Isolation and Anti-oxidant Potentials of Parahydroxybenzaldehyde from the Methanol Leaf Extract of *Aspilia Africana* (Pers.) C.D. Adams (Asteraceae)

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ABSTRACT

The leaf of *Aspilia africana* (Pers) C. D. Adams (Asteraceae) is widely used in ethno medicinal practices in tropical Africa because of its ability to stop bleeding, promote rapid healing of wounds and treat infertility. It has also been used in the management of problems related to cardiovascular diseases, lumbago, venereal diseases inflammations, tumours and parasitic infections. This study was carried out on the leaf methanol extract to determine the phytochemical and anti-oxidant properties. The methanol extract was subjected to preliminary phytochemical screening, which showed the presence of saponins, tannins, flavonoids, carbohydrate and other secondary metabolites. The methanol extract was partitioned with n-hexane, dichloromethane and n-butanol. The bio-assay guided silica gel column chromatographic separation of the butanol fraction furnished pale white crystals (18 mg), mp. 116-118 ^{o}C, characterized as parahydroxy benzaldehyde on the basis of spectral analysis (UV, IR, ^{1}H NMR). The anti-oxidant assay using DPPH scavenging method, showed that the isolated compound had higher activity than n-butanol fraction and methanol extract with IC_{50} values of 73.5 µg/mL, 88.90 µg/mL and 95.50 µg/mL respectively compared to standard drugs (Vitamin C; 37.5 µg/mL and Vitamin E; 48.0 µg/mL).. The n-hexane and dichloromethane fractions did not show any antioxidant activity. This also confirmed the basis for the local use of the plant in treating/managing cardiovascular diseases, wounds, tumors inflammations and parasitic infections

Keywords: *Aspilia Africana*, Parahydroxybenzaldehyde, Antioxidant activity, DPPH Assay.

1.0 INTRODUCTION

The recent growth in the knowledge of free radicals and reactive oxygen species (ROS) in biology is producing a medical revolution that profiles free radical chemistry. Free radical reactive oxygen is generated in human body by various endogenous systems, exposure to different physicochemical conditions or pathological states (Lobo *et al*, 2010). It is ironic that oxygen, an element indispensable for life under certain situations has deleterious effects on the human body. Most of the potentially harmful effects of oxygen, are due to the formation and activity of a number of chemical compounds known as ROS, (reactive oxygen species), which has a tendency to donate Oxygen to other substances (Bagchi and Puri, 1998).

A free radical is chemical specie capable of independent existence that contains an unpaired election in an atomic orbital (Cheeseman and Slater, 1993). Free radicals and other ROS are derived either from normal essential metabolic processes in the human body or from external sources such as exposure to x-rays, ozone, cigarette smoking, air pollution, and industrial chemicals (Bagchi and Puri, 1998). Early research on the role of antioxidants in biology focused on their use in preventing the oxidation of unsaturated fats, which is the cause of rancidity (German, 1999). However, Vitamins A, C and E were identified as the antioxidants; this revolutionized the field and led to the realization of the importance of antioxidants in the biochemistry of living organism (Muller *et al*, 2007). An antioxidant donates an electron to a rampaging free radical to neutralize it, thus reducing its capacity to damage. Some low molecular weight antioxidants such as glutathione, ubiquinol and uric acid produced during normal metabolism in the body can safely interact with free radicals to terminate their harmful effect (Shi *et al*, 2008). Antioxidant can take the form of enzymes in the body, vitamins, food supplements and nutritional additions. In view of the increasing risk factors of human to various deadly diseases, there has been a global trend towards the use of natural substances present in medicinal and dietary plants as therapeutic antioxidants. More so in the wake of numerous questions raised over the use of synthetic antioxidants due to their potential health risks and toxicity (Valentino *et al*, 2002).

When antioxidant levels in the human body are lower than that of free radicals – due to poor nutrition, toxin exposure or other factors – oxidation wreaks havoc in the body. The effects are numerous and include; accelerated ageing, damaged or mutated cells, broken-down tissue, the activation of harmful genes within DNA and an overload immune system. In addition, accumulated evidence indicates that oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases. Some pathologies arising during bacterial infections can be attributed to oxidative stress and generation of reactive species (Pohanka, 2013).

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Free radicals, especially reactive oxygen species (ROS), have been identified to play a crucial role in the pathogenic processes in many human disorders including cancer and microbial infectious diseases (Dzoyema *et al*, 2017). These days some herbs and shrubs are known which contain natural antioxidants that can prevent oxidative stress and aid the chemo suppression of diseases that have their etiology and pathophysiology in reactive oxidative species. One of such herbs is *Aspilia africana* which grows ubiquitously in the savannah and forested zones of Africa; used in the treatment of fever, haemorrhage, bacterial infections, wounds, cardiovascular diseases and inflammations (Burkill, 1985; Duke, 1992; Petacci *et al*., 2012; Ken, 2014; Johnson *et al*., 2016). This research study was carried out with the aim of prospecting for antioxidant activity in the methanol extract, fractions and isolated chemical compound from the plant based on its local uses in treating/managing cardiovascular diseases, wounds, tumors inflammations and parasitic infections.

2.0 MATERIALS AND METHODS

2.1 Materials

Melting point was determined using Electrothermal Melting Apparatus (USA); refractive index was obtained using Abbe Digital Refractometer (AE11767, Taiwan) and the UV analysis was carried out using Spectro UV-VIS 2700 Dual Beam, 200-1100 nm, Labomed, Inc (USA). The FT-IR analysis (neat) was performed on Shimadzu FTIR 8400S Transform Infrared Spectrophotometer (Japan) while ^{1}H NMR was done on JEOL AS400 (400 MHz) spectrometer, (Japan) using DMSO-d_6 (peak at 2.5 ppm) as solvent, TMS as internal standard and run at 25º C. The chemical shifts were reported as δ ppm relative to TMS.TLC silica gel $60F_{254}$ pre-coated (Aluminum) plates were obtained from Merck, Germany. All solvents used were of analytical grade from Sigma-Aldrich (USA).

2.1Collection, Identification and Extraction of leaf Plant material

The fresh plant was collected from a farm in Zaria, Northern Nigeria and identified by Mr Umar Gallah, taxonomist in the Herbarium Unit of the Department of Biological Science, Ahmadu Bello University, Zaria. A voucher (Specimen No 1146) was deposited at the Herbarium. The plant was sorted, graded, dried under shade and pulverized using a mortar and pestle for extraction. The pulverized leaf plant material (500 g) was macerated, using methanol for 72 hours at room temperature. The extract was filtered and evaporated into dryness *in-vacuo*, weighed and then stored in a refrigerator for subsequent use. 50 % of this extract was dissolved in water and partitioned with n-hexane dichloromethane and n-butanol to obtain n-hexane, dichloromethane and butanol fractions. The methanol extract was subjected to phytochemical screening to detect the bioactive constituents using standard methods (Harborne, 1984; Trease and Evans, 2002; Sofowora, 2008).

2.2 Isolation

The n-butanol soluble fraction ((2 g; which had higher antioxidant activity) was ground with silica gel (60 – 120 mesh) and loaded onto a column (3.5 x 50 cm) packed with silica gel (60 – 120 mesh) and eluted with n-hexane : ethylacetate : methanol solvent system (starting with 100% n-hexane) in increasing polarity order ratio (Richard, 1998). Thin layer chromatography (TLC) was used to monitor the outcome. Eluates (215 fractions) of 10 mL aliquot were collected. Based on TLC report, fractions with similar R_f were pooled together to obtain 11 major fractions (D1 – D11). The TLC profiles of fraction D4 (eluates 37-45; solvent system, ethyl acetate: methanol; 97: 3) revealed a single blue spot when sprayed with 10% H_2SO_4 in ethanol and heated at 110°C. The fraction was further purified with sephadex LH-20 using CH_2Cl_2-Methanol and then evaporated to dryness in vacuo and recrystallized in methanol to yield pale white crystal (18 mg) coded as ECJ37-45. The structure was elucidated using mp, UV, FTIR and NMR and by comparison with reported data in the literature.

2.3.1 2, 2-Dipheny -1- picrylhydrazyl hydrate (DPPH) Assay

DPPH (4mg) was dissolved in methanol (100mL) to obtain 0.004% (w/v) stock solution of DPPH. The absorbance of the methanol solution of DPPH was read at 517 nm and recorded as blank. The procedure was repeated for n-hexane.

2.3.2 DPPH Assay with crude extract, fractions and the isolated compound

Extract/fraction/isolated compound (2 mg) was dissolved in 50 mL of methanol. Serial dilutions were carried out to obtain concentrations of 20, 40, 60, 80 and 100µg/mL respectively for each of the samples. 2 mL of each of the diluted solution was incubated with 2 mL of 0.004% DPPH in methanol at room temperature in a dark cupboard. After incubating for 30 minutes, the absorbance was read for each concentration against a blank at 517 nm. The percentage inhibition of free radical DPPH was calculated as follows:

% inhibition of DPPH radical = $\frac{Ablank - Asample}{Ablank}$ x 100

A_{blank} is the absorbance of the control reaction (DPPH solution without test sample)

A_{sample} is the absorbance of DPPH incubated with the sample

The concentration producing 50% inhibition ($1C_{50}$) was calculated using a graph of percentage inhibition against concentration of extract/fraction/isolated compound (Guangdong *et al*., 2008).

2.3.3 DPPH Assay with Standard Antioxidants

Standard antioxidant drugs used were Vitamin C (Emzor, Nigeria) and Vitamin E (Archy, Nigeria). The tablet dosage form of Vitamin C was used while Vitamin E was in a gelatin capsule form. The estimated weights of the formulations that would provide a stock solution of 2mg/ml were determined by proportionality (100 mg of Vitamin C was dissolved in 50 mL of solvent). Serial dilutions of vitamins solutions were made using methanol for Vitamin C and n-hexane for vitamin E to obtain solution of concentrations of 20, 40, 60, 80 and 100 µg/mL. 2mL of the different concentrations of the vitamins solutions was incubated with 2 mL of 0.004% DPPH for 30 minutes in a dark cupboard. The absorbance was then detected and the percentage inhibition calculated for each of the vitamins. The IC_{50} for the standard drugs was determined and comparisons were made with those of the samples.

3.0 RESULTS AND DISCUSSIONS

3.1 Phytochemical Studies

The results of phytochemical screening showed the presence of terpenoids, tannins saponins, flavonoids, phenols and carbohydrates. Compound ECJ37-45 was obtained as a pale white crystal, 18 mg, mp 116-118^{o}C (Litt 115-116^{o}C; Chemical Book, 2016); TLC, R_f 0.45 (n-Hexane: Ethyl acetate 4:5) and 0.77 ($CHCl_3$: Methanol 9:1), Refractive index was determined to be 1.57 (Litt 1.57; Chemical Book, 2016). It was found to be soluble in chloroform and acetone but sparingly soluble in water. The UV analysis of ECJ37-45 showed $\lambda_{(max)}$ at 256 nm (c = 0.895) showing the presence of a benzene ring (Philips, 2016).

The FT-IR analysis indicated peaks at 3419 cm^{-1} showing the presence of phenolic hydroxyl group, 2847cm^{-1} assigned to the C-H band of aldehyde group and the band appearing at 1731 cm^{-1} due to the aldehyde (CHO) group. A lower stretching frequency at 1632 cm^{-1} also observed was attributable to the Carbonyl group due to internal hydrogen bonding caused by the presence of phenolic hydroxyl group in the molecule (Shriner *et al*., 2004; Silverstein *et al*., 2005; Pouramini and Moradi, 2012). ^{1}H NMR δ_H (ppm): showed resonances assigned as follows: at δ_H 10.00 (H of OH; [A]), 8.58 (H of CHO [B]), 8.09 (2H on benzene ring ortho to CHO [C]); 7.51 ppm, (2H on benzene ring ortho to OH [D]); (Pouramini and Moradi, 2012).

This clearly showed that the OH group was para to the CHO group on the benzene ring. The combination of the spectral data which compared aptly with the reported spectral data of parahydroxybenzaldehyde provided a structure of ECJ37-45 as shown in figure 1. Thus ECJ37-45 was identified as parahydroxybenzaldehyde.

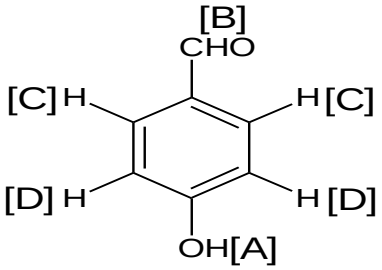

Figure 1: The structure of ECJ37-45 (Parahydroxybenzaldehyde)

3.2 Anti-Oxidant Studies

2, 2-Dipheny -1- picrylhydrazyl hydrate (DPPH) is characterized as a stable free radical by virtue of delocalization of the spare electron over the molecule as a whole, so that the molecule do not dimerize, as would be the case with most other free radicals.

This delocalization of electron is what gives rise to the deep violet colour solution, characterized by an absorption band centered at about 517 nm (Molyneux, 2004). On incorporation of the DPPH solution with plant samples and vitamins drugs (that is, methanol extract (MEOH), butanol fraction (BUOH), the isolated compound (ECJ37-45) as well as vitamins C and E), the purple colour of DPPH was bleached to pale yellow. This colour change was due to rapid and sufficient scavenging of the free electron thus neutralizing DPPH radical, leaving the inherent yellow colour of the non-radical form (1, 1-diphenyl-2-picryl hydrazine). The incubation period of 30 minutes was used to provide sufficient time for reaction between DPPH and the antioxidants. Prolonged incubation time was avoided as it might result in an unstable solution since DPPH is a strong antioxidant and has already been dissolved in an alcoholic solvent (methanol) prior to usage. The incubated solutions were kept in a cupboard to avoid exposure to light which has been shown to decrease the absorbance of DPPH at 517 nm (Prakash, 2001). Molyneux (2004), suggested that presentation of the results of antioxidant efficiency should involve the use of a standard due to doubt concerning the direct determination of DPPH obtained from calibration curve (Leitao *et al*, 2002). Thus vitamin C and E were employed to compare and check the correctness

of the procedure. Based on the recommendation by Blios, (1959), methanol solutions of the plant samples and vitamin C were used for the analysis to facilitate extraction of the antioxidant compounds from the sample. For vitamin E, n-hexane solution was used. This is due to the oily nature of the vitamin which makes it insoluble in polar solvents like methanol but miscible with non-polar solvent like n-hexane (Blios, 1959).

The radical scavenging indices of the plant samples and standard antioxidant indicate the reducing power of the respective drugs on DPPH radical. It was noted that the absorbance of DPPH at 517 nm decreased as the concentration of the added free radical scavengers (plant samples and the standard drugs) increased indicating that DPPH reagent was being reduced. The result of the reduction reaction is presented in Table 1. The Percentage Inhibition (PI %) or Radical Scavenging Activity (RSA %) and the IC_{50} values of the plant samples and standard drugs are displayed in Table 2. The RSA% is an indicator of the antioxidant activity of the plant samples and the standard drugs (Buijnster *et al*, 2001; Guangrong *et al*., 2008; Ahmad *et al* 2014). The IC_{50} was extrapolated from the graph of percentage inhibition against concentration for the plant samples and vitamins (Figure 2). The BUOH demonstrated a marginal higher antioxidant activity than MEOH as reflected in their IC_{50} of 88.90 µg/mL and 95.50 µg/mL respectively. ECJ37-45 (the isolated compound) however showed a much higher activity of 73.5 µg/mL, though not as high as those of Vitamins C and E at 37.5 µg/mL and 48.0 µg/mL respectively as shown in Table 2. Previous studies on the extract of the leaves of *A. Africana* showed the plant possessed antioxidant activity and parahydroxybenzaldehyde has been reported to possess antioxidant property (Oboh, 2006; Faleye and Abiodun, 2012; Dzoyema *et al*., 2017). This research study has confirmed this and has further shown that parahydroxybenzaldehyde is one of the compounds responsible for the antioxidant activity in the plant. This is the first report of isolation of parabenzaldehyde from *Aspilia Africana.*

TABLE 1: Absorbance of Samples incubated with DPPH at different Concentrations (λ_{max} 517nm)

	SAMPLE				
CONC	**MEOH**	**BUOH**	**ISOLATED COMPD**	**VIT C**	**VIT E**
0µg/mL	0.00	0.00	0.00	0.00	0.00
20µg/mL	1.262	1.771	1.162	1.011	1.051
40µg/mL	1.192	1.588	1.092	0.688	0.941
60µg/mL	1.045	1.323	0.945	0.385	0.734
80µg/mL	0.824	1.081	0.624	0.254	0.431
100µg/mL	0.688	0.864	0.501	0.142	0.132

Table 2: Radical Scavenging Activity (Percentage Inhibition) of Samples at different Concentrations and IC_{50} of Samples

	SAMPLE				
CONC	MEOH	BUOH	Isolated Compd	Vit C	Vit E
0 µg/mL	0.00	0.00	0.00	0.00	0.00
20 µg/mL	13.44	10.15	20.30	30.66	38.86
40 µg/mL	18.24	19.43	25.10	52.81	45.26
60 µg/mL	28.33	32.88	35.19	73.59	57.30
80 µg/mL	43.48	45.15	57.20	82.58	74.93
100 µg/mL	52.81	56.16	65.64	90.26	92.32
IC_{50} (µg/mL)	95.50	88.90	73.50	37.50	48.00

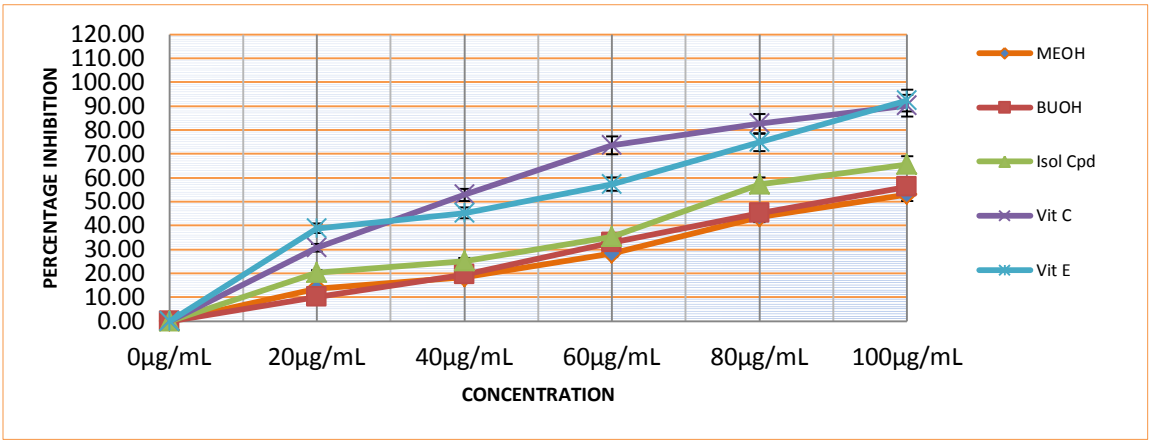

Figure 2: Radical Scavenging Activity (Percentage Inhibition) of Samples at different Concentrations

4.0 CONCLUSION

The results of this study have shown that the methanol extract and its butanol fraction of *A africana* as well as the isolated compound which has been identified as parahydroxybenzaldehyde have demonstrated antioxidant property which compared favourably with that of standard antioxidant drugs. This has lent credence to the use of *A. africana* in treating/managing cardiovascular diseases, wounds, tumors, inflammations and parasitic infections. The results also provide a baseline information confirming the use of parahydroxybenzaldehyde (which isolation from this plant is reported here for the first time) to treat oxidative stress.

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SUPPLEMENTAL MATERIALS

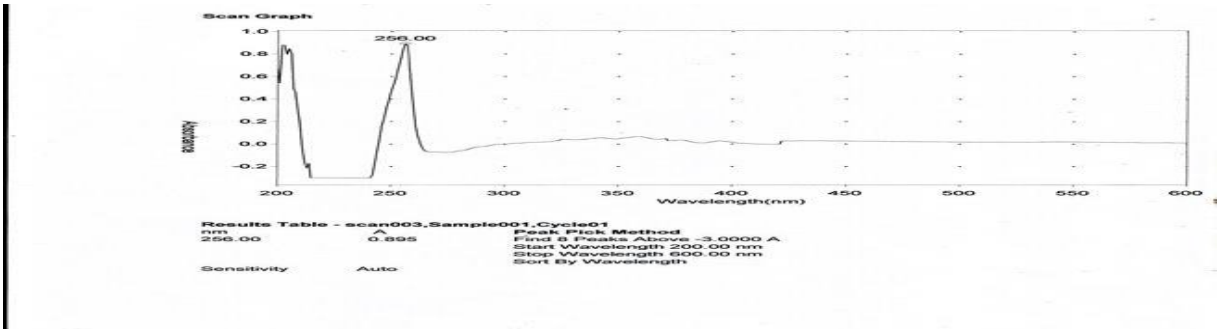
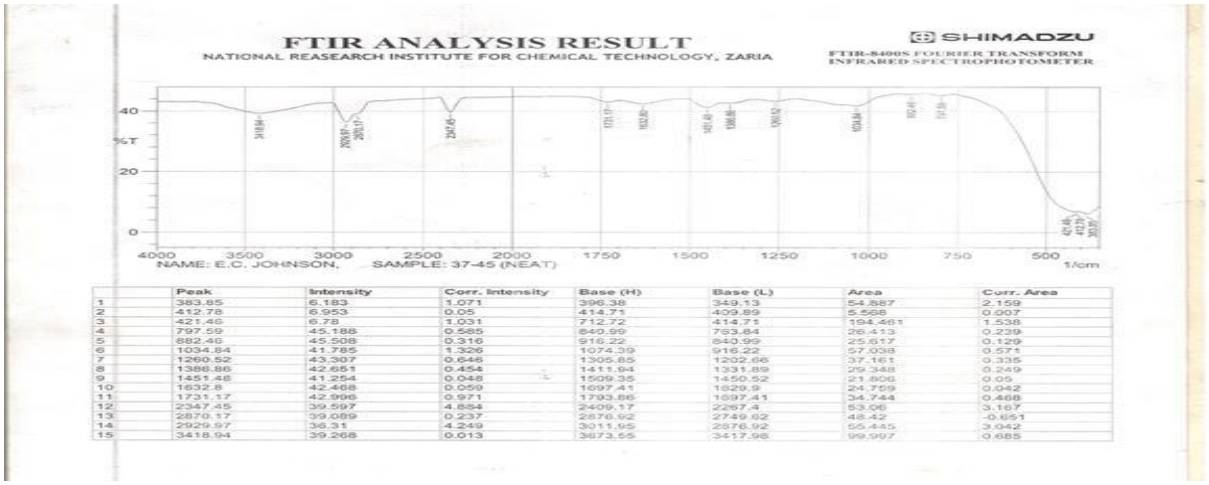


Figure 3 UV Spectrum ECJ37-45



	Peak	Intensity	Corr. Intensity	Base (H)	Base (L)	Area	Corr. Area
1	383.85	6.183	1.071	396.38	349.13	54.887	2.159
2	412.78	6.953	0.05	414.71	409.89	5.568	0.007
3	421.46	6.78	1.031	712.72	414.71	194.461	1.538
4	797.59	45.188	0.585	840.99	783.84	26.413	0.239
5	882.46	45.508	0.316	916.22	840.99	25.617	0.129
6	1034.84	41.785	1.326	1074.39	916.22	57.008	0.571
7	1260.52	43.307	0.646	1305.85	1202.66	37.161	0.335
8	1386.86	42.651	0.454	1411.94	1331.89	29.348	0.249
9	1451.48	41.254	0.048	1509.35	1450.52	21.806	0.05
10	1632.8	42.468	0.059	1697.41	1629.9	24.759	0.042
11	1731.17	42.996	0.971	1793.86	1697.41	34.744	0.468
12	2347.45	39.597	4.884	2409.17	2297.4	53.06	3.167
13	2870.17	39.089	0.237	2876.92	2749.02	48.42	-0.651
14	2929.97	36.31	4.249	3011.95	2876.92	55.445	3.042
15	3418.94	39.268	0.013	3673.55	3417.98	99.997	0.685

Figure 4 FTIR Spectrum of ECJ37-45

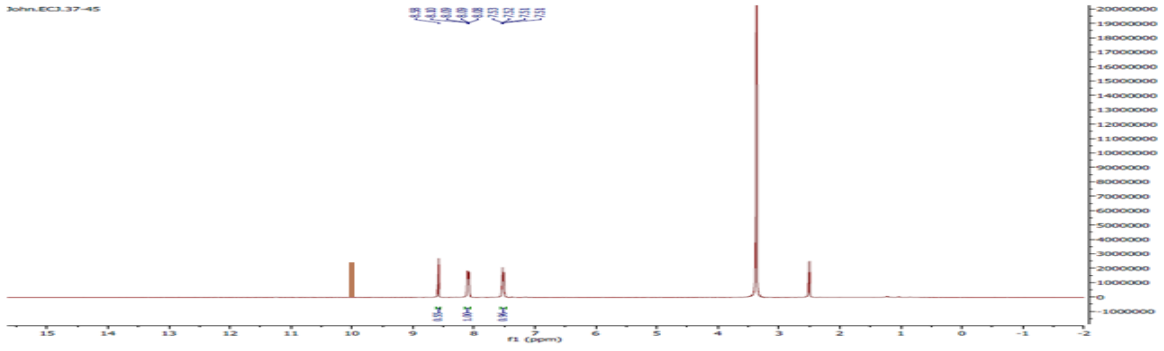


Figure 5 ^{1}H-NMR (400MHz) CD_3OD Spectrum of ECJ37-45