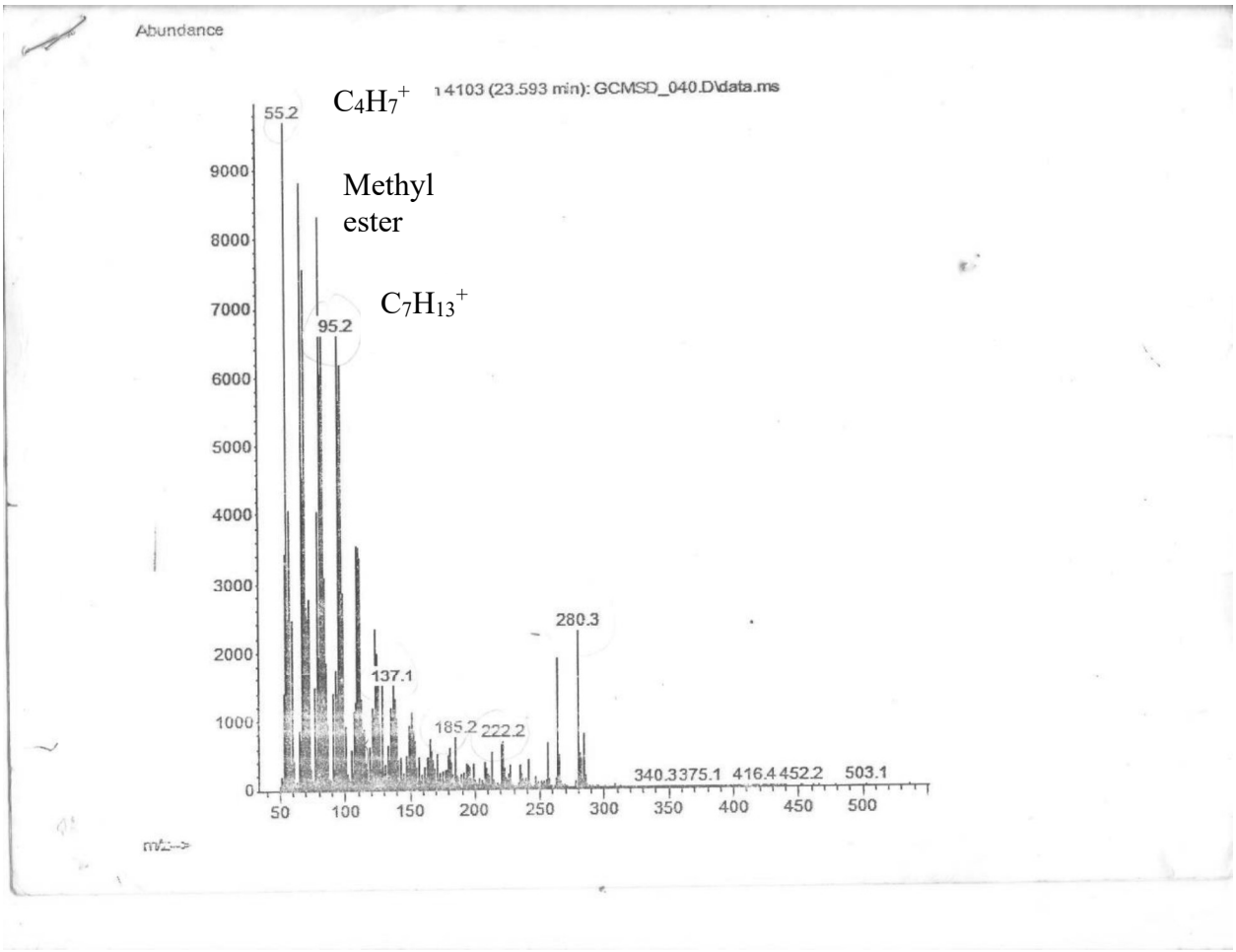

FIGURE 2; Mass – spectrum of SSME 82

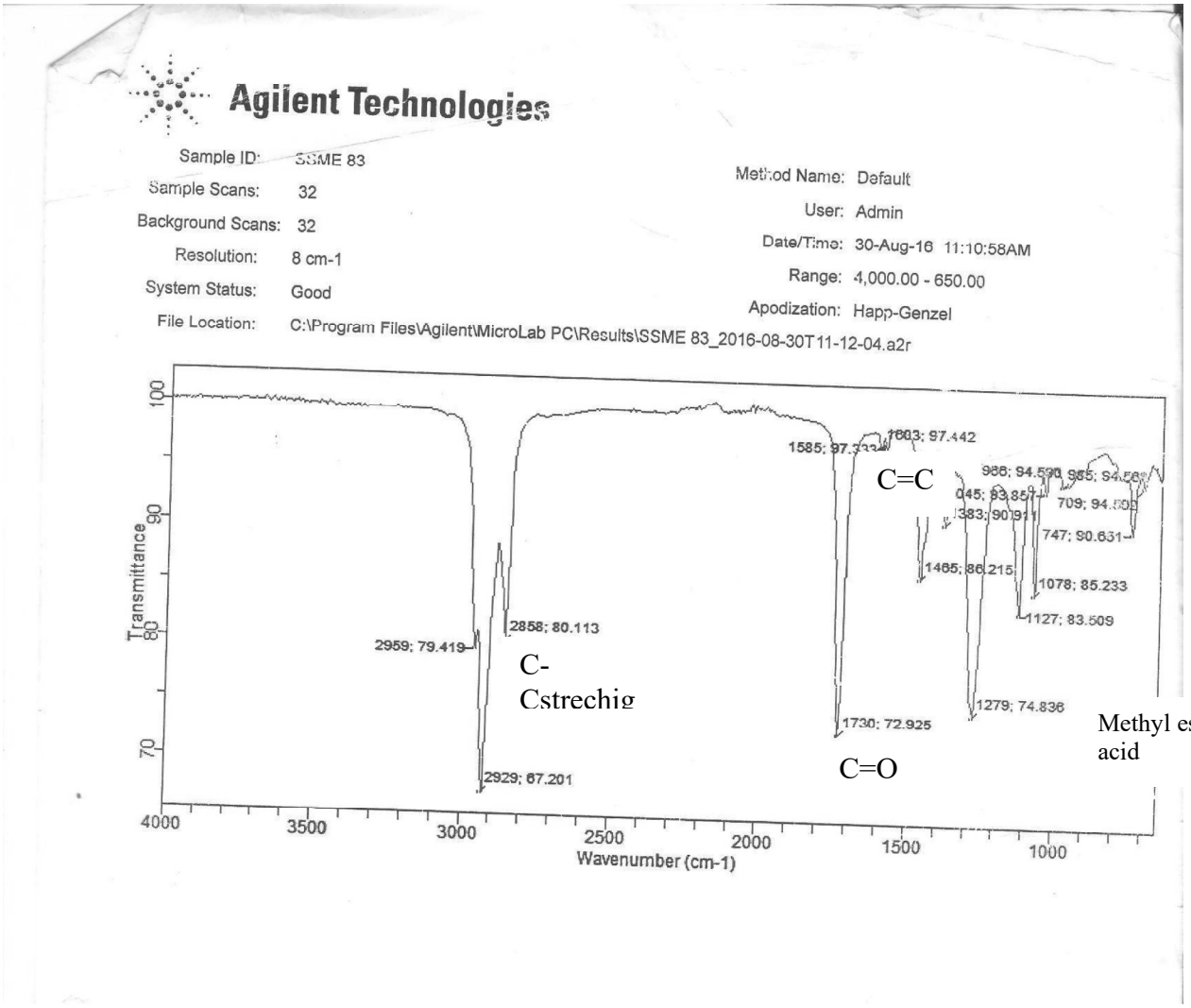

FIGURE 3: FT- IR Spectrum of SSME 82

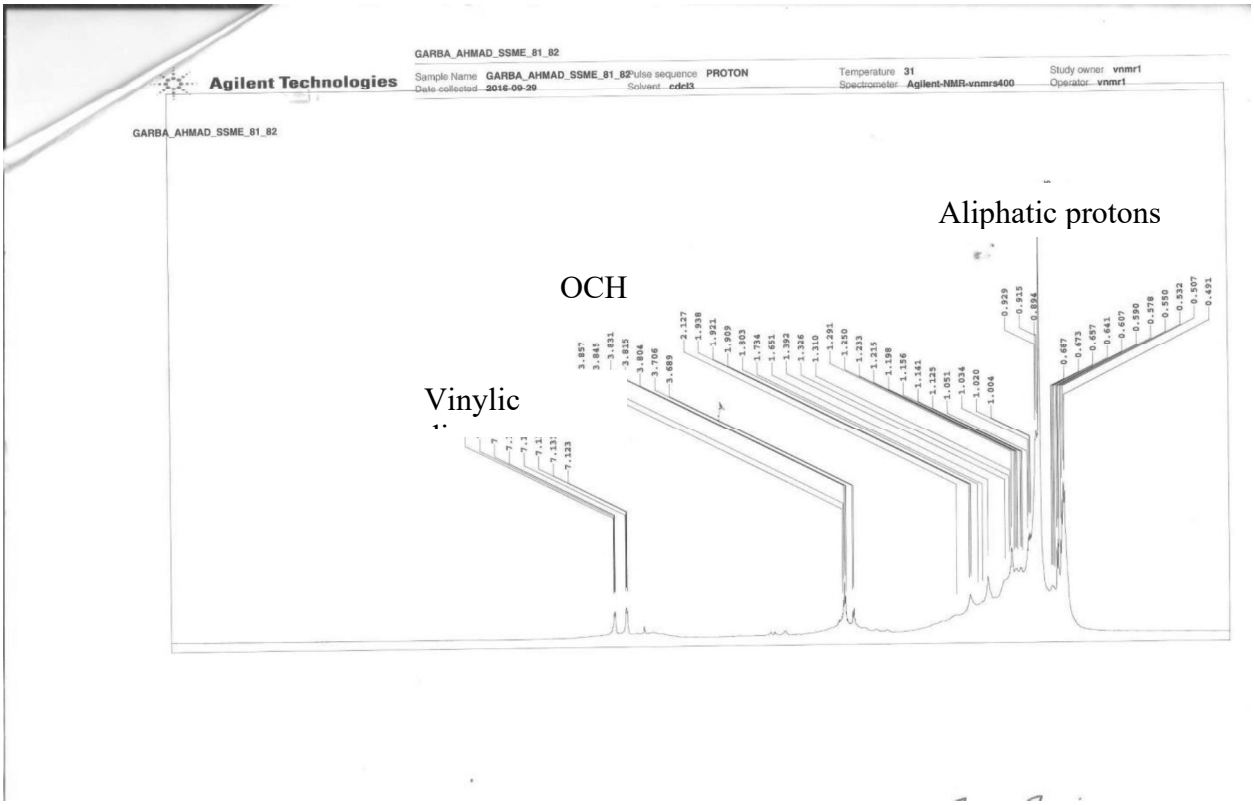

Figure 4; ^{1}H – NMR of SSME 82

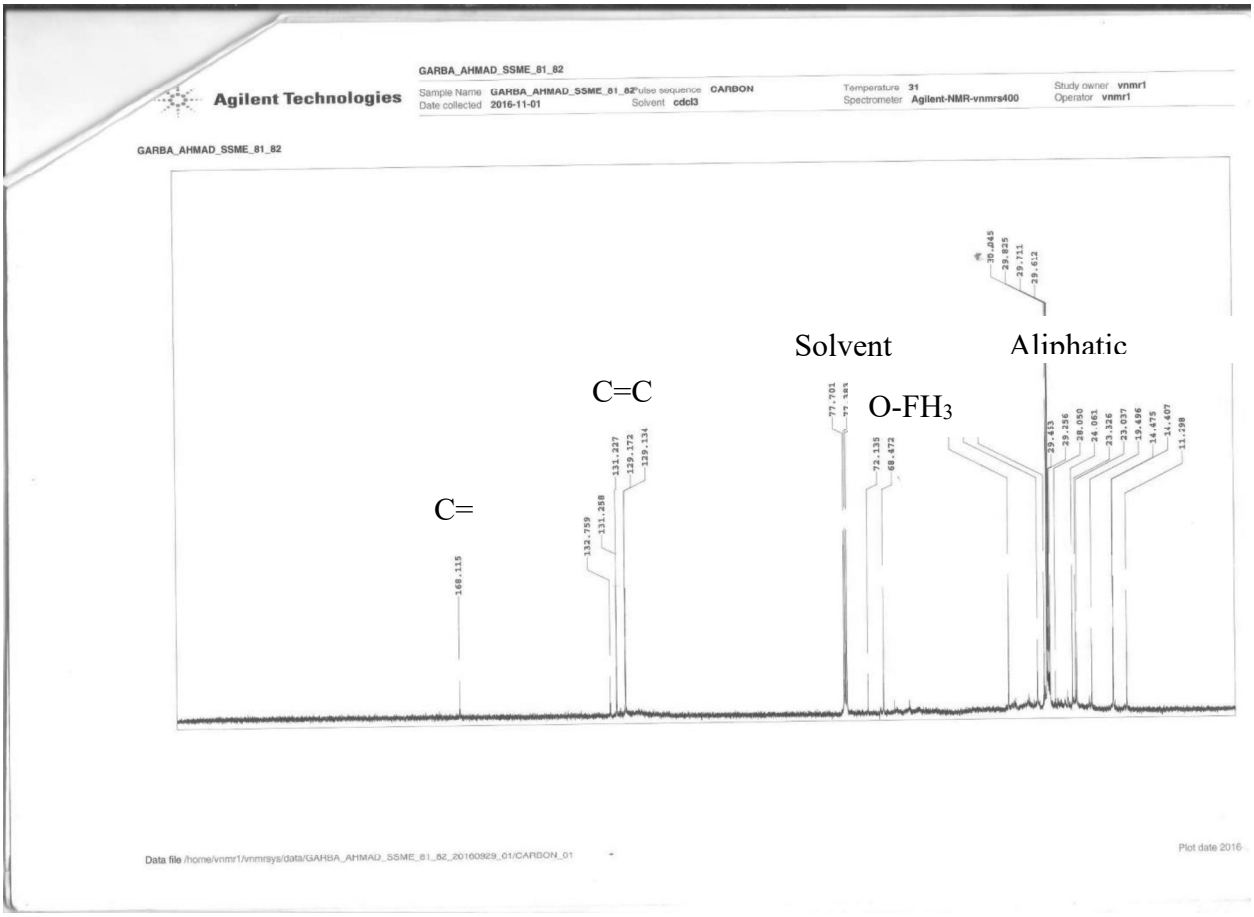

Figure 5; ^{13}C – NMR of SSME 82

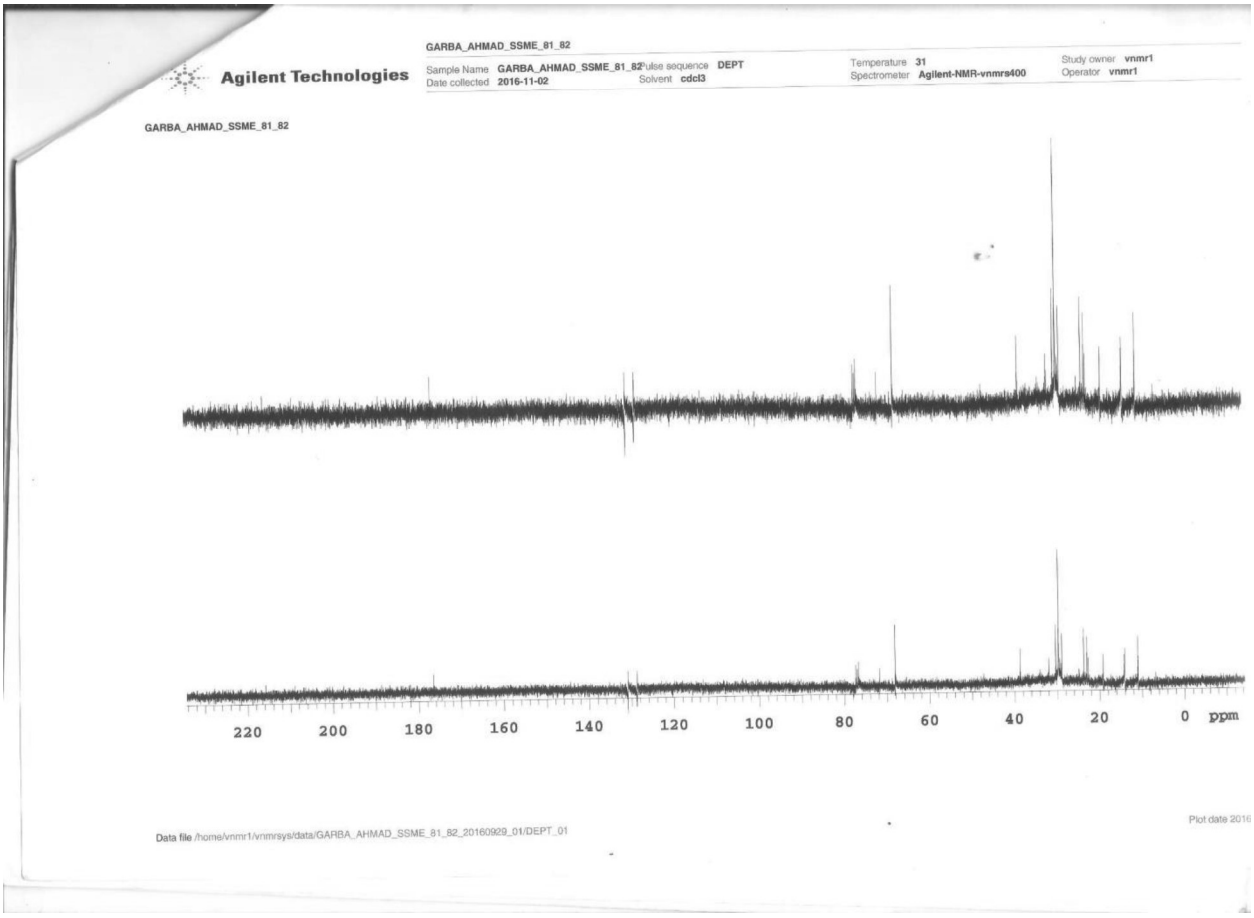

Figure 6 DEPT spectrum of SSME 82

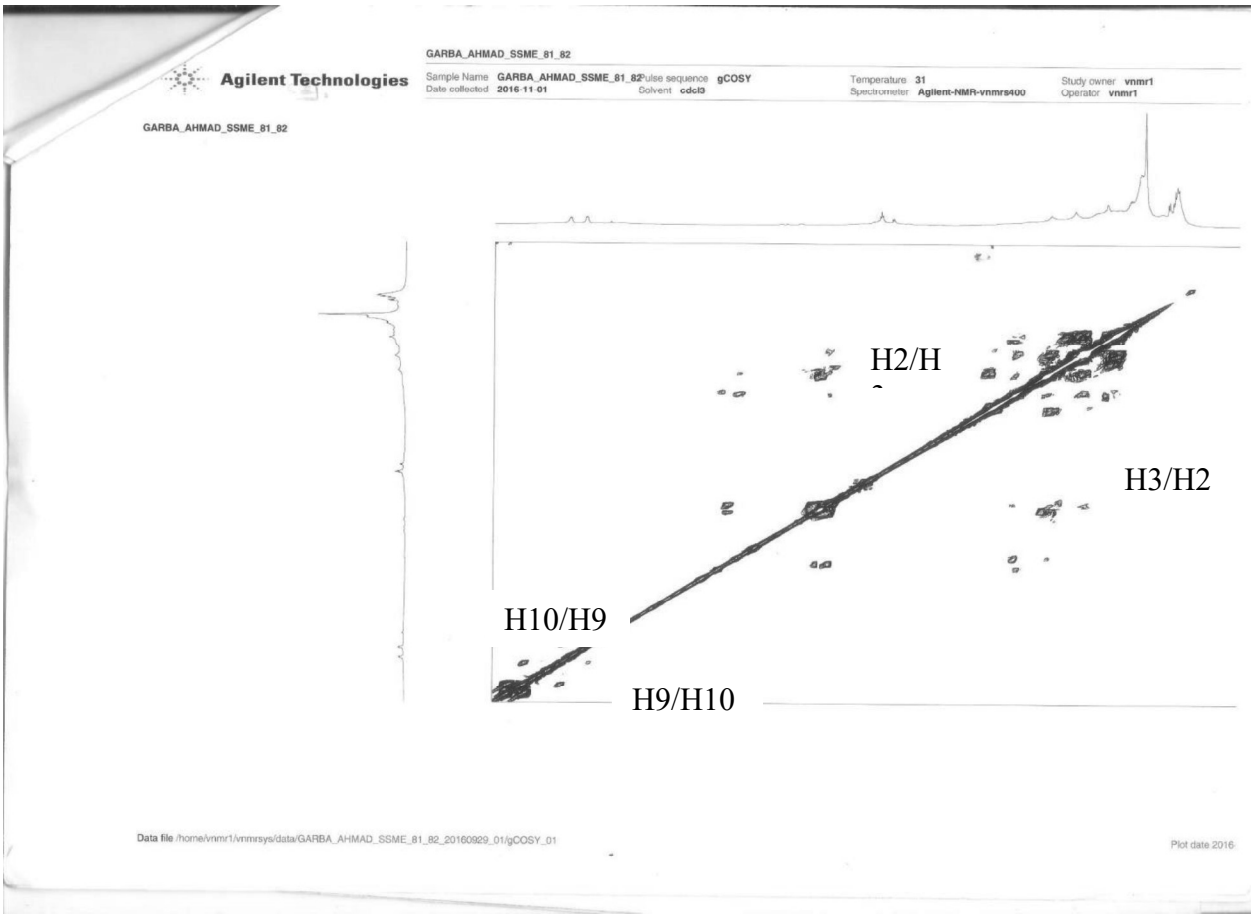

Figure 7; COSY Spectrum of SSME 82

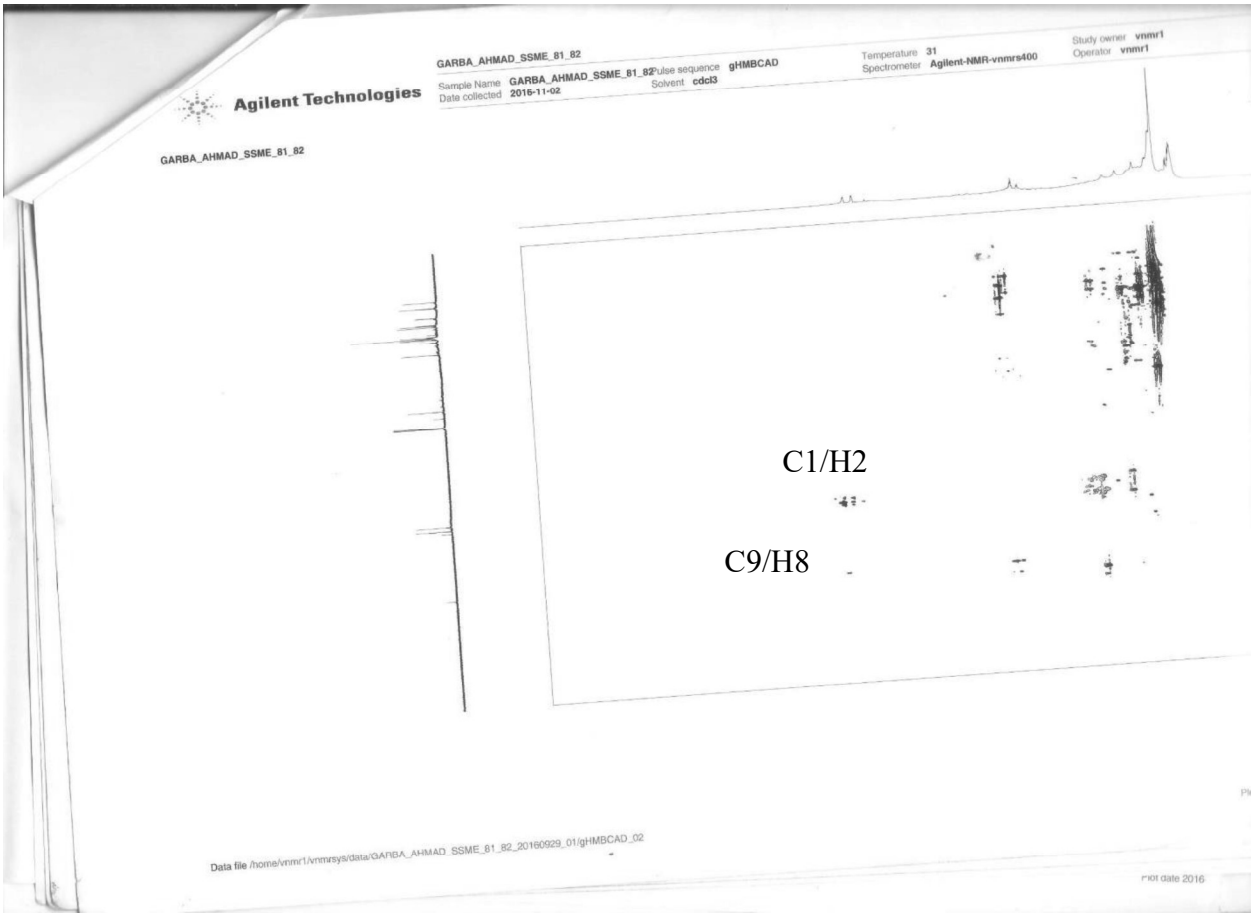

Figure 8; HMBC Spectrum of SSME 82