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Anti-Tubercular Glycolipids from the leaves of *Sterculia setigera* Del. (Sterculiaceae)

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Abstract
Natural products form one avenue in the search for new anti-tubercular agents with many groups undertaken screening of natural products as the preliminary step to finding new lead compounds. The anti-TB activity of ethno pharmacologically used *Sterculia setigera* leave was investigated by bioactivity-guided fractionation against virulent strains of *Mycobacterium tuberculosis* (H_{37}Rv (ATCC27294)) *in-vitro*, using the Alamar Blue Assay. Repeated purifications of the active fractions using combination of normal and reverse phase chromatography led to the isolation of a fraction with an interesting anti-TB activity (minimum inhibitory concentration of 15.13 µg/ ml). Preliminary spectroscopic studies shows that the most active fraction is a mixture of isomeric glycolipids based on evidence obtained from ^{1}H, ^{13}C nmr spectra. Effort is currently ongoing to separate the mixture for complete individual structure elucidation. This preliminary report is evident to the fact that glycolipids are the anti-mycobacterial agents in *S. setigera* leaves. This is the first report on the occurrence of biologically active glycolipids from *S. setigera* leaves.

Keywords: ethnopharmacology, antimycobacterial activity, *Sterculia setigera*, natural products, glycolipids, drug discovery

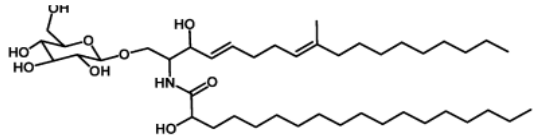
1. Introduction
Historically tuberculosis (TB) is one of the oldest and most pervasive diseases in history. Worldwide, TB is caused by *Mycobacterium tuberculosis* (MTB) and to a lesser degree *M. bovis* and *M. africanum, and* continues to be a major disease of global importance (Okunade *et al*. 2004) [11]. Mycobacterium tuberculosis, the bacterium that causes this disease, is protected from the host by a unique cell wall. It is a highly infective airborne and chronic disease usually infecting the lungs, although other organs are also involved. Although the number of tuberculosis (TB)-related deaths appears to have stabilized at around 2million per annum, the incidence of new infections is rising, largely owing to the HIV epidemic (Gutierrez-Lugo and Bewley, 2008) [7].

There are many challenges to eradicating TB, not least of which are the complexity associated with the disease, such as latency and drug resistance (Gutierrez-Lugo and Bewley, 2008) [7]. The drug used in the present day six-month combination therapy for treating tuberculosis was discovered 40 years ago. New drugs for tuberculosis are urgently needed to shorten the duration of therapy and to effectively treat drug-resistant TB and to eliminate the latent state. Such new anti-mycobacterium compound must possess suitable pharmacological targets that could affect cell wall synthesis, bacterial survival and be able to inhibit the mechanism by which these organisms overcome the early immune response of the host (Mc Kinney *et al*., 2000). The WHO estimates that if efforts do not change, by 2020, nearly 1 billion additional people will be newly infected with tuberculosis, 200 million people will become sick, and 35 million will die of the disease. (WHO, 2002).

Natural products form one avenue in the search for new anti-tubercular agents with many groups undertaken screening of natural products from higher plants, fungi and marine organisms as the preliminary step to finding new lead compounds (Okunade *et al*., 2004, Copp, 2003) [11, 5]. Naturally occurring compounds from higher plants, fungi and marine organisms that demonstrated significant inhibitory activity in the *in vitro* bioassays against *mycobacterium tuberculosis* and other mycobacterial species have been reported (Copp, 2003, Newton *et al*., 2000) [5, 10].

Findings from recent researches to identify phytochemicals that are inhibitory *in vitro* against *Mycobacterium* pertaining to C_6 (glycolytic) metabolism proves MTB populations present during infection are physiologically heterogenic, and therapeutic agents selected to inhibit the glycolytic phase may not affect the slow and non-growing phase that predominates in lung granulomas; hence the need for prolonged therapy (Okunade *et al*., 2004) [11]. A new class of secondary metabolites in the chemotherapy of tuberculosis with anticipated resolution of an infection in an expedited manner are glycolipids. One of the most important discoveries of all time was the isolation of carbohydrate antibiotic called Streptomycin, which disrupts bacterial protein synthesis. Recent researches have shown that carbohydrates joined through glycosidic linkages to lipids and to proteins called glycolipids and glycoproteins have functions that span the entire spectrum of activities in the cell. Glycolipids and Glycoproteins in the cell are known to be the agents by whom cells interact with other cells and with invading bacteria and viruses (Solomon and Fryhle, 2007) [16]. Indeed, most proteins are glycoproteins, of which the carbohydrates content can vary from less than 1% to greater than 90%. Glycolipids are built with one or two sugar residues, mainly glucose and galactose, in either α-or β-configuration (Fig. I, II &III), and attached to different lipid backbones. They are found in cell membranes of bacteria, fungi, plants and animals in the form of steryl glycosides, glucosylceramides, and diacylglycerol

glycosides. Glycolipids, together with some lipids, are considered structural and static components of the lipid bi-layer of biological membranes. Several key biological processes require the presence of amphipathic compounds such as glycolipids, whose structure shows both hydrophilic and hydrophobic groups. They constitute the backbone of biological structures such as cell membranes in both eukaryotic and prokaryotic cells, assuring the transport and exchange of materials.



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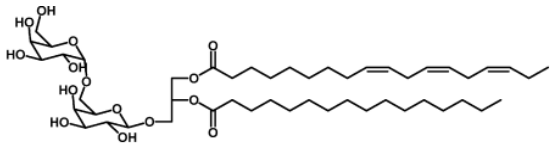

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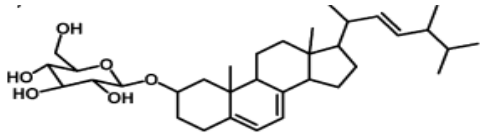


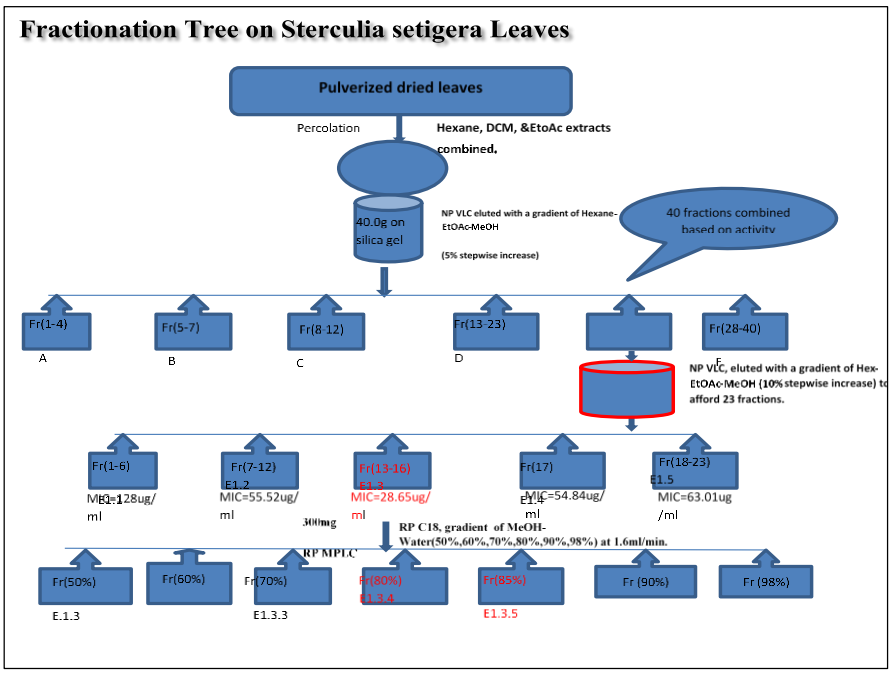
(III)

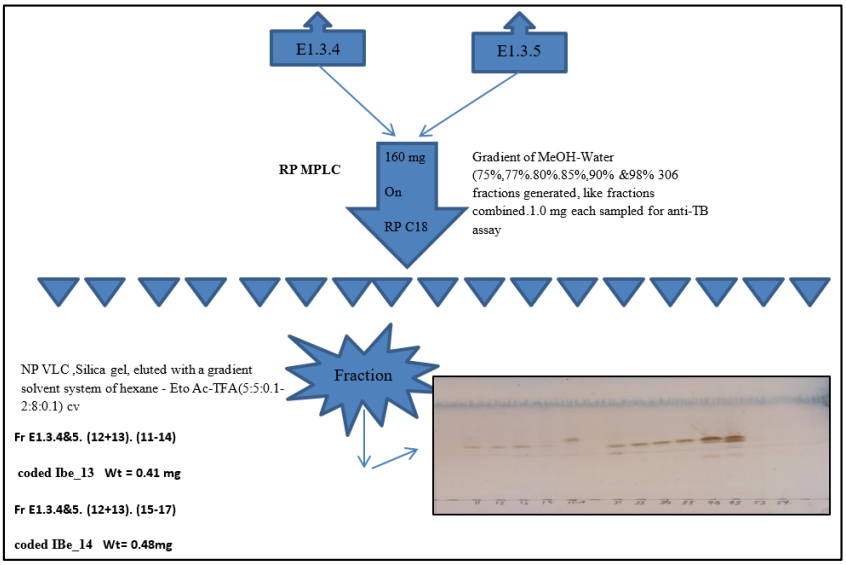
Some examples of these compounds in superior cells are: phosphatidylcholine, sphingosine, and glycolic acids, which are well-known. Besides being a part of the structure, they have been characterized as functional and dynamic components in eukaryotic cells that help in their flow regulation, as they are part of the micro regions in the membranes known as “lipid rafts”. Glycolipids have also demonstrated their biological activity in compounds such as ceramides, glucosylceramides, sphingolipids, glycosphingolipids and sphingosines, where they mediate anti-proliferative responses such as inhibition of cell growth, proliferation and differentiation, interruption of the cell cycle, signal transduction, senescence transformation, inflammation and apoptosis. The balance between the intra-cellular levels of each glycolipid, for example sphingolipids, is controlled by their producing enzymes. These enzymes must be strictly regulated, given their crucial role in the death or survival of the cell. They have become the objective in the fight against cancer and other degenerative diseases, since they take part in the initiation and progression phases and affect the outcome of anti-neoplastic therapies (Ruckhäberle *et al*. 2008).

The goal of this study is to identify phytochemicals that possess *in vitro* cidal effect or potent inhibitory activity against M*ycobacterium tuberculosis* in the study plant. A long term goal of this research is to identify novel natural products that have the potential of becoming new drug leads for anti-Tb drug development.

2. Material & Methods

The Pulverized leaves of *Sterculia setigera* was extracted using percolation method. The anti-TB activity of the leaves extract was investigated by bioactivity-guided fractionation against virulent strains of *Mycobacterium tuberculosis* ($H_{37}Rv$ (ATCC27294)) *in vitro*, using the Alamar Blue Assay (MABA). The method has the advantage of being simple and do not require radioactive substrates and less risk of contamination as it is carried out in a containment lab (Collins and Franzblau, 1997) [2]. Extensive purifications of the extract was carried by bioactivity guided fractionation using combination of normal and reverse phase chromatography and monitored with TLC. Scheme 1 present details of the fractionation steps leading to isolation of the pure fraction which gave a single spot on chromatogram (Ibe_1) and the result of the bioactivity guided fractionation has been reported (Babalola *et al.*,2012).





Scheme 1

3. Results and Discussion

Preliminary examination of the ^{1}H and ^{13}C spectra of the most active isolate Ibe_1 suggests a mixture of two closely related long chain fatty acids with glycoside unit (glycolipids). The ^{1}H and ^{13}C NMR spectra of the sample as shown in Tables 1&2 and Appendices 1-V show the presence of two characteristic ester carbons (171.76 ppm and 168.89 ppm).The intensity of the carbon signal at 171.76 triple that of the signal at 168.89, which suggests that it is the major compound in a mixture of two esters. The presence of 3 signals of anomeric carbons suggests the presence of sugars (99.10 ppm, 99.00 ppm and 98.97 ppm) and indicates the presence of glycoside linkage. This is in agreement with reported value for ^{13}C signals of β-D-galactose and of β-D-glucose (Breitmaier &Voelter, 1988). It can be established from the available evidence that Ibe_1 is a mixture of isomeric glycolipids and the major component of the mixture is proposed to be (2S)-1-O-(pentadecane)-glyceryl-β-D-galactopyranoside by simulation using chem Draw (IV). The sugar moiety in these glycolipids could either be glucose or rhamnose.

Table 1: ^{1}H NMR Spectrum of Ibe -1

Signal position	^{1}H (ppm)	Multiplicity
1	8.5	S
2	4.88	Broad & Singlet(s)
3	4.81	S
4	4.16-4.06	M
5	3.69	
6	3.63	
7	3.60	
8	3.59-3.32	
9		
10	3.36	
11	3.32	
12	3.32	
13	2.67	
14	2.59	M
15	2.08	
16	2.04	
17	1.90	
18	1.58	
19	1.30	
20	1.26	
21	1.25	
22	0.93	T
23	0.92	
24	0.90	

Table 2: ^{13}CNMR Spectrum of Ibe _1

Signal position	^{13}C (ppm)	Inference (assignment based on literature value)
1.	171.76	Ester carbon (-COO)(intensity is -3x that of signal (168.89ppm) Ester carbon (-COO)
2.	168.89	
3.	99.10	Anomeric carbons
4.	99.00	
5.	98.97	
6.	74.16	Sugar carbon signals
7.	74.13	
8.	72.44	
9.	71.19	
10.	70.86	
11.	69.66	
12.	69.64	
13.	68.78	
14.	65.34	
15.	64.48	
16.	62.97	
17.	62.63	
18.	60.19	
19.	39.77	
20.	33.06	Aliphatic carbon signals
21.	31.63	
22.	29.34	
23.	29.32	
24.	29.29	
25.	29.26	
26.	29.03	
27.	24.50	
28.	22.29	
29.	16.47	
30.	13.00	

Glycolipids are major constituents of the chloroplast membrane in plant kingdom. The biological functions as well as occurrence and distribution of galactolipids is an area of intense interest and investigation. Ibe_1 was previously reported for an interesting anti-TB activity (100% inhibition against the virulent strain of Mycobacterium tuberculosis (MIC of 15.13 µg/ ml). The cytotoxicity of these compounds evaluated was found non-toxic to vero cells and human hepatocellular carcinoma (HepG2) cell lines (Babalola *et al*., 2012) [1].

It has been shown in a study that glycolipids analogues have a promising inhibitory effect on Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) (Colombo *et al*., 2000; Colombo *et al*., 2001) [3, 4].

In different study, two new glyceroglycolipids - (2S)-2,3-O-di-(hexadecanoyl)glycerol-β-D-galactopyranoside and (2S)-3-O-(9,12-octadecadienoyl)-glyceryl-β-D-galactopyranoside isolated from *Euphorbia nicaeensis* (Euphorbiaceae) exhibited interesting anti-inflammatory activity (Francesca *et al*., 2004). Some glycolipids work as bioprobes, controlling a variety of functions in mammal cells. They participate in mechanisms of inter-cellular recognition and immune response (Osada, 1998) [12]. Glycolipids and their derivatives are of great interest in different health disorders due to their variety of biological functions and their potential for therapeutic uses. Some of these activities are: the anti-carcinogenic effects reported for lung, cervical, breast, and brain cancer (Isoda *et al*., 1997; Preetha *et al*., 2005) [8, 13]. Glycolipids have also demonstrated their biological activity in compounds such as ceramides, glycosyl ceramides, sphingolipids, glycosphingolipids and sphingosines, where they mediate anti-proliferative responses such as inhibition of cell growth, proliferation and differentiation, interruption of the cell cycle, signal transduction, senescence transformation, inflammation and apoptosis. Several other studies have validated biological activity of this class of metabolites such as antibacterial, antifungal, antiviral, anti-carcinogenic, and immune-modulating (Colombo *et al*., 2000; Colombo *et al*., 2001; Francesca *et al*., 2004; Cortes-Sanchez *et al*., 2013) [3, 4, 6].

Interestingly, pharmacological activity reported in some members of the family- *Sterculiaceae* were also attributed to long chain fatty acids (Reid *et al.*, 2005) [14]. Saravanakumar *et al*., (2008) [15] investigated the red marine alga, *Polysiphonia virgata* (*Rhodomelaceae*) for anti-mycobacterial activity against *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, isolating a mixture of long chain fatty acids as the main active components. In a related study, Oleic acid exhibited 100% inhibitory activity at 25 µg/ml against *Mycobacterium tuberculosis* $H_{37}RV$ in BACTEC method, followed by lauric acid, myristic and linoleic acids (98-100% inhibition) at 50 µg/ml. Palmitic and stearic acid showed no significant inhibition (Saravanakumar *et al*., 2008) [15].

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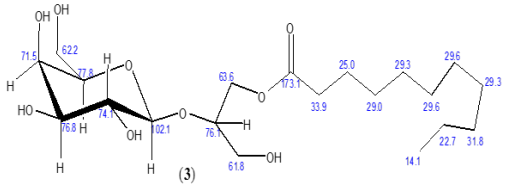
- Yobe State University, Damaturu

4. Conclusion

Natural products continue to play a most significant role in the drug discovery and development process (Newman and Cragg, 2007) [9], and plants are recognized as useful source of highly active antimycobacterial metabolites (Gibbons, 2003). Bioactivity guided fractionation led to the isolation of a fraction (Ibe_1) which was found to be a mixture of two isomeric glycolipids. Preliminary spectroscopic studies showed that the fraction is a mixture of two isomeric glycolipids (^1H, ^{13}C NMR), and the major component of the mixture is proposed to be ((2S)-1-O-(pentadecane)-glyceryl-β-D-galactopyranoside) by simulation.

Effort is ongoing to re-isolate in an appreciable quantity and separate the mixture for complete individual structure elucidation. This preliminary report establishes that the anti-tubercular agents in *S. setigera* leaves are glycolipids and this is the first report on the occurrence of biologically active glycolipids from *S. setigera* leaves.



(3)

(IV)

5. References

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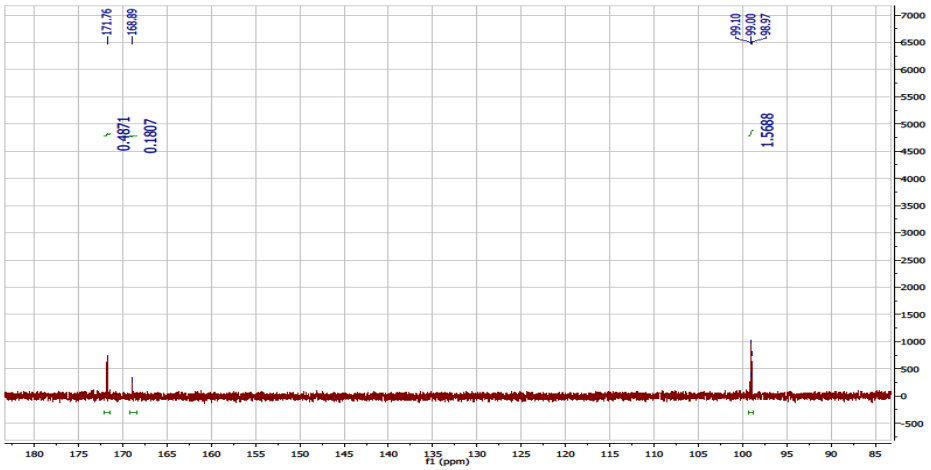
Appendixes



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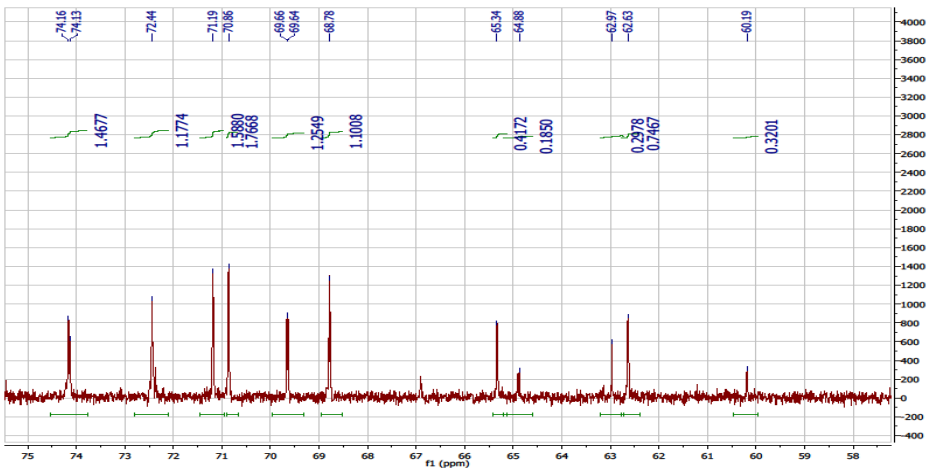

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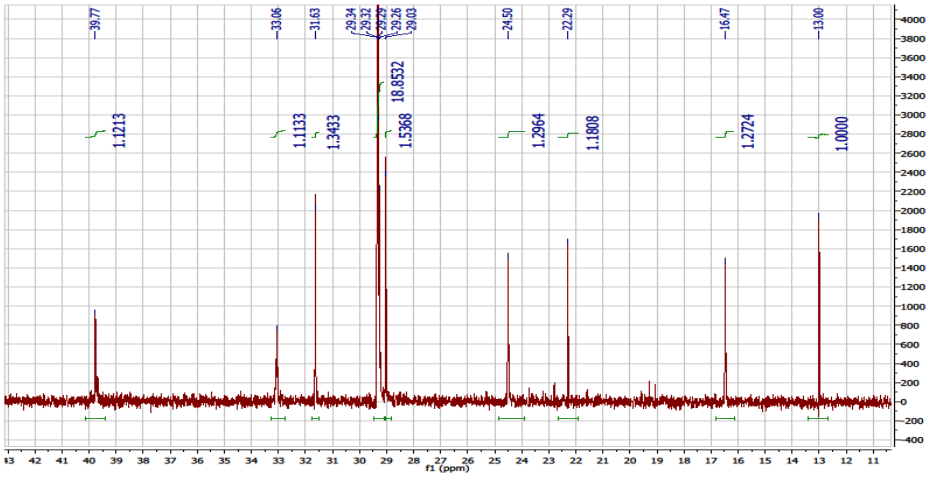

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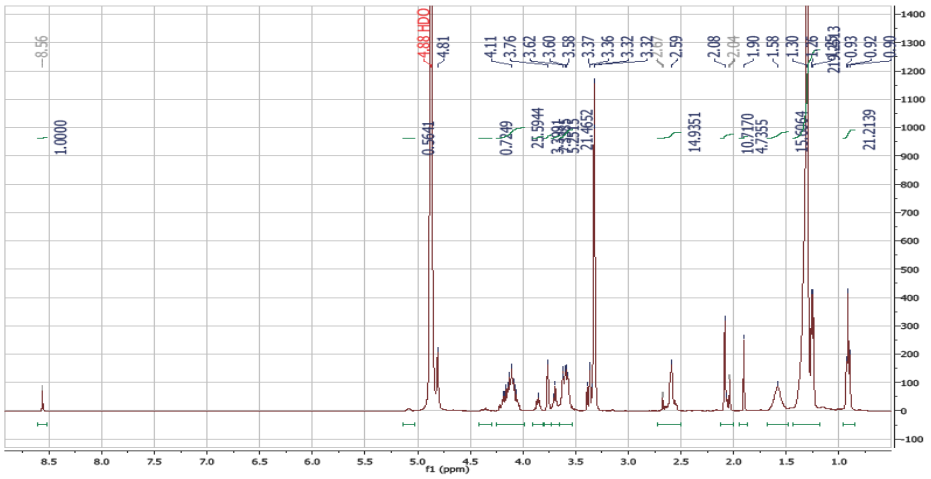

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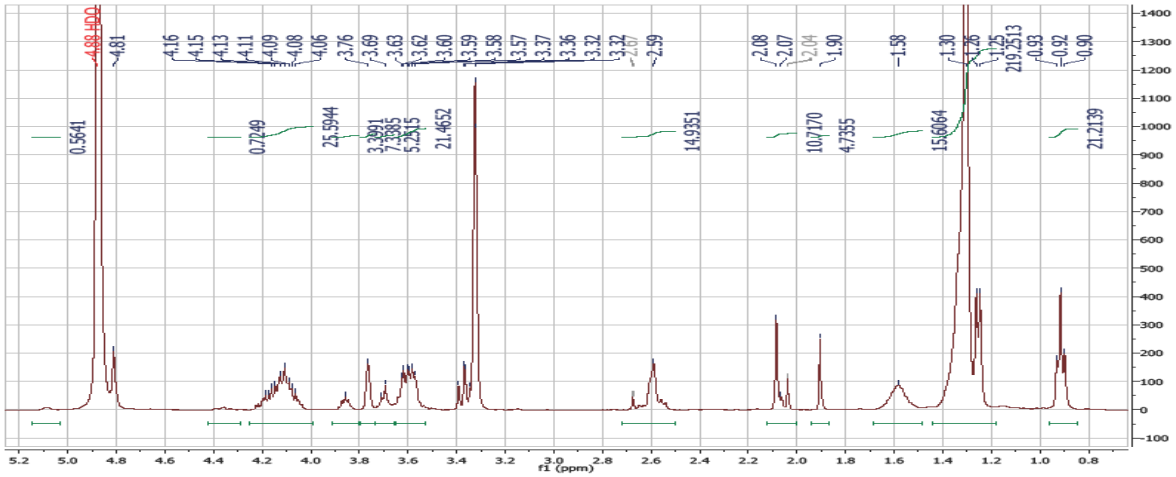

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