Synergistic Bioactivity Testing of a Medicinal Herb From the United States Virgin Islands

Jaime Heimbegner, B. S.
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Synergistic Bioactivity Testing of Extracts from a Medicinal Herb of the United States Virgin Islands

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Sweet Briar College

Jaime Lee Heimbegner

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Prof. John J. Beck	Thesis Project Faculty Advisor	04 April '04 Date
Prof. Jill N. Granger		4/9/04 Date
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~ DEDICATION ~

I would like to dedicate this work to my family, friends, and teachers who have played and integral role in its completion. I could not have made it through without their knowledge, guidance, and support. Thanks for always being there for me when I needed you. You are all much appreciated and I will never forget you.

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Synergistic Bioactivity Testing of Extracts from a Medicinal Herb of the United States Virgin Islands

Abstract: The research herein reports on the antibacterial activity of the medicinal herb lemongrass (*Cymbopogon flexuosus*) and possible synergistic activity between its extracts and the known antibiotics penicillin and ampicillin. The novel bioactive compounds of this medicinal herb are well known, thus this research focuses on confirming the plant's antibacterial activity, and more importantly, identifying possible synergistic activity. Disc susceptibility testing was performed against the bacteria *Bacillus subtilis* and *Staphylococcus aureus* to obtain background and synergistic bioactivity data. Critical analysis of the results led to the conclusion that an enhancement in bioactivity is exhibited by combining several plant extracts of *Cymbopogon flexuousus* with the known antibiotics penicillin and ampicillin. However, due to the lack of consensus pertaining to the issue of drug interaction, there are several equally valid models for evaluation of drug interaction. Since the definitions of drug synergism critically depend on the reference model for interaction, this research only reports the demonstration of enhanced bioactivity.

~ INTRODUCTION ~

Medicinal herbs contain substances known to modern and ancient civilizations for their healing properties. For centuries medicinal herbs have been an important source of remedies for assorted illnesses and injuries.[1] Since the beginning of their usage, medicines have advanced from the simple employment for treatment of ailments to the isolation, identification, and synthesis of their active components so that they can be marketed as commercial drugs. One example is the use of willow tree bark as an effective treatment for reducing fever and relieving pain. The evolution of willow bark as a drug began in the eighteenth century with the isolation of the active component, salicin, by Johann Buchner.[2] Salicin was subsequently converted into salicylic acid via hydrolysis and oxidation, and proved to be a very successful fever reducing agent.[3] The use of salicylic acid, however, often led to severe gastrointestinal toxicity, which was later overcome when salicylic acid was converted into acetylsalicylic acid via acetylation.[3] In the mid-nineteenth century, laboratory synthesis of the compound began and it was marketed under the trade name aspirin. Today, aspirin is the most widely used synthetic drug, with Americans alone consuming eighty million pills a day.[2]

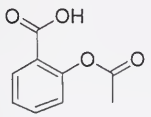


Figure 1. Aspirin

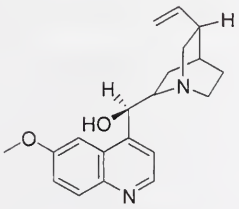
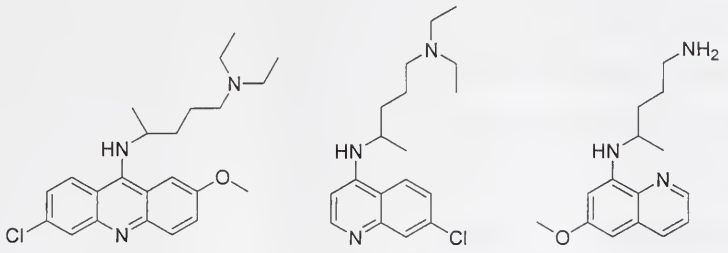


Figure 2. Quinine

Another example of the evolution of a drug in this manner is the use of the bark of cinchona trees to reduce fever, relieve pain, and to induce uterine contractions during labor.[4] Cinchona bark was brought to Europe from South America in the mid-seventeenth century and the active component, quinine, was isolated in the early nineteenth century by French chemists J. B. Caventou and P. J. Pelletier.[4] In the mid-nineteenth century laboratory synthesis of quinine was achieved by

American chemists R. B. Woodward and W. E. Doering; and before the recent development of more effective synthetic drugs such as quinacrine, chloroquine, and primaquine (See Figure 3), quinine was the specific agent in the treatment of malaria.[4]



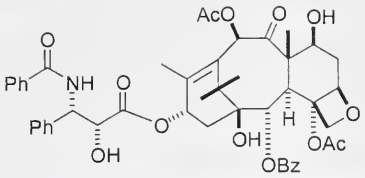
Quinacrine Chloroquine Primaquine

Figure 3

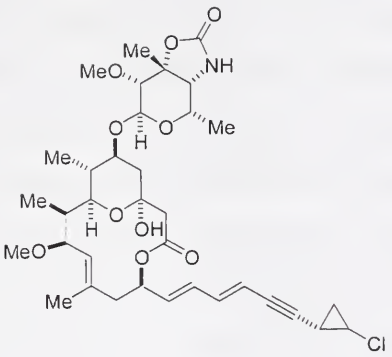
Following World War II there was a considerable investment by companies in the search for new drugs from plants.[5] However, after 1960 interest in the search began to fade and by 1974 only one pharmaceutical company in the United States was investigating plant-derived drugs.[5] By 1980, the total pharmaceutical budget of the U.S. had grown from $723 million to $2 billion, yet not a single U.S. company was researching potential drugs from higher plants.[5] The decrease in interest can be attributed to the introduction of automation, robotics, and personal computers to the drug discovery scene.[6] The introduction of this technology made chemistry the rate-limiting step in drug discovery programs.[6] Less than a decade later, there was a renewed interest in investigating plants as a likely source of new commercial drugs. One reason for the dramatic reversal of attitude can be attributed to the successful trials on Taxol® and other plant compounds that have shown encouraging activity against cancer and diseases such as HIV. Some companies continue to investigate natural products as a source of life-improving pharmaceuticals.[6] Bayer, Merck, and Wyeth are major drug companies that have remained committed to natural product

drug discovery.[6] With the current increase of new viruses and diseases, the desire to find new drugs from plants is at is peak.

In addition to the advent of new illnesses, the importance of new drug discovery from plants is critical due to the rapid destruction of the world's vegetation, threatening the extinction of many species. As our natural resources are being consumed the window of opportunity for the discovery of new drugs from plants diminishes. It has been estimated that the number of plant species on Earth is well over 250,000, of which only about one percent have been investigated in any depth in terms of their bioactive potentials.[5] With many active compounds yet to be discovered and fully evaluated, the investigation of natural resources in an effort to discover, manipulate, and synthesize new medicine, is of great importance to the pharmaceutical industry. It is estimated that twenty-five percent of prescription drugs contain plant-derived active ingredients and an even greater percentage is based on semi-synthetic or wholly synthetic ingredients originally isolated from plants.[3] According to a recent survey by the National Cancer Institute, 61% of the 877 small-molecule new chemical compounds introduced as drugs worldwide during 1982-2002 can be traced to or were inspired by natural products.[7] In 1985 2,618 new structures were isolated from plants, most of which were beyond the imagination of the most inventive chemists.[5] Molecules such as Taxol® and callipeltoside A (Figure 4) are prime examples of compounds whose structural complexity still manage to keep chemists in awe. In addition to their complex structures these compounds are also of great interest to chemists because of the cytotoxic activity they exhibit against cancer cells.



Taxol®



Callipeltoside A

Figure 4

Many chemists have been eager to research medicinal plants that exhibit antibacterial activity due to the recent health problem of increased bacterial resistance to antibiotics. Despite the fact that antibiotics dramatically transformed medical care by reducing illness and death from infectious diseases, the bacteria that they inhibit have developed a resistance to them over the decades. Excessive and inappropriate use of antibiotics promote this resistance, which occurs when the bacteria change in some way that reduces or eliminates the effectiveness of drugs or other agents designed to cure or prevent infections.[8] As a result of the widespread use of antibiotics, virtually all common bacterial infections in the United States, and throughout the world, are becoming resistant towards treatment, and for this reason *antibiotic resistance has become one of the world's most pressing public health problems.*[8]

In order to help combat antibiotic resistance, chemists have started to take a great deal of interest in drug synergism. Drug synergism is generally defined as the pharmacological effect of a drug combination that is greater than the effect of either agent alone. Because no clear consensus exists relative to the issue of drug interaction, there are several equally valid models for the evaluation of drug interaction.[9] As a consequence there are several definitions of what

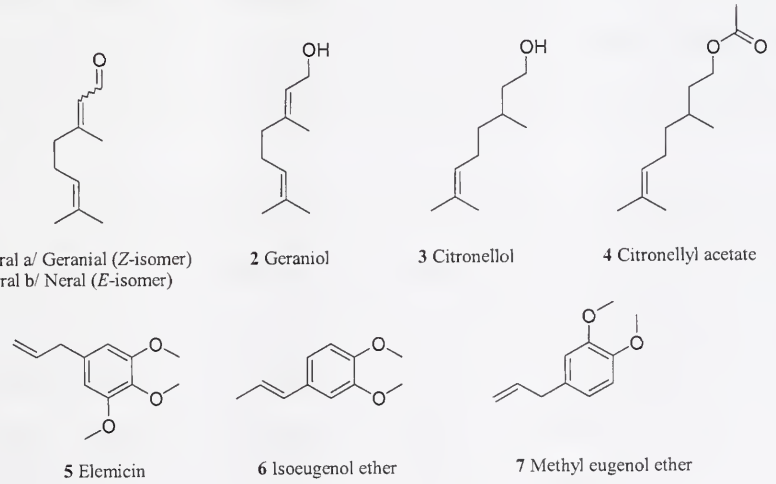
type of drug interaction constitutes drug synergism. However, there are two more common definitions of drug synergism; one states that drug synergism occurs when two drugs are combined so that the action of one drug aids or enhances the action of another.[10] Using this definition, the effect of the drug combination does not need to exceed the sum of the individual effects in order to be considered synergistic but must only be greater than the effect of either agent alone. The second definition states that drug synergism occurs when the combined effect of two drugs exceeds the sum of their individual effects.[10] By this definition, in order for a drug combination to be considered synergistic the effect of the combination *must* exceed the sum of the individual effects. Regardless of the reference model of drug interaction used, a prime example of drug synergism is the enhancement that occurs when certain drugs, such as the opiates codeine and morphine, are consumed with alcohol. The effect produced by the combination of the two is greater than the effect produced by either substance individually. Therefore, in theory, drug synergism between known antibiotics and plant extracts with antibiotic activity would allow for the use of minimal amounts of antibiotics, which in turn would result in reduced antibiotic resistance.

In order to find combinations of drugs that work synergistically, chemists rely on a relatively new method of testing both known and unknown compounds called synergistic testing. Synergistic testing examines the bioactivity of a plant extract (or any compound) and determines if the bioactivity is increased when mixed with a known drug and/or compound. The application of this method for the testing of both known and unknown herbal extracts not only allows researchers to identify new drug mixtures for treatment of illnesses and diseases but also to identify combinations of known drugs and natural products that can be particularly effective for combating bacteria that have become resistant to known antibiotics.

An example of a product that exhibits drug-drug synergy is the dietary supplement "Fen-phen." Fen-phen is the off-label combination of the appetite suppressants fenfluramine and phentermine. The term "off-label" refers to the use of a drug for purposes not specifically approved by the Food and Drug Administration's (FDA). The synergism of this combination of drugs results in the suppression of appetite and body weight, the reduction of brain serotonin levels, pulmonary vasoconstriction, and heart valve disease.[11] The rationale for this combination of drugs was that they exerted independent actions on brain satiety mechanisms so that it was possible to use lower dosages of each drug and yet retain a common action on suppressing appetite while minimizing adverse drug effects.[11]

In an effort to contribute to the research and discovery of active components of plants and their pharmaceutical uses, the research herein investigated a plant that has demonstrated medicinal properties and performed synergistic testing with its bioactive components and the known antibiotics penicillin and ampicillin. A search of the chemical literature indicates that synergistic activity between plant extracts has been investigated[12] but no literature references on possible synergistic activity between plant extracts and known pharmaceuticals were found. In an investigation of multi-drug resistance pumps, Stermitz *et. al.* found that two plant-derived compounds that do not exhibit bioactivity by themselves potentiated growth inhibitory activity of the natural antibacterial alkaloid berberine.[13] This discovery by Stermitz *et. al.*, and the promising results of the synergistic testing discussed in this thesis, are significant to the pharmaceutical industry because they have opened the door for the discovery of a new category of drugs that can be used to combat the ever-increasing threat of antibiotic resistance.

The synergistic testing performed in this investigation involved a plant commonly known as lemongrass (*Cymbopogon flexuosus*). Lemongrass is a medicinal herb from the family Gramineae that is widely cultivated in the tropics and subtropics.[14] It has exhibited antimicrobial activity in previous research[15] and has been used to treat a variety of conditions such as acne and flatulence.[16] Lemongrass oil has been found to be an important source of citral (**1** in Figure 5.) which is used for the production of ionones and vitamin A.[17] The chemical literature reports that this family of plants has a history of containing compounds that could explain the plants' purported bioactivities and in a few instances the structures for known compounds are identified (see Figure 5). Due to the extensive research performed by others to characterize the novel bioactive compounds of lemongrass, this research is able to investigate the plant's antibacterial activity and more importantly possible synergistic activity with known antibiotics.



1a Citral a/ Geranial (*Z*-isomer)
1b Citral b/ Neral (*E*-isomer)

2 Geraniol

3 Citronellol

4 Citronellyl acetate

5 Elemicin

6 Isoeugenol ether

7 Methyl eugenol ether

Figure 5

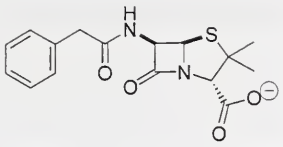


Figure 6. Penicillin G

The two known antibiotics combined with the plant extracts for synergistic testing in this investigation were penicillin and ampicillin. Penicillin was used as one of the co-biotics in an effort to provide possible alternate drug therapy treatment to patients with mild reactions to penicillin. The first drug-quality penicillin was produced in 1940 and has since become the drug of choice for most common bacterial infections due to its high activity rate and lack of toxicity.[18] Due to the specificity of penicillin's bioactivity, ampicillin was used as a second co-biotic in order to provide a broader range of activity.[19] Ampicillin is a semi-synthetic antibiotic and a member of the beta-lactam family. It is active against all penicillin-sensitive bacteria and has shown to be a versatile and relatively well-tolerated antibiotic.[19] Both penicillin and ampicillin hinder the cell wall synthesis of sensitive bacteria; hence they are classified as bactericidal.[18, 19]

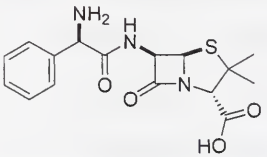


Figure 7. Ampicillin

The synergy of the plant extracts and the known antibiotics were evaluated by testing their bioactivity against bacteria plated on agar. The strains of bacteria used were *B. subtilis* and *Staphylococcus aureus*. *B. subtilis* is a gram-positive, rod-shaped bacterium found in soil and decomposing plant residue.[20] It is a non-pathogenic bacterium to humans, animals, and plants. *S. aureus* is also a gram-positive bacterium, but, unlike *B. subtilis*, it is a spherical-shaped bacterium, and is commonly found on the skin or in the mucous membranes of healthy people.[21] It can cause minor skin infections and food poisoning but can also cause serious and sometimes fatal infections such as bloodstream infections, surgical wound infections, and pneumonia.[22]

Bacteria are classified as either gram-positive or gram-negative. These two main groups of bacteria can be distinguished through a staining technique devised in 1884 by the Danish physician Hans Christian Joachim Gram. Gram found that when different types of bacteria were stained with aniline dye methyl violet followed by an iodine solution, the bacteria could be divided into two groups according to whether or not the addition of alcohol removed the dye (Gram-negative group) or remained fixed to the cells (Gram-positive group).[23]

Gram-positive bacteria, such as those used in this investigation, are characterized as having a cell wall structure comprised mainly of peptidoglycan.[24] Peptidoglycan is a unique polymer that provides much of the strength and rigidity possessed by bacterial cell walls.[24] It is a linear polymer with a backbone that consists of alternating subunits of N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). Attached to each NAM subunit is a side chain of four amino acids.[25] Gram-positive bacteria do not possess an outer membrane.

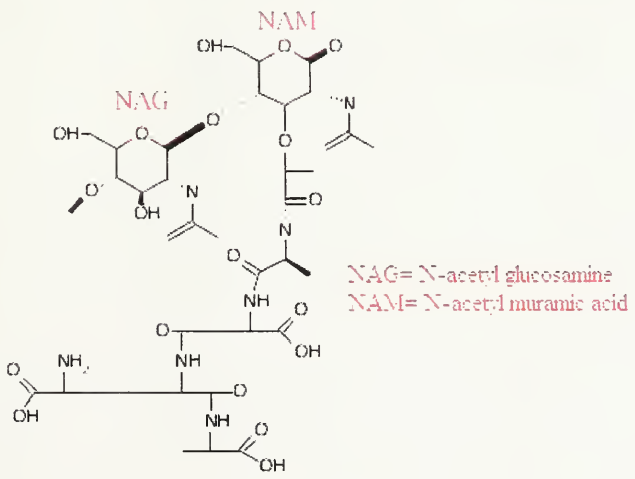


Figure 8. Structure of peptidoglycan

Gram-negative bacteria are characterized as having a cell wall structure comprised mainly of lipopolysaccharides.[24] Lipopolysaccharides are large, complex molecules that contain both lipids and carbohydrates.[25] The cell wall of gram-negative bacteria is a thinner structure than that of gram-positive bacteria, with an outer layer that is more like a cytoplasmic membrane.[24]

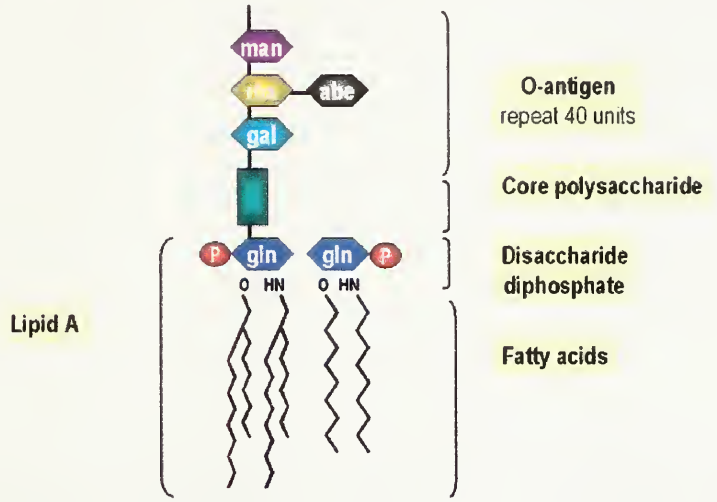


Figure 9. Structure of a lipopolysaccharide

~ RESULTS AND DISCUSSION ~

Due to the humid climate of the United States Virgin Islands (USVI), the plant material of lemongrass (*Cymbopogon flexuosus*) was oven-dried (40.5 °C), as opposed to air-dried, to prevent molding. Once received, the oven-dried plant material was extracted via the 3 x 24 h extraction method with methanol and concentrated *in vacuo*. The crude extract was tested for bioactivity and exhibited antibacterial activity against the two bacteria to be used for the entire investigation, *Bacillus subtilis* and *Staphylococcus aureus*. The bioassays were duplicated and the averages of the zones of inhibition are shown in Table 1. All replications can be found in Appendix A-01. As the data in Table 1 shows, the crude extract of lemongrass was only partially active against the two bacteria. *S. aureus* was observed to be more susceptible to the crude plant extract than *B. subtilis* based on the larger zones of inhibition exhibited. Previous research investigating the antibacterial activity of lemongrass supports these results.[26] The chemical literature not only shows that lemongrass exhibits antibacterial activity against the two bacteria, but it also shows that *S. aureus* is more susceptible to the plant extract.[27]

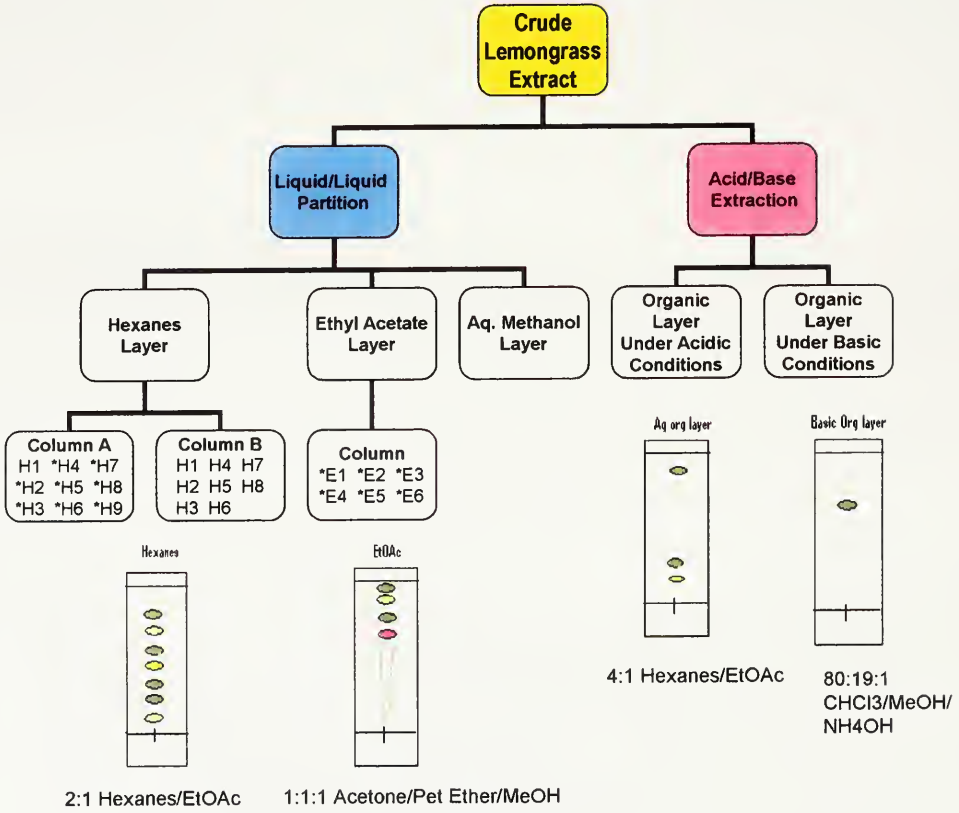
Table 1. Average zones of inhibition (mm) for lemongrass crude extract

Bacillus subtilis					***Staphylococcus aureus***				
Blank	**Penicillin (ctrl)**	**10µg**	**100µg**	**1000µg**	**Blank**	**Penicillin (ctrl)**	**10µg**	**100µg**	**1000µg**
0	31.3	0	*4	8.5	0	32.8	0	8.5	12

* Average of 8 mm and 0 mm because one of the discs fell off during testing

A portion of the crude extract was subjected to an acid/base extraction, and another portion was subjected to a liquid/liquid extraction. The process used for the extraction of lemongrass is shown in Scheme 1. The acid/base extraction yielded two fractions, an organic layer collected under acidic aqueous conditions and an organic layer collected under basic aqueous conditions.

Scheme 1. Extraction of Lemongrass



Despite no literature being found that suggested the presence of alkaloids in *Cymbopogon flexuosus*, alkaloids have been found in other plants of the *Cymbopogon* genera.[27] For this reason, the acid/base extraction was performed to rule out the possibility of alkaloid content in *C. flexuosus*. Alkaloids can occur naturally in either the N-oxide or free amine form.[28] Examples of these two forms and the protonated form are given in Figure 10.

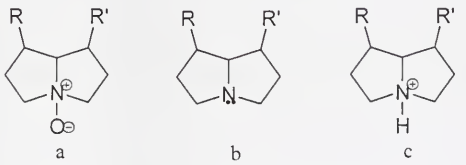
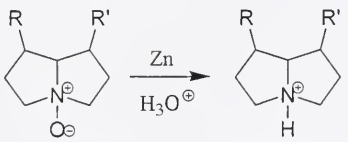


Figure 10. Generic alkaloid in the (a) N-oxide, (b) free amine, and (c) protonated forms

When brought under acidic conditions by addition of hydrochloric acid (HCl), all alkaloids in the free amine form are protonated and reside in the aqueous layer. Upon addition of zinc dust, alkaloids in the N-oxide form are reduced to free amines, and subsequently protonated, so that they too can be moved to the aqueous layer (see Scheme 2). The addition of zinc dust is an integral step in the extraction process because only the alkaloids present in the free amine form can be extracted; any alkaloids left in the N-oxide form would remain in the aqueous layer. All other compounds of non-alkaloidal nature would remain in the organic layer during acidic aqueous conditions. Ammonium hydroxide is added to the solution to deprotonate the alkaloids to the free amine form, causing them to move back into the organic layer for collection. Thin layer chromatography (TLC) of the organic layer extracted under acidic conditions and the organic layer extracted under basic



Scheme 2. Zinc reduction

conditions indicated that no alkaloids were present in *C. flexuosus* as viewed under UV light (254 nm) and vanillin developing spray. Iodoplatinic acid would have been better to use as a developing spray but was not utilized because it was not available at the time.

The liquid/liquid extraction yielded three fractions, a hexanes layer, an ethyl acetate layer, and a methanol/water layer. As anticipated, the TLC of the organic layer extracted under acidic conditions during the acid/base extraction was similar to the TLC of the crude hexanes layer from the liquid/liquid partition. In addition, the TLC of the organic layer extracted under basic conditions during the acid/base extraction was similar to the TLC of the ethyl acetate layer from the liquid/liquid partition. Despite that different eluent systems were used to obtain the TLC's, the hexanes and ethyl acetate layers appeared by TLC to contain the same compounds found in the organic layers obtained from the acid/base extraction. As a result, the acid/base fractions were set aside and only the fractions obtained from the liquid/liquid partition were subjected to bioactivity testing. A well-separated TLC of the methanol layer could not be obtained despite numerous solvent systems used, so it was also subjected to bioactivity testing.

The results of the bioactivity testing of the liquid/liquid partition fractions are as follows: The crude hexanes layer exhibited moderate bioactivity only against *B. subtilis* at concentrations of 100 and 1000 µg; the crude ethyl acetate layer exhibited bioactivity against *B. subtilis* at concentrations of 100 and 1000 µg and against *S. aureus* at a concentration of 1000 µg; and the methanol/water layer did not exhibit any bioactivity against either bacteria. All bioassays were performed in triplicate and the averages of the results are provided in Table 2. All replications can be found in Appendix A-02. Because the crude hexanes and ethyl acetate layers exhibited bioactivity they were subjected to synergistic testing with penicillin. Interestingly, for both samples, the greater the amount of plant extract loaded onto the penicillin disc the more

significant the decrease exhibited in bioactivity. This type of behavior was termed "reverse synergy" due to its apparent reversal of bioactivity. The bioassays were performed in triplicate and the averages of the results are provided in Table 3. All replications can be found in Appendix A-03.

Table 2. Average zones of inhibition (mm) of lemongrass crude layers from the liquid/liquid partition

	Bacillus subtilis					*Staphylococcus aureus*				
Fraction	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**
Hexanes	0	26	0	**8.6**	**10**	0	33.5	0	0	0
Ethyl acetate	0	33	0	**6.6**	**11.6**	0	34.6	0	0	**12.2**
Methanol/H_2O	0	33.7	0	0	0	0	32.3	0	0	0

Table 3. Average zones of inhibition (mm) for synergistic testing of lemongrass crude layers from the liquid/liquid partition (reverse synergy)

	Bacillus subtilis				*Staphylococcus aureus*			
Fraction	**Blank**	**Penicillin (ctrl)**	**Pen./ 100 µg**	**Pen./ 1000 µg**	**Blank**	**Penicillin (ctrl)**	**Pen./ 100 µg**	**Pen./ 1000 µg**
Hexanes	0	34.7	34	29.7	0	34.3	32.7	31.8
Ethyl acetate	0	33.3	30.3	26.3	0	32.7	31	28.3

The compounds of the hexanes layer and the ethyl acetate layer were partially separated via flash column chromatography then subjected to bioactivity testing. Due to the minimal amount of product in each fraction obtained from the column of the hexanes layer, the fractions were tested for bioactivity against only one bacterium. All fractions of the hexanes layer exhibited bioactivity against *S. aureus* except for one, H1. The fractions that exhibited bioactivity are marked in Figure 1 with an asterisk. Table 4 provides the averages of the results and all replications can be found in Appendix A-04. It was interesting to note that the crude hexanes layer did not exhibit bioactivity against *S. aureus* but almost all of the fractions of the

crude hexanes layer column did. This difference in bioactivity is most likely due to the removal of extraneous plant material by column chromatography, which is present in greater amounts than the bioactive components of the plant, or possibly compounds acting as inhibitors in combination.

Table 4. Average zones of inhibition (mm) for hexanes layer fractions

	Staphylococcus aureus				
Fraction	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**
H1a	0	35.5	0	0	0
H2a	0	35.5	0	10	8.5
H3a	0	36	0	11	12.5
H4	0	37	0	12	10
H5	0	34.5	0	11	13
H6	0	35.5	0	10.5	17
H7	0	34.5	0	9	13
H8	0	38	0	7.5	11.5
H9	0	36.5	0	8	8.5

To sufficiently continue the investigation, a second aliquot of the crude hexanes layer was subjected to flash column chromatography to obtain more product. The resultant fractions from these columns were different due to varying fraction collection patterns. Due to limited product availability, the fractions from the two columns run on the hexanes layer were combined based on the R_f values of the compounds by TLC visualized by vanillin developing spray. Fractions H1-H4 were combined to make fraction 1 (H1b), fractions H5 and H6 were combined to make fraction 2 (H2b), and fractions H7-H9 were combined to make fraction 3 (H3b). Essentially, all spots (3 total) located on the top third of the silica plate were combined, all spots (2 total) located in the middle third

Figure 11. Division of Fractions by TLC

of the plate were combined, and all spots (2 total) located in the bottom third of the plate were combined (see Figure 2). The new fractions were loaded onto discs preloaded with the antibiotics penicillin (10 IU) and ampicillin (10 μg) then subjected to synergistic testing.

Because the column fractions were only tested against *S. aureus*, analysis of the bioactivity data against *B. subtilis* was determined from data obtained for the crude hexanes layer and since *S. aureus* has proven to be more susceptible to the lemongrass plant extracts than *B. subtilis*. *S. aureus* has typically been more susceptible to the lemongrass extracts by 2-3 mm of inhibition, which can be observed in Tables 1-3. The first fraction (H1b) demonstrated an increase in bioactivity against *B. subtilis* when combined with penicillin or ampicillin, and did not exhibit an increase or decrease in bioactivity against *S. aureus*. Table 4 shows that the H3a fraction, the most bioactive of those combined to make the H1b fraction, had a zone of inhibition of 12.5 mm at a concentration of 1000 μg. In order for the compound to demonstrate additive bioactivity with penicillin it would have to exhibit a zone of inhibition of approximately 43.5 mm. Since 1000 μg of the first fraction (H1b) combined with penicillin only had a zone of inhibition of 36.3 mm against *B. subtilis*, it can be concluded that additive activity was not demonstrated but rather enhanced bioactivity of penicillin. In terms of deciphering the results to determine possible synergistic activity, the results can be interpreted in two different ways depending on the definition of synergism used as the basis of analysis. If one defines synergistic activity as the combined effect of two agents exceeding the sum of their individual effects,[10] then synergistic activity was not demonstrated. However, if one defines synergistic activity as the action of one drug aiding or enhancing the action of another,[10] then synergistic activity was demonstrated. Since the definition of synergism has not been absolutely established as observed

from the chemical literature, it will only be suggested that "enhanced bioactivity" was exhibited when the plant extract was combined with the known antibiotics.

It is interesting to note that when the zones of inhibition were evaluated by the areas and not by the diameters, there was a clear enhancement in bioactivity that demonstrated more than additive bioactivity and was congruent with both definitions of synergistic activity. Using the formula πr^2, the area of bioactivity for the H3a fraction was 122.72 mm^2 and 754.77 mm^2 for the penicillin. Their sum is 877.49 mm^2, which is less than that of the 1034.92 mm^2 area of bioactivity exhibited when they were tested in combination. When tested together the area of bioactivity was 157.42 mm^2 greater than the sum of their individual bioactivities, which suggests that additive and synergistic activity cannot be accurately quantified by linear measurement. The results of this new method of measurement poses an interesting question as to which method is the most accurate when evaluating inhibition of bacteria.

The same type of analysis was applied when determining the type of bioactivity the H1b fraction demonstrated when combined with ampicillin and tested against *B. subtilis*, as well as when determining the type of bioactivity demonstrated by fractions H2b and H3b. The second fraction (H2b) exhibited enhanced bioactivity against *B. subtilis* when combined with penicillin or ampicillin, and exhibited reverse synergy against *S. aureus* when combined with penicillin. This type of drug interaction is also referred to as drug antagonism, which occurs when the pharmacological effect of one drug (agonist) is reduced by a second drug (antagonist).[9] When combined with ampicillin and tested against *S.aureus*, fraction H2b did not demonstrate any change in bioactivity. The third fraction (H3b) exhibited enhanced bioactivity against *B.subtilis* when combined with penicillin or ampicllin, and against *S.aureus* when combined with penicillin. Reverse synergy was exhibited against *S.aureus* when fraction H3b was combined

with ampicillin. Tables 5 and 6 provide the averages of the results and all replications can be found in Appendices A-05 and A-06.

Table 5. Average zones of inhibition (mm) for synergistic testing of the hexanes fractions

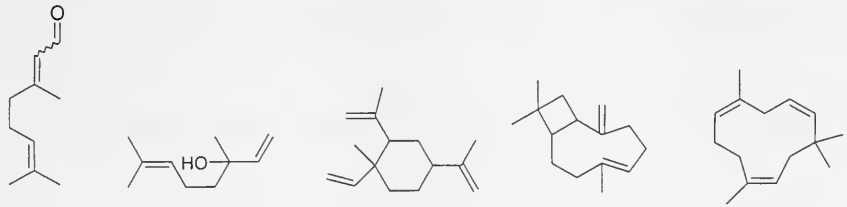
	Bacillus subtilis									
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
H1b	0	31	30	**33**	**36.3**	0	35.3	30	32.5	**39.6**
H2b	0	33	30	**35.6**	**38.7**	0	32	31	34	**38**
H3b	0	29.7	**31.7**	**35**	**40**	0	32	31.7	35.3	**39.3**

Table 6. Average zones of inhibition (mm) for synergistic testing of the hexanes fractions

	Staphylococcus aureus									
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
H1b	0	34	34.3	34	34	0	31.7	31.7	31.7	31
H2b	0	33	32.7	32.3	32	0	30	30	30	30
H3b	0	32.3	31.7	32	**33.7**	0	33	31.7	29.3	29.3

Because there are many taxa of *Cymbopogon flexuosus*, it was not possible to identify the taxon or the chemical composition of the plant used in this preliminary investigation.[29] A study of four taxa of *C. flexuosus* revealed that they possess distinct chemical characteristics when compared to each other, but when considered together with their differences in morphology, they warrant recognition as distinct taxa.[29] The study determined that linalool (**8**), β-elemene (**9**), β-caryophyllene (**10**), and α–humulene (**11**) are the only constituents common to all four *C. flexuosus* taxa, whereas nine constituents are common to three of the plants and twenty-one constituents to only two of the plants.[29] Although the taxa and chemical composition could not be identified, ^{1}H NMR spectra of several fractions of the hexanes layer bear resemblance to the

^{1}H NMR spectrum of citral, **1**, a mixture of the isomers neral and geranial, which have been found to be bioactive against both *B. subtilis* and *S. aureus* (see **1** in Figure 5).[30, 31] The ^{1}H NMR spectra of many hexanes fractions suggest the presence of citral due to vinyl signals present between 5-6 ppm and more importantly the aldehydic signal present just before 10 ppm.



1 **8** **9** **10** **11**

Figure 12. (**1**) citral , (**8**) linalool, (**9**) beta-elemene, (**10**) beta-caryophyllene, (**11**) alpha-humulene

Previous research comparing extraction methods for marker compounds in the essential oil of lemongrass by gas chromatography indicated that the hexanes extract of a solvent extraction contained the highest concentration of citral.[32] The results of the previous research supports the hypothesis that the hexanes layer of the liquid/liquid partition performed in this investigation contains citral. Another study that supports this hypothesis investigated the composition of the essential oil of *C. flexuosus*, in which a gas chromatogram of the hexanes fraction indicated the presence of a possible metabolic precursor of citral, 3,7-dimethyl-1-octene.[33]

Proton NMR data of the hexanes fractions also suggest the presence of compounds **8** and **10**. Comparison of the spectra shows signals present at approximately 6 ppm, indicating the presence of an internal alkene. These signals have a more complex splitting pattern than those of

a terminal alkene, which should be present around 5 ppm. The terminal alkene signals along with the other signals could not be identified in the spectra of the hexanes fractions due to the presence of other signals in the same chemical shift range. Although the ^{1}H NMR data does not positively identify the presence of compound **8** in the hexanes layer, the occurrence of the terminal alkene signal and the fact that the ^{1}H NMR data of the ethyl acetate and methanol layers do not bear any semblance to the linalool spectrum, provide evidence of compound **8** occurring in the hexanes layer.

The ^{1}H NMR data also suggests the presence of the compound **10** in the hexanes layer because of the doublet at approximately 4.8 ppm, which distinctly indicates the presence of a terminal alkene. The other signals could not be distinguished due to the interference of other signals. Proton NMR data was unavailable in the chemical literature for compounds **9** and **11**, and as a result their spectra could not be compared to the ^{1}H NMR spectra obtained from this investigation. In future research these compounds will be isolated and characterized by NMR but based on their structures it is anticipated that they would reside mainly in the hexanes layer as well, since both are relatively large and non-polar molecules. However, as is the case for the other compounds discussed, it is foreseeable that any compounds that are relatively non-polar will reside in both the hexanes and ethyl acetate layers. As shown in Figure 10, compound **9** contains three terminal alkenes, which are anticipated to have chemical shifts of approximately 5 ppm. Compound **11** contains three internal alkenes, which are anticipated to have chemical shifts of approximately 6 ppm.

All fractions of the ethyl acetate layer exhibited bioactivity against both bacteria and are marked in Scheme 1 (page 13) with an asterisk. The bioassays were performed in triplicate and the averages of the results are provided in Table 7. A table of all replications can be found in

Appendix A-07. Bacitracin was used instead of penicillin as a control for the background bioactivity testing of the ethyl acetate fractions due to the unavailability of penicillin at that particular time. Substituting bacitracin for penicillin was of no consequence because it was only used as a control and not for synergistic testing with the plant extracts.

Table 7. Average zones of inhibition (mm) for ethyl acetate layer fractions

	Bacillus subtilis					*Staphylococcus aureus*				
Fraction	**Blank**	**Bacitracin (ctrl)**	**10µg**	**100µg**	**1000µg**	**Blank**	**Bacitracin (ctrl)**	**10µg**	**100µg**	**1000µg**
E1	0	9.7	0	0	12.3	0	17.7	0	0	0
E2	0	9.3	0	0	12.6	0	18	0	0	7.7
E3	0	10	0	11	13.3	0	18.7	0	0	0
E4	0	10	0	12.7	15.3	0	17.3	0	10	11
E5	0	9.7	0	0	14.7	0	17	0	0	7.7
E6	0	9.7	0	8.7	11.3	0	18.7	0	0	8

Following the same procedure used for the hexanes layer, the fractions of the ethyl acetate layer were loaded onto discs preloaded with penicillin and ampicillin then subjected to synergistic testing. The bioassays were duplicated and the averages of the results are provided in Tables 8 and 9. All replications can be found in Appendices A-08 and A-09. Some fractions exhibited enhanced bioactivity, some fractions exhibited a decrease in bioactivity, and some fractions did not exhibit a change in bioactivity.

Fractions E1, E3, E4, and E5 exhibited enhanced bioactivity against *B. subtilis* when combined with penicillin or ampicillin, and fraction E2 exhibited enhanced bioactivity only when combined with ampicillin. Fraction E4 exhibited enhanced bioactivity against *S. aureus* when combined with penicillin or ampicillin, and fraction E3 exhibited enhanced bioactivity when combined only with penicillin. Fraction E5 exhibited enhanced bioactivity against *S. aureus* when combined with penicillin, but unlike the other combinations the greater the amount

of plant extract added the less enhanced the bioactivity exhibited. Additive activity was not observed because the zones of inhibition of the combinations were not equal to the sum of the individual bioactivities of the controls and the fractions. But again, if a surface area analysis is performed instead of a linear analysis it is evident that the enhancement in bioactivity demonstrated was more than additive and congruent with both definitions of synergistic activity.

Fraction E6 exhibited a decrease in bioactivity against *B. subtilis* when combined with penicillin or ampicillin, and fraction E2 exhibited a decrease in bioactivity only when combined with penicillin. Fraction E2 exhibited a decrease in bioactivity against *S. aureus* when combined with penicillin or ampicillin, and fraction E5 exhibited a decrease only when combined with ampicillin. It was interesting to note that when tested against *S. aureus* all of these combinations exhibited reverse synergy except the combination of fraction E6 with penicillin. Despite that the bioactivity increased the greater the amount of plant extract added, the bioactivity of the penicillin combined with 1000 µg of fraction E6 was still less than that of the penicillin control. Fractions E2 and E6 did not exhibit an increase or decrease in bioactivity against *S. aureus* when combined with penicillin or ampicillin, and fraction E3 exhibited an increase in bioactivity only when combined with penicillin.

Table 8. Average zones of inhibition (mm) for synergistic testing of the ethyl acetate fractions

	Bacillus subtilis									
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
E1	0	31.5	30	35.5	**39.5**	0	31.5	**33.5**	**36.5**	**40**
E2	0	32.5	31	31	30	0	29.5	29.5	**32**	**35.5**
E3	0	32	30	32.5	**41**	0	31	**33.5**	**37**	**39.5**
E4	0	30	29.5	31	**35.5**	0	32	29	30.5	**33.5**
E5	0	34.5	26.5	30	**35.5**	0	32.5	32.5	33.5	**42**
E6	0	33	31.5	32	32.5	0	33	32	31	31.5

Table 9. Average zones of inhibition (mm) for synergistic testing of the ethyl acetate fractions

	Staphylococcus aureus									
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
E1	0	33.5	34	33.5	33	0	32	31	31.5	31.5
E2	0	34	33.5	33	32.5	0	32.5	30.5	32.5	31.5
E3	0	30	**31.5**	**33.5**	**33.5**	0	31.5	32	32	32
E4	0	29.5	**30.5**	**30.5**	24	0	32	31	29.5	**42.5**
E5	0	31	**35.5**	**34**	**32.5**	0	31.5	31	31	29.5
E6	0	33.5	33	33	33	0	31.5	31.5	32	31.5

~ CONCLUSION ~

Experiments by other researchers have investigated possible synergistic activity of plant-derived compounds and reports mention the demonstration of synergistic activity but do not provide the model of drug interaction on which the analysis was based.[12,34, 35] Because no clear consensus exists pertaining to the definition of drug synergism, this investigation merely concludes that the combination of a number of lemongrass (*Cymbopogon flexuosus*) extracts with the antibiotics penicillin and ampicillin demonstrates some form of both enhanced and reduced bioactivity. Whether or not synergistic activity was demonstrated by these samples is dependent on the reference model used for drug interaction. If drug synergism is defined as the action of one drug aiding or enhancing the action of another[10], then drug synergism was demonstrated in this investigation. If it is defined as the combined effect of drugs that exceeds the sum of their individual effects[10], then drug synergism was not demonstrated in this investigation.

Critical analysis of the results led to the conclusion that combining plant extracts of *Cymbopogon flexuousus* with the known antibiotics penicillin and ampicillin did not result in clear additive activity. This conclusion was based on the fact that when the zones of inhibition were evaluated linearly by measuring the diameters, the bioactivities of the combined plant extracts and antibiotics were not equal to the sum of their individual bioactivities. However, it is interesting to note that when the zones of inhibition were evaluated by the areas and not by the diameters, there was a clear enhancement in bioactivity that demonstrated more than additive bioactivity and was congruent with both definitions of synergistic activity. This method of analysis will be addressed in a forthcoming paper.

~ EXPERIMENTAL ~

General. All solvents used were purchased from Aldrich Chemical Company[36] and were distilled prior to use. Final plant extracts were dried over Na_2SO_4 prior to concentration. NMR spectra were obtained on a JEOL ECX400 spectrometer in $CDCl_3$ and internally referenced to residual $CHCl_3$ (7.24 ppm, ^1H). Plant material was collected and dried by Toni Thomas, an extension agent of the University of the Virgin Islands co-op. extension service. All microbial agents were purchased from Carolina Biological Supply Company.[37] Blank discs (6 mm), agars, and nutrient broth were purchased from VWR Scientific Products.[38] Antibiotic discs (penicillin/ampicillin, 6mm) were purchased from Ward's Natural Science Company.[39]

Initial Extraction Process. Weighed and milled plant material was placed in an Erlenmeyer flask and soaked in MeOH for 24 h. The MeOH was decanted into a round-bottom flask, and the process repeated twice more. The combined extracts were concentrated *in vacuo*, weighed, and set aside for subsequent extractions.

Liquid/Liquid Extraction Process. Concentrated plant extract (330 mg) was dissolved in 80% MeOH/H_2O (100 mL), and a small amount of hexanes (10 mL) then transferred to a separatory funnel to be diluted in hexanes (total volume 100 mL) and partitioned. The methanolic solution was rinsed with hexanes (2 x 40 mL) and the combined extracts of the hexanes layer were concentrated *in vacuo*. The 80% MeOH/H_2O solution was diluted to 30% MeOH/H_2O and rinsed with EtOAc (3 x 40 mL). The combined extracts of the EtOAc layer were dried over Na_2SO_4 and concentrated *in vacuo*. The remaining MeOH/H_2O layer was concentrated *in vacuo* as well.

TLC of the crude hexanes and EtOAc layers was performed on precoated silica gel 60 F254 plates and column chromatography was carried out using 230-400 mesh silica gel. The eluent system used to separate the hexanes layer was 2:1 hexanes/EtOAc and the eluent system used to separate the EtOAc layer was 1:1:1 acetone/pet ether/MeOH. A TLC of the crude hexanes layer yielded two streaked spots with R_f values 0.37 and 0.71. A TLC of the crude ethyl acetate layer yielded only one streaked spot with an R_f value of 0.70. All spots were observed by UV (254 nm) detection.

Acid/Base Extraction Process of Lemongrass. Concentrated plant extract (1.22 g) was diluted in EtOAc (50 mL) and 0.1 M HCl (45 mL) and was then transferred into a separatory funnel. The layers were allowed to separate then partitioned. The aqueous layer was extracted with EtOAc (2 x 40 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. To the resultant acidic layer was then added zinc dust (5 g) and the mixture was allowed to stir at rt for approximately 3 h after which time the mixture was filtered through a pad of Celite. The mother liquor was then made basic (~ pH 9) using 25% NH_4OH and rinsed with EtOAc (3 x 40 mL). The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. A TLC of the organic layer collected under acidic conditions was run in 4:1 hexanes/EtOAc and showed four spots observed by UV (254 nm) detection with the R_f values 0, 0.17, 0.29, and 0.88. A TLC of the basic organic layer was run in 80:19:1 $CHCl_3$/MeOH/NH_4OH and yielded only one spot with an R_f of 0.7.

Preparation of Agars. *Nutrient Agar*- A 1L bottle containing deionized water (500 mL) and Nutrient Agar (11.5 g) was heated and stirred until the agar dissolved. The solution was allowed

to boil for 1 min, and was then autoclaved at 121 °C for 15 min. After completing the autoclave cycle the solution was allowed to cool, and the agar was poured into sterile Petri dishes that were covered and left overnight. The following morning the Petri dishes were turned upside down and refrigerated. *Sabouraud Dextrose Agar-* A 1 L bottle containing deionized water (500 mL) and Sabouraud Dextrose agar (32.5 g) was heated and stirred until the agar dissolved. The solution was allowed to boil for 1 min, and was then autoclaved at 121°C for 15 min. After completing the autoclave cycle the solution was allowed to cool, and the agar was poured into sterile Petri dishes that were covered and left overnight. The following morning the Petri dishes were turned upside down and refrigerated.

Preparation of Nutrient Broth. A 1 L bottle containing deionized water (500 mL) and Nutrient Broth (4 g) was heated and stirred until the powder dissolved then allowed to boil for 1 min. The mixture was autoclaved at 121 °C for 15 minutes. After completing the autoclave cycle the solution was allowed to cool to rt then refrigerated.

Preparation of Starter Plates. A Petri dish filled with the appropriate agar for the microbial agent to be plated (Nutrient Agar for *Staphylococcus aureus*, Sabouraud Dextrose for *Bacillus subtilis*) was removed from the refrigerator and allowed to warm to rt. An ethanol/flame sterilized inoculating hoop was used to remove a small amount of bacteria from a vial, which was spread across one edge of the agar plate a few times. The inoculating hoop was sterilized once more by ethanol/flame and used to scrape approximately 2-3 lines from the starter colony in another direction to start a new colony. The inoculating hoop was used to scrape 2-3 lines from the second colony to start a third colony. The cultured Petri dish was allowed to incubate upside

down at 37 °C for approximately 18 h and stored in the refrigerator upon removal from the incubator.

Preparation of Starter Cultures. Nutrient broth (5 mL) was transferred into a large autoclaved vial and allowed to warm to rt. An ethanol/flame sterilized inoculating hoop was used to scrape a colony from the starter plate and transfer the microbial agent into the large vial containing the nutrient broth. The hoop was rotated numerous times to ensure that the tip of the hoop came in contact with the bottom of the vial. The inoculated broth was incubated at 37 °C for 4-6 h and gently agitated approximately every 30 min.

Process of Loading Discs. The concentrated plant extract (20 mg) was diluted with the appropriate amount of solvent (2 mL) to make a 10 mg/mL solution referred to as Solution A. One half milliliter of Solution A was transferred into a vial and diluted to 10 mL with the same solvent to yield a 500 µg/mL solution referred to as Solution B. The discs were placed on a watch glass and loaded one drop at a time while directing a stream of air at the discs for drying purposes. For discs containing 10 µg of the plant extract the discs were loaded with 20 µL of Solution B. For discs containing 100 µg of the plant extract the discs were loaded with 10 µL of Solution A. For discs containing 1 mg of the plant extract the discs were loaded with 100 µL of Solution A. After loading, the discs were placed in a vacuum dessicator and concentrated *in vacuo* for a minimum of 20 min.

Preparing Culture Dishes. Petri dishes, filled with the appropriate agar for the microbial agent to be plated, were removed from the refrigerator and allowed to warm to rt. Using an autoclaved

Eppendorf pipet, 100 µL of the starter culture was transferred into the center of the Petri dish. An ethanol/flame sterilized spreader was used to disperse the bacteria evenly across the agar and the bacterial solution allowed to dry for no more than 5 min. Using autoclaved tweezers, the discs were applied to the agar a considerable distance from each other (~ 2.5 cm) and from the sides of the Petri dish (~1 cm). The dish was turned upside down and incubated at 37 °C for approximately 18 h.

Measurement of the Zones of Inhibition. The cultures were removed from the 37 °C incubator after approximately 18 h. Using a ruler, the zone of inhibition (in mm.) for each disc was determined by measuring the diameter of the area in which bacterial growth was inhibited.

Synergistic Testing Process. The discs to be used for synergistic testing were prepared in the same manner as outlined in the experimental with the exception that the extracts were loaded onto discs preloaded with penicillin (10 IU) or ampicillin (10 µg). The process of synergistic testing was identical to the process used for background bioactivity testing.

Storage of Microbes. All microbes were stored in a 37 °C incubator.[40]

~ FUTURE RESEARCH ~

This investigation was initiated as a pilot study for many future investigations. The next investigation will be the synergistic testing of the pure bioactive components of the *Cymbopogon flexuosus* taxa used in this investigation. Through an extensive literature search, all compounds within the plant that are known to exhibit bioactivity will be purchased and subjected to synergistic testing with penicillin, ampicillin, and possibly other antibiotics such as streptomycin. All other compounds will be isolated and characterized, and those that exhibit bioactivity will be subjected to synergistic testing with known antibiotics as well.

Another type of synergistic testing to be investigated is the possible synergy between compounds within the plant. Plants usually present defenses as a suite of compounds, not as individual ones.[37] It is thought that minor constituents found in low percentages may act as synergists, enhancing the effectiveness of the major constituents through a variety of mechanisms.[37] The results of an investigation of possible synergistic activity between compounds within the plant can then be applied to further synergistic testing of plant extracts and antibiotics. After thoroughly investigating possible synergistic activity between lemongrass plant extracts and antibiotics, possible synergistic activity between the plant extracts and fungicides can be investigated. Lemongrass has been found to exhibit antifungal activity, with citral as one of the fungicidal constituents.[15,39]

These investigations will be performed on other medicinal herbs from the United States Virgin Islands, particularly worrywine (*Stachytarpheta jamaicensis*), beggar's tick (*Bidens bipinnata*), inflammation bush (*Verbesina alata*), and noni (*Morinda citrifolia*).

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37. Carolina Biological Supply Company. P.O. Box 6010. Burlington, NC 27216-6010.

38. VWR International. P.O. Box 5015. Bristol, CT 06011-8960.

39. Ward's Natural Science. P.O. Box 92912. Rochester, NY 14692-9012.

40. Microbiological Techniques. **http://web.ukonline.co.uk/webwise/spinneret/microbes/tech1.htm** Accessed on 14 February 15, 2004.

~ Appendix A ~

Tables of Replications

A-01 Replications of Bioactivity Testing of Crude Lemongrass Extract

A-02 Replications of Bioactivity Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Bacillus subtilis*

A-03 Replications of Bioactivity Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Staphylococcus aureus*

A-04 Replications of Synergistic Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Bacillus subtilis*

A-05 Replications of Synergistic Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Staphylococcus aureus*

A-06 Replications of Bioactivity Testing of Hexanes Fractions against *Staphylococcus aureus*

A-07 Replications of Synergistic Testing of Hexanes Fractions and Penicillin against *Bacillus subtilis*

A-08 Replications of Synergistic Testing of Hexanes Fractions and Ampicillin against *Bacillus subtilis*

A-09 Replications of Synergistic Testing of Hexanes Fractions and Penicillin against *Staphylococcus aureus*

A-10 Replications of Synergistic Testing of Hexanes Fractions and Ampicillin against *Staphylococcus aureus*

A-11 Replications of Bioactivity Testing of Ethyl Acetate Fractions against *Bacillus subtilis*

A-12 Replications of Bioactivity Testing of Ethyl Acetate Fractions against *Staphylococcus aureus*

A-13 Replications of Synergistic Testing of Ethyl Acetate Fractions and Penicillin against *Bacillus subtilis*

A-14 Replications of Synergistic Testing of Ethyl Acetate Fractions and Ampicillin against *Bacillus subtilis*

A-15 Replications of Synergistic Testing of Ethyl Acetate Fractions and Penicillin against *Staphylococcus aureus*

A-16 Replication of Synergistic Testing of Ethyl Acetate Fractions and Ampicillin against *Staphylococcus aureus*

~ APPENDIX A ~

*The results of the replications below are shown in the following manner:

Replication 1, Replication 2, Replication 3

A1. Replications of Bioactivity Testing of Crude Lemongrass Extract

Bacillus subtilis					*Staphylococcus aureus*				
Blank	**Penicillin (ctrl)**	**10µg**	**100µg**	**1000µg**	**Blank**	**Penicillin (ctrl)**	**10µg**	**100µg**	**1000µg**
0, 0	35, 27.5	0, 0	8, 0	8.5, 8.5	0, 0	31.5, 34	0, 0	8, 9	11, 13

A2. Replications of Bioactivity Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**
Hexanes	0, 0, 0	24, 27, 27	0, 0, 0	8.5, 9, 8.5	9, 11, 10
Ethyl acetate	0, 0, 0	34, 30, 35	0, 0, 0	6.5, 7, 6.5	13, 11, 11
Methanol/H_20	0, 0, 0	32.5, 32.5, 36	0, 0, 0	0, 0, 0	0, 0, 0

A3. Replications of Bioactivity Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**
Hexanes	0, 0, 0	33.5, 33.5, 33.5	0, 0, 0	0, 0, 0	0, 0, 0
Ethyl acetate	0, 0, 0	34, 35, 35	0, 0, 0	0, 0, 0	12, 11.5, 13
Methanol/H_20	0, 0, 0	27, 33, 37	0, 0, 0	0, 0, 0	0, 0, 0

A4. Replications of Synergistic Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Bacillus subtilis*

	Bacillus subtilis			
Fraction	**Blank**	**Penicillin (ctrl)**	**Pen./ 100 µg**	**Pen./ 1000 µg**
Hexanes	0, 0, 0	33, 35, 36	33, 35, 34	30, 29, 30
Ethyl acetate	0, 0, 0	27, 35, 38	27, 32, 32	27, 23, 29

A5. Replications of Synergistic Testing of Crude Liquid/Liquid Partition Layers of Lemongrass against *Staphylococcus aureus*

	Staphylococcus aureus			
Fraction	**Blank**	**Penicillin (ctrl)**	**Pen./ 100 µg**	**Pen./ 1000 µg**
Hexanes	0, 0, 0	34, 34, 35	32, 34, 32	32.5, 32, 31
Ethyl acetate	0	31, 34, 33	31, 32, 30	28, 28, 29

A6. Replications of Bioactivity Testing of Hexanes Fractions against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Penicillin (ctrl)**	**10 µg**	**100 µg**	**1000 µg**
H1	0, 0	36, 35	0, 0	0, 0	0, 0
H2	0, 0	35, 36	0, 0	9, 11	9, 8
H3	0, 0	35, 37	0, 0	10, 14	10, 15
H4	0, 0	36, 38	0, 0	10, 12	10, N/A
H5	0, 0	35, 34	0, 0	10, 12	13, 13
H6	0, 0	36, 35	0, 0	10, 11	18, 16
H7	0, 0	34, 35	0, 0	9, 9	13, 13
H8	0, 0	38, 38	0, 0	8, 7	11, 12
H9	0, 0	36, 37	0, 0	7, 9	10, 7

A7. Replications of Synergistic Testing of Hexanes Fractions and Penicillin against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**
H1	0, 0, 0	32, 29, 32	34, 31, 25	35, 34, 30	38, 37, 34
H2	0, 0, 0	33, 33,33	31, 28, 31	36, 35, 36	39, 37, 40
H3	0, 0, 0	32, 30, 27	35, 32, 28	37, 35, 33	39, 41, 40

A8. Replications of Synergistic Testing of Hexanes Fractions and Ampicillin against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
H1	0, 0, 0	35, 35, 36	30, 30, N/A	31, 34, N/A	43, 38, 38
H2	0, 0, 0	31, 33, 32	31, 32, 30	33, 35, 34	40, 39, 35
H3	0, 0, 0	32, 32, 32	31, 32, 32	35, 36, 35	39, 40, 39

A9. Replications of Synergistic Testing of Hexanes Fractions and Penicillin against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**
H1	0, 0, 0	34, 34, 34	34, 35, 34	34, 35, 33	34, 35, 33
H2	0, 0, 0	33, 32, 34	33, 32, 33	33, 31, 33	32, 31, 33
H3	0, 0, 0	31, 32, 34	32, 31, 32	33, 31, 32	35, 33, 33

A10. Replications of Synergistic Testing of Hexanes Fractions and Ampicillin against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
H1	0, 0, 0	31, 32, 32	31, 32, 32	31, 32, 32	31, 31, 31
H2	0, 0, 0	30, 30, 30	30, 30, 30	30, 30, 30	30, 30, 30
H3	0, 0, 0	35, 32, 32	31, 32, 32	29, 29, 30	29, 30, 30

A11. Replications of Bioactivity Testing of Ethyl Acetate Fractions against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Bacitracin (ctrl)**	**10µg**	**100µg**	**1000µg**
E1	0, 0, 0	10, 10, 9	0, 0, 0	0, 0, 0	12, 12, 13
E2	0, 0, 0	9, 10, 9	0, 0, 0	0, 0, 0	12, 12, 14
E3	0, 0 ,0	10, 10, 10	0, 0, 0	11, 12, 10	12, 15, 13
E4	0, 0, 0	10, 10, 10	0, 0, 0	12, 14, 12	15, 16, 15
E5	0, 0, 0	10, 10, 9	0, 0, 0	0, 0, 0	19, 15, 10
E6	0, 0, 0	10, 9, 10	0, 0, 0	9, 9, 8	11, 13, 10

A12. Replications of Bioactivity Testing of Ethyl Acetate Fractions against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Bacitracin (ctrl)**	**10µg**	**100µg**	**1000µg**
E1	0, 0, 0	18, 17, 18	0, 0, 0	0, 0, 0	0, 0, 0
E2	0, 0, 0	18, 18, 18	0, 0, 0	0, 0, 0	8, 7, 8
E3	0, 0, 0	19, 18, 19	0, 0, 0	0, 0, 0	0, 0, 0
E4	0, 0, 0	17, 18, 17	0, 0, 0	10, 10, 10	11, 12, 10
E5	0, 0, 0	17, 17, 17	0, 0, 0	0, 0, 0	7, 8, 8
E6	0, 0, 0	19, 18, 19	0, 0, 0	0, 0, 0	8, 8, 8

A13. Replications of Synergistic Testing of Ethyl Acetate Fractions and Penicillin against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**
E1	0, 0	32, 31	29, 31	37, 34	39, 40
E2	0, 0	33, 32	31, 31	31, 31	30, 30
E3	0, 0	33, 31	30, 29	32, 32	35, 36
E4	0, 0	30, N/A	31, 28	32, 30	36, 35
E5	0, 0	34, 35	28, 25	30, 30	36, 35
E6	0, 0	33, 32	34, 31	33, 34	42, 42

A14. Replications of Synergistic Testing of Ethyl Acetate Fractions and Ampicillin against *Bacillus subtilis*

	Bacillus subtilis				
Fraction	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
E1	0, 0	31, 32	34, 33	36, 37	40, 40
E2	0, 0	30, 29	30, 29,	32, 32	35, 36
E3	0, 0	31, 31	32, 35	37, 37	40, 39
E4	0, 0	34, 30	29, 29	29, 32	32, 35
E5	0, 0	33, 32	34, 31	33, 34	42, 42
E6	0, 0	33, 33	32, 32	31, 31	32, 31

A15. Replications of Synergistic Testing of Ethyl Acetate Fractions and Penicillin against *Staphylococcus aureus*

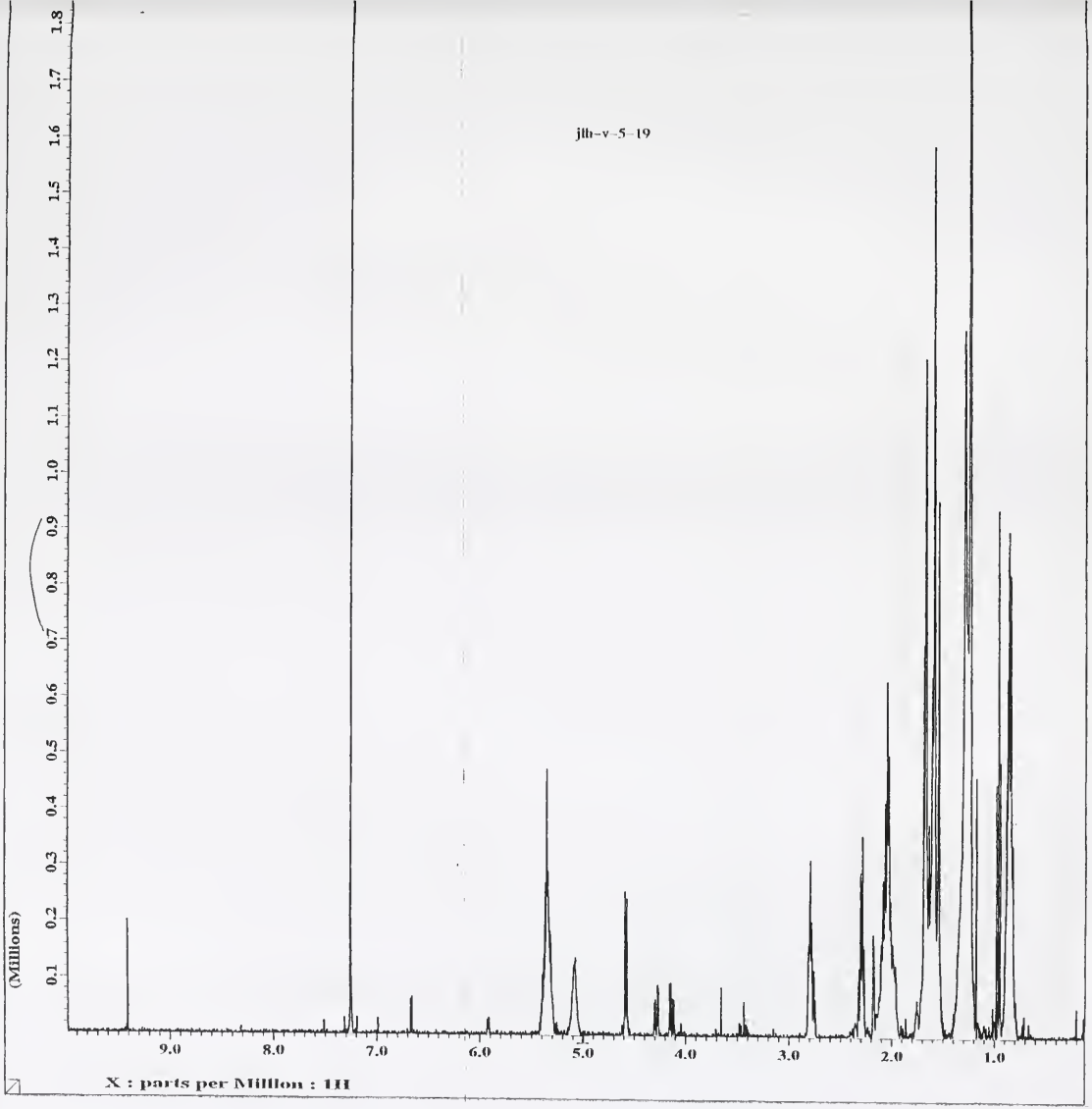
	Staphylococcus aureus				
Fraction	**Blank**	**Pen. (ctrl)**	**Pen./ 10µg**	**Pen./ 100µg**	**Pen./ 1000µg**
E1	0, 0	33, 34	34, 34	33, 34	33, N/A
E2	0, 0	34, 34	33, 34	34, 32	33, 32
E3	0, 0	31, 29	32, 31	35, 32	35, 32
E4	0, 0	30, 29	30, 31	31, 30	22, 26
E5	0, 0	30, 32	36, 35	35, 33	33, 32
E6	0, 0	33, 34	33, 33	33, 33	33, 33

A16. Replication of Synergistic Testing of Ethyl Acetate Fractions and Ampicillin against *Staphylococcus aureus*

	Staphylococcus aureus				
Fraction	**Blank**	**Amp. (ctrl)**	**Amp./ 10 µg**	**Amp./ 100µg**	**Amp./ 1000µg**
E1	0, 0	32, 32	31, 31	31, 32,	31, 32
E2	0, 0	32, 33	30, 31	33, 32	31, 32
E3	0, 0	32, 31	32, 32	32, 32	32, 32
E4	0, 0	32, 32	31, 31	30, 29	24, 25
E5	0, 0	32, 31	32, 30	32, 30	28, 31
E6	0, 0	31, 32	31, 32	32, 32	31, 32

~ Appendix B ~

Lemongrass ^{1}H NMR Spectra

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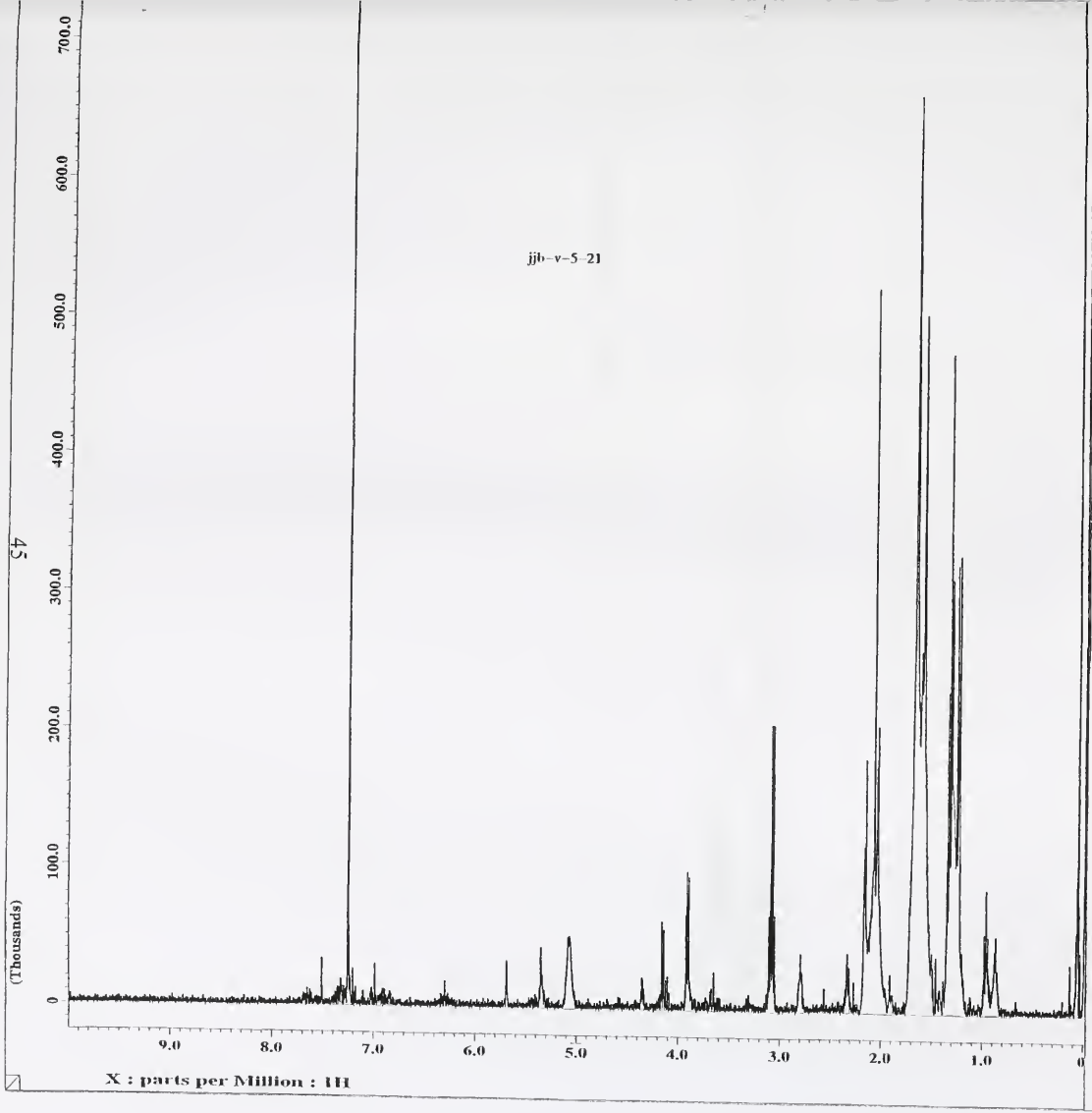

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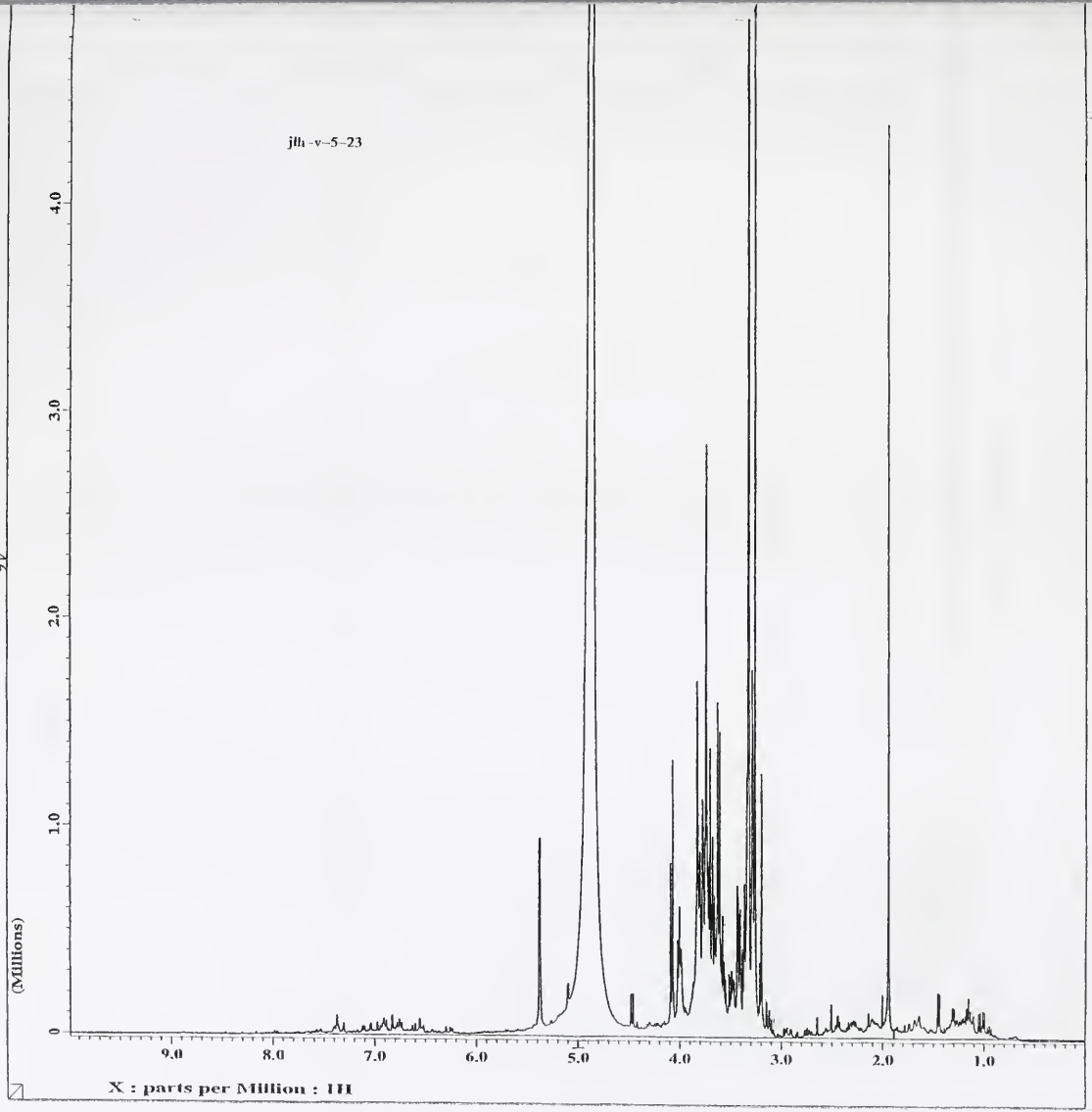

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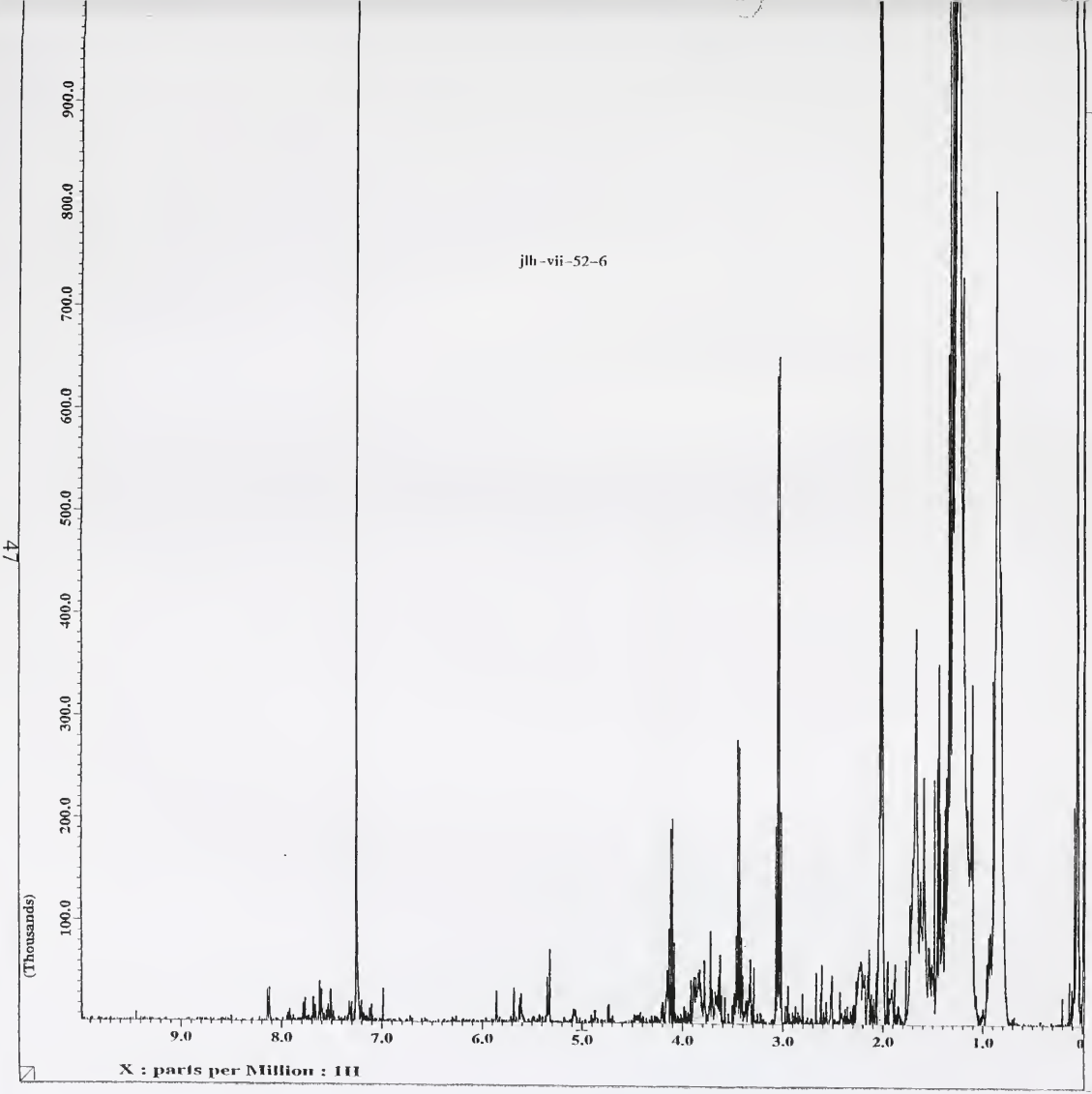

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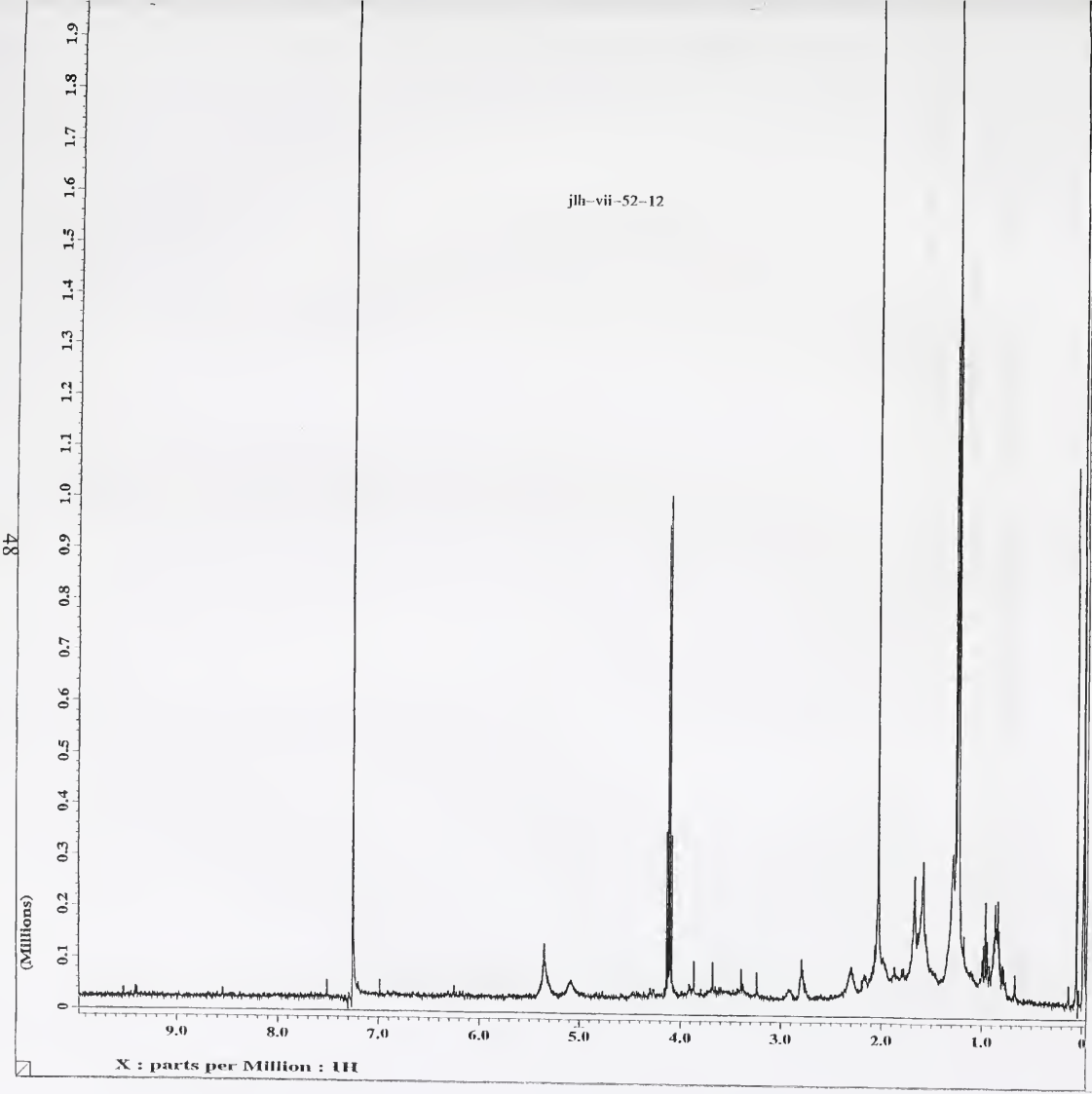


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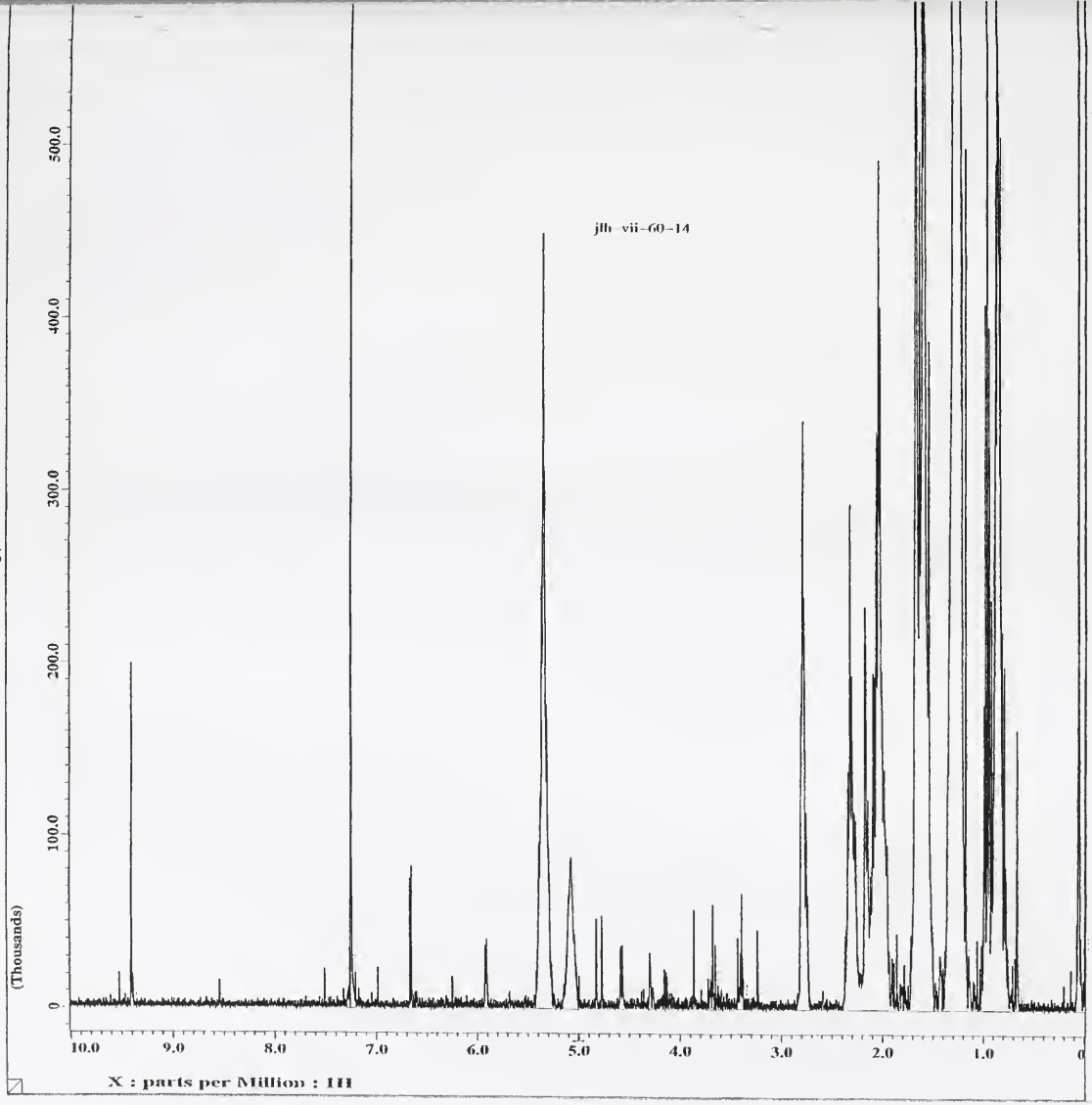

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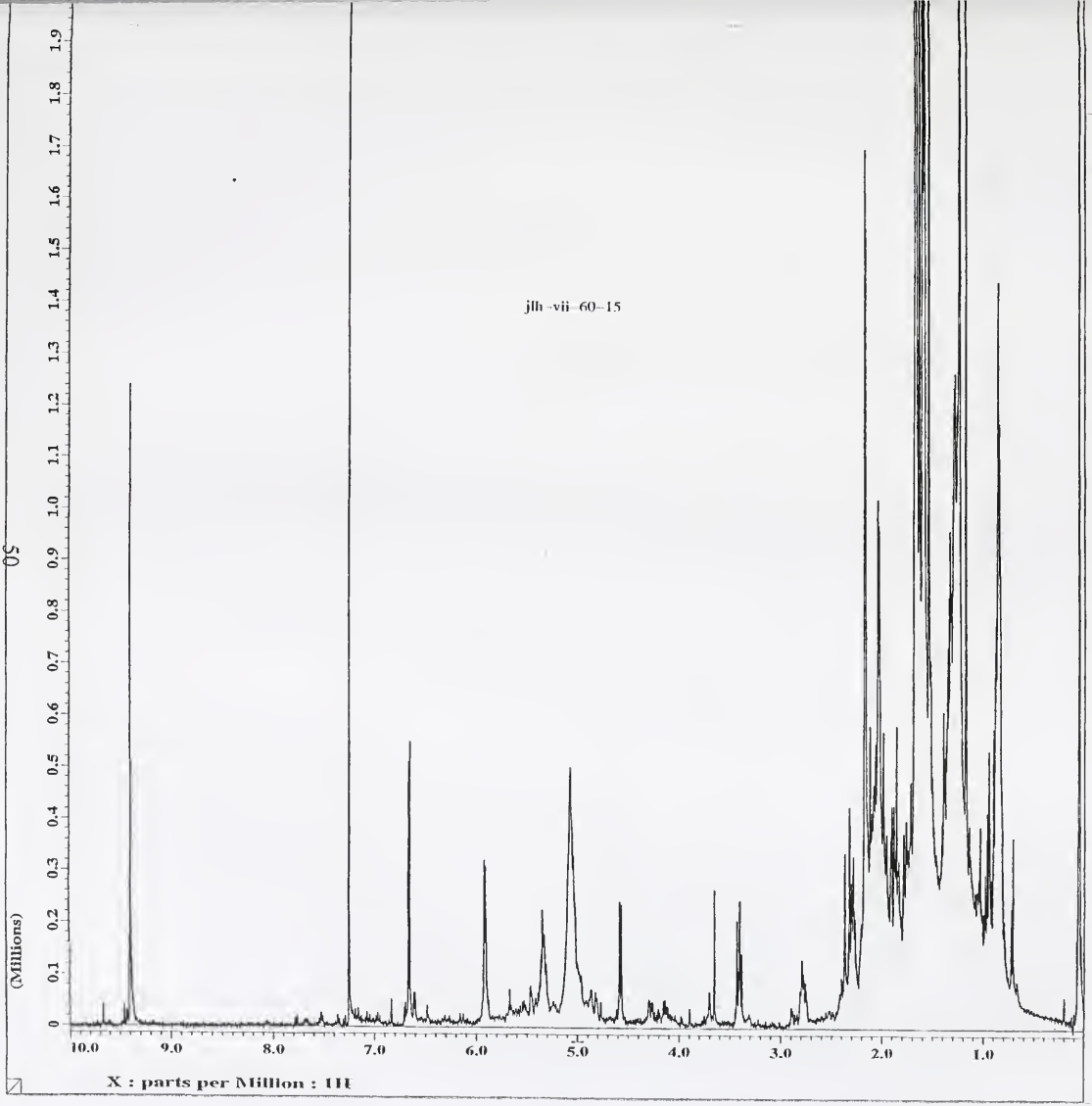

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Derived from: jlh-vii-60-15 1h.1
File Name           = jlh-vii-60-15 1h.3
Author              = SBC Researchers
Sample ID           = jlh-vii-60-15
Content             = LG Hex. Fracs 19-28
Creation Date       = 10-FEB-2004 15:33:23

Revision Date       = 10-FEB-2004 15:38:23
Spec Site           = ECX 400

Spec Type           = DELTA2_NMR
Data Format         = 1D COMPLEX
Dimensions          = X
Dim Title           = 1H
Dim Size            = 13107
Dim Units           = [ppm]
Field_strength      = 9.389766[T] (400[MHz]
X_acq_duration      = 2.18365952[s]
X_domain            = 1H
X_freq              = 399.78219838[MHz]
X_offset            = 5[ppm]
X_points            = 16384
X_prescans          = 1
X_resolution        = 0.45794685[Hz]
X_sweep             = 7.5030012[kHz]
Irr_domain          = 1H
Irr_freq            = 399.78219838[MHz]
Irr_offset          = 5[ppm]
Tri_domain          = 1H
Tri_freq            = 399.78219838[MHz]
Tri_offset          = 5[ppm]
Mod_return          = 1
Scans               = 8
Total_scans         = 8

X_90_width          = 13.7205[us]
X_acq_time          = 2.18365952[s]
X_angle             = 45[deg]
X_atn               = 3.00032[dB]
X_pulse             = 6.86025[us]
Irr_mode            = Off
Tri_mode            = Off
Dante_presat        = FALSE
Initial_wait        = 1[s]
Relaxation_delay    = 5[s]
Repetition_time     = 7.18365952[s]
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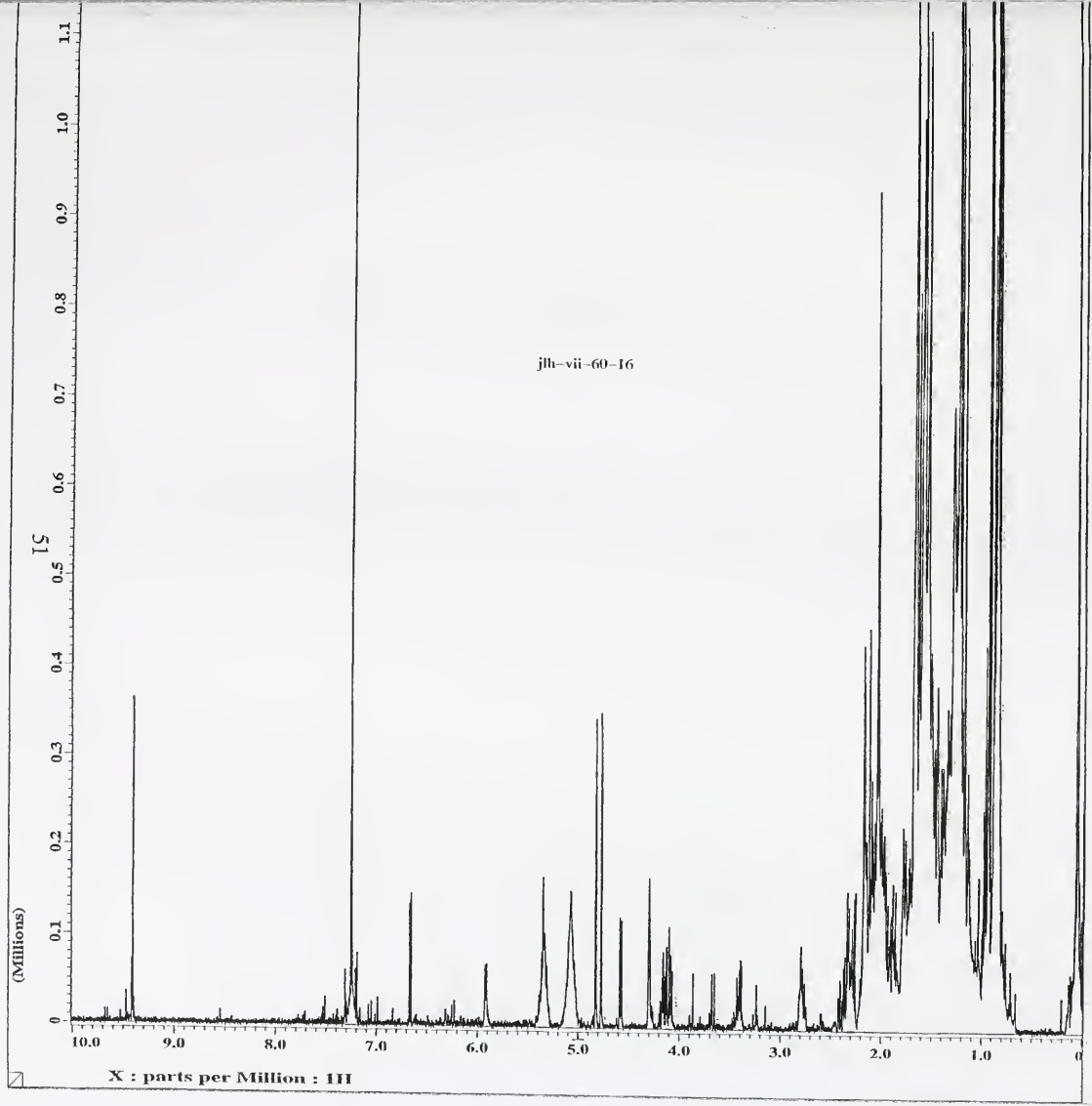


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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-16 1h.1
File Name           = jlh-vii-60-16 1h.3
Author              = SBC Researchers
Sample ID           = jlh-vii-60-16
Content             = fracs. 29-33
Creation Date       = 28-OCT-2003 14:24:58

Revision Date       =  5-FEB-2004 17:18:41
Spec Site           = ECX 400

Spec Type           = DELTA2_NMR
Data Format         = 1D REAL
Dimensions          = X
Dim Title           = 1H
Dim Size            = 13107
Dim Units           = [ppm]
Field_strength      = 9.389766[T] (400[MHz]
X_acq_duration      = 2.18365952[s]
X_domain            = 1H
X_freq              = 399.78219838[MHz]
X_offset            = 5[ppm]
X_points            = 16384
X_prescans          = 1
X_resolution        = 0.45794685[Hz]
X_sweep             = 7.5030012[kHz]
Irr_domain          = 1H
Irr_freq            = 399.78219838[MHz]
Irr_offset          = 5[ppm]
Tri_domain          = 1H
Tri_freq            = 399.78219838[MHz]
Tri_offset          = 5[ppm]
Mod_return          = 1
Scans               = 8
Total_scans         = 8

X_90_width          = 13.7205[us]
X_acq_time          = 2.18365952[s]
X_angle             = 45[deg]
X_atn               = 3.00032[dB]
X_pulse             = 6.86025[us]
Irr_mode            = Off
Tri_mode            = Off
Dante_preset        = FALSE
Initial_wait        = 1[s]
Relaxation_delay    = 5[s]
Repetition_time     = 7.18365952[s]
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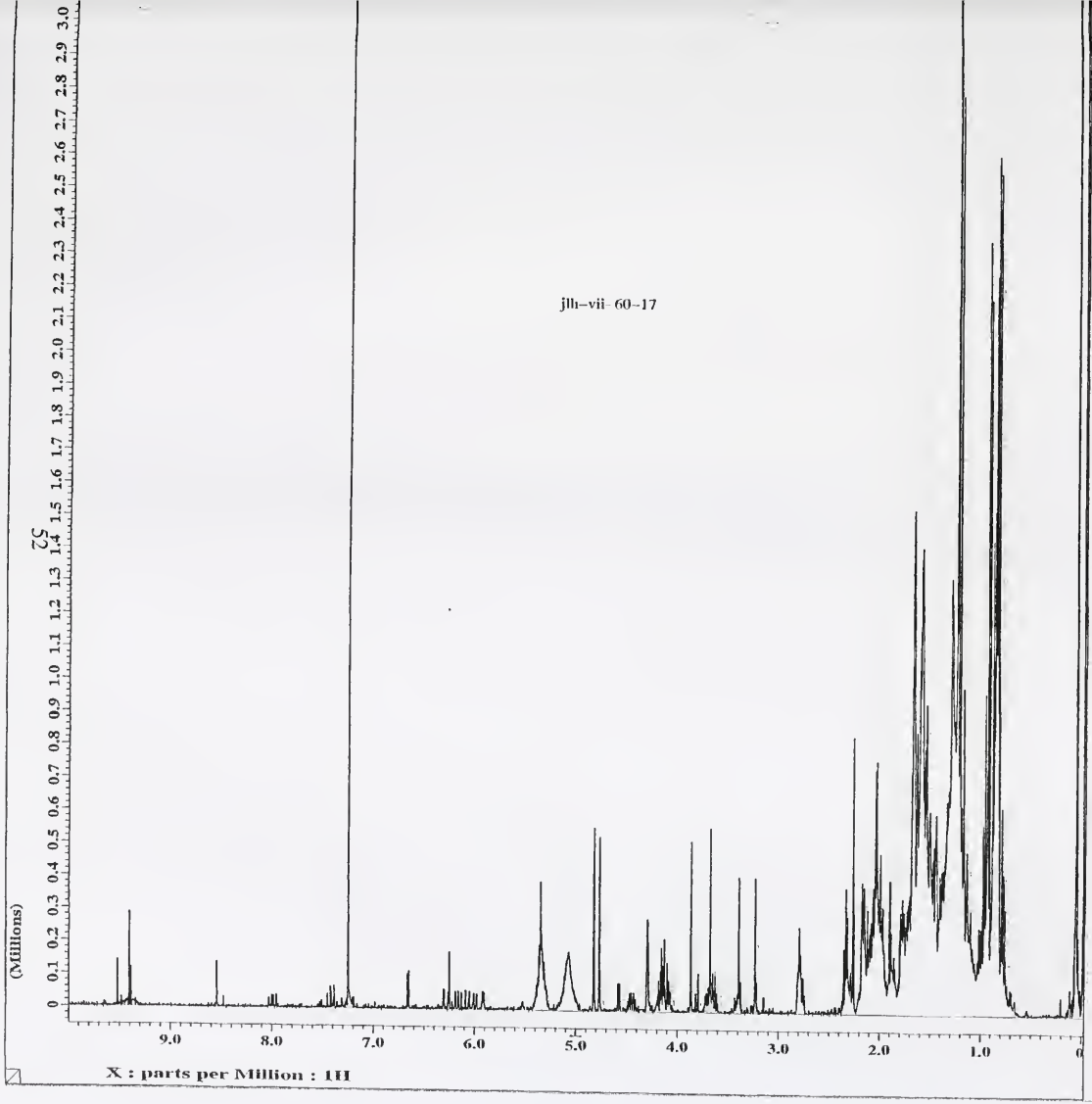

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```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-17 1h.1
File Name          = jlh-vii-60-17 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-60-17
Content            = fracs. 34-44
Creation Date      = 28-OCT-2003 14:38:04

Revision Date      =  5-FEB-2004 17:19:36
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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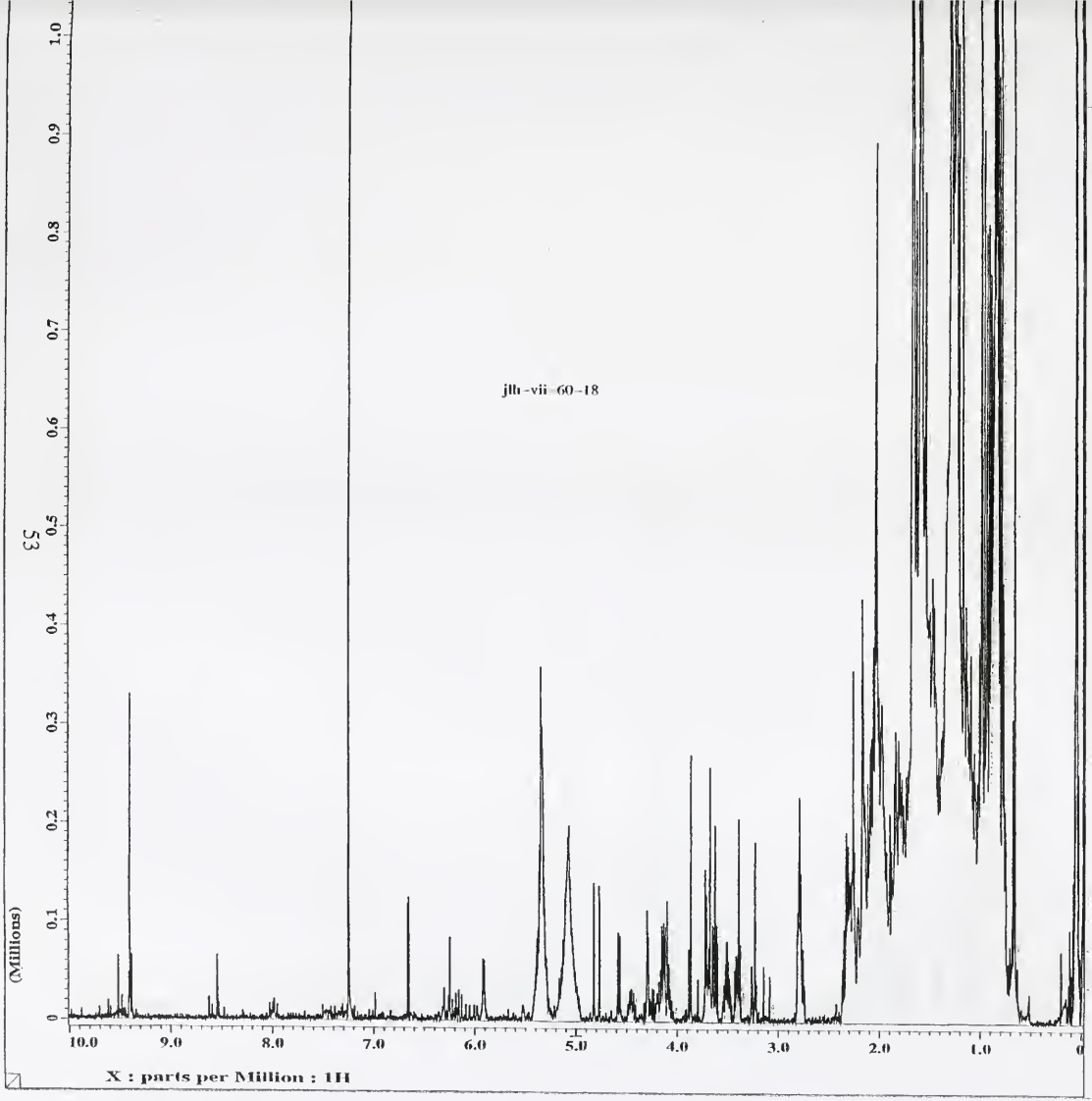

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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-18 1h.1
File Name          = jlh-vii-60-18 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-60-18
Content            = fracs. 45-72
Creation Date      = 28-OCT-2003 14:55:38

Revision Date      =  5-FEB-2004 17:20:36
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_preset       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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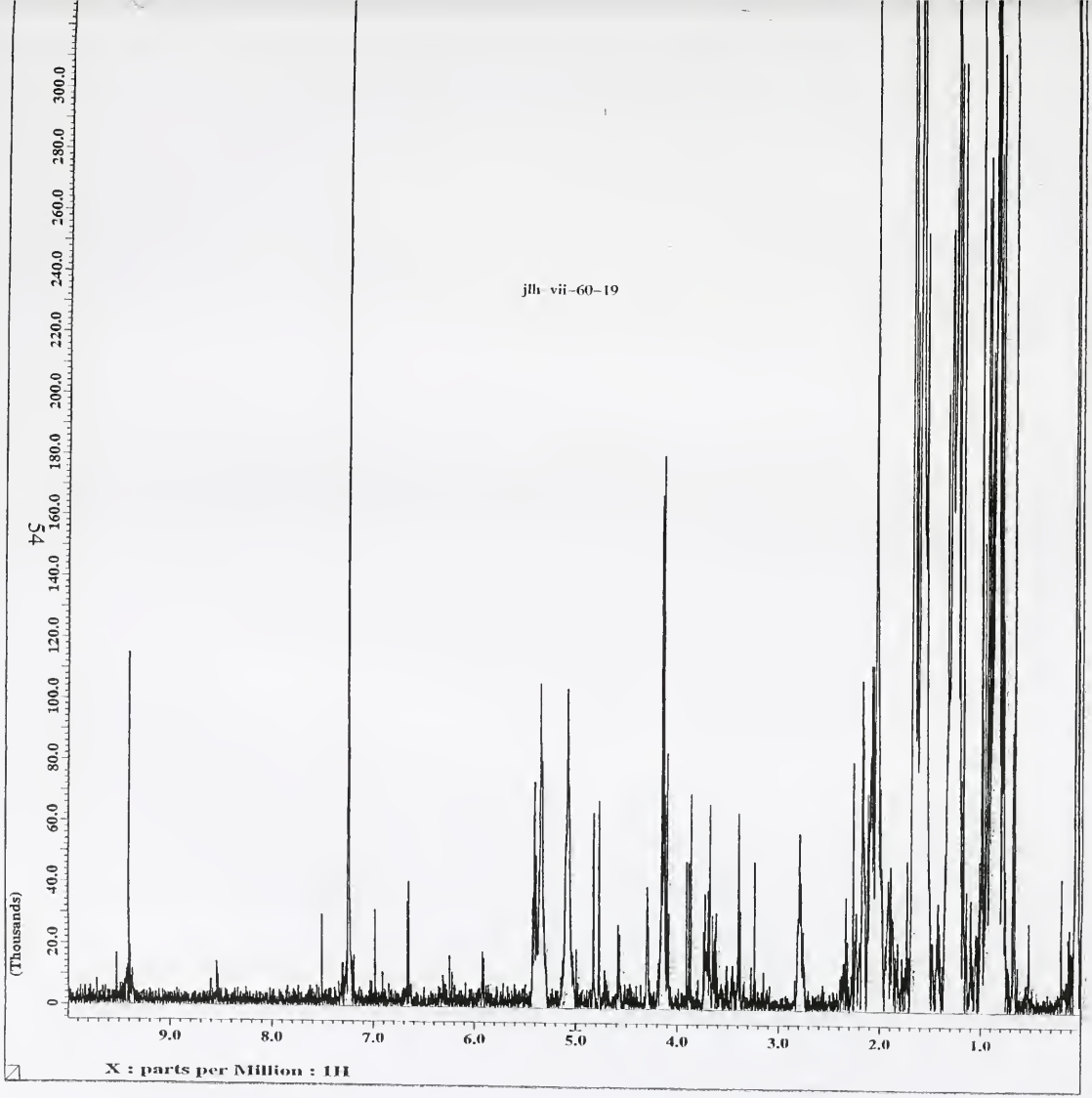


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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-19 1h.1
File Name          = jlh-vii-60-19 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-60-19
Content            = fracs. 73-92
Creation Date      = 28-OCT-2003 15:06:43

Revision Date      =  5-FEB-2004 17:22:36
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X acq_duration     = 2.18365952[s]
X domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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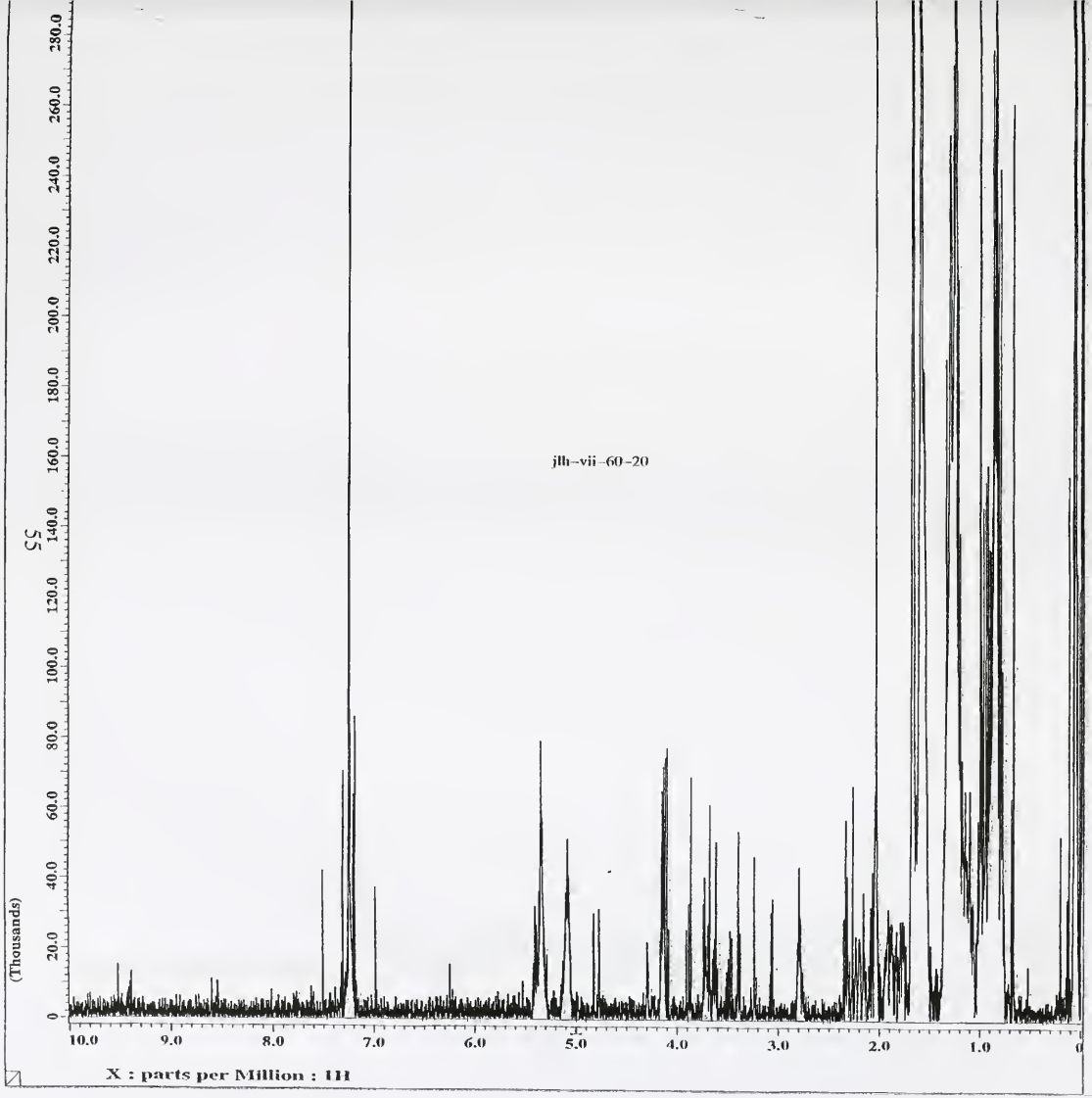


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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-20 1h.1
File Name          = jlh-vii-60-20 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-60-20
Content            = fracs. 93-107
Creation Date      = 28-OCT-2003 15:27:59

Revision Date      =  5-FEB-2004 17:23:34
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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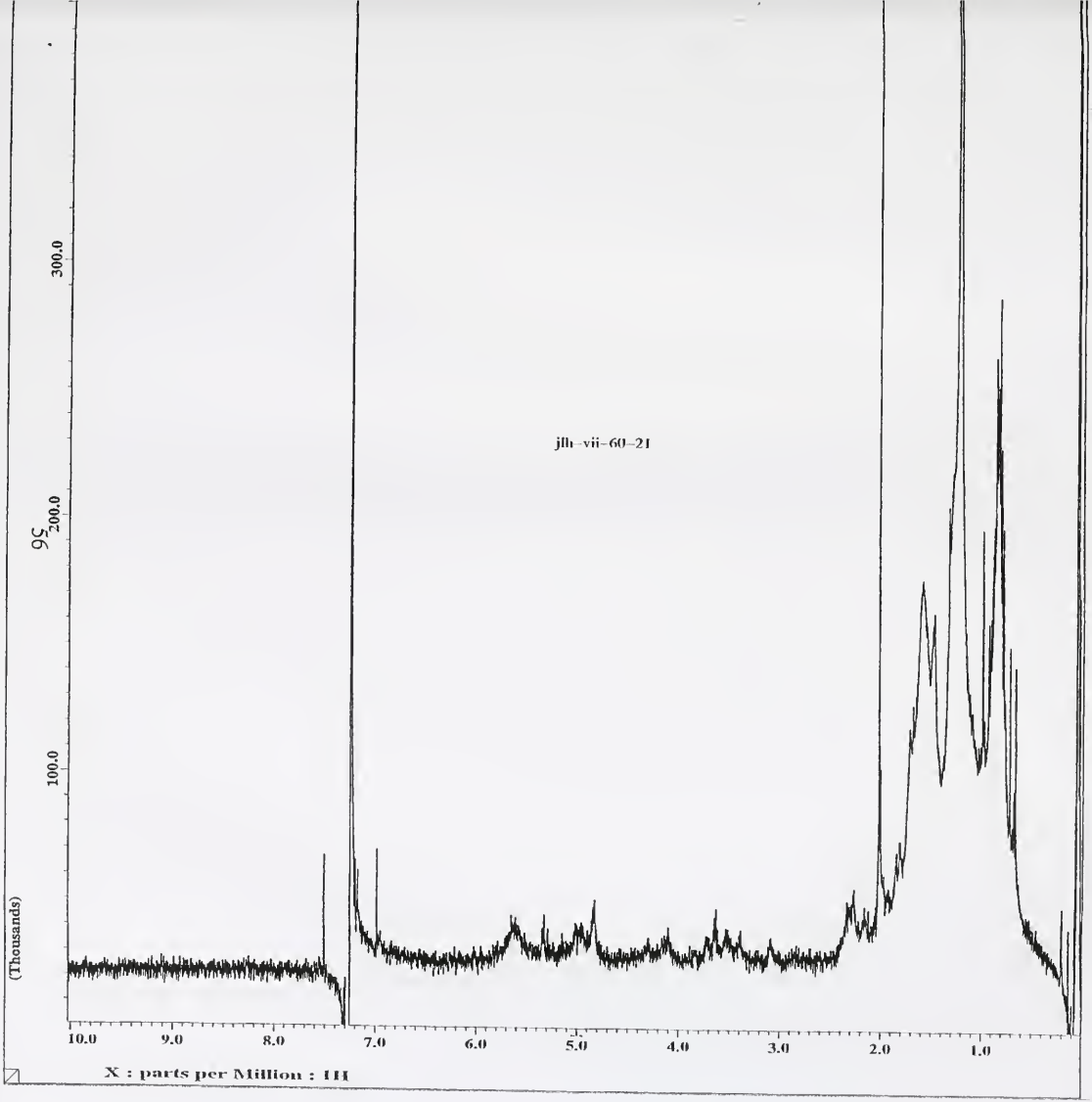

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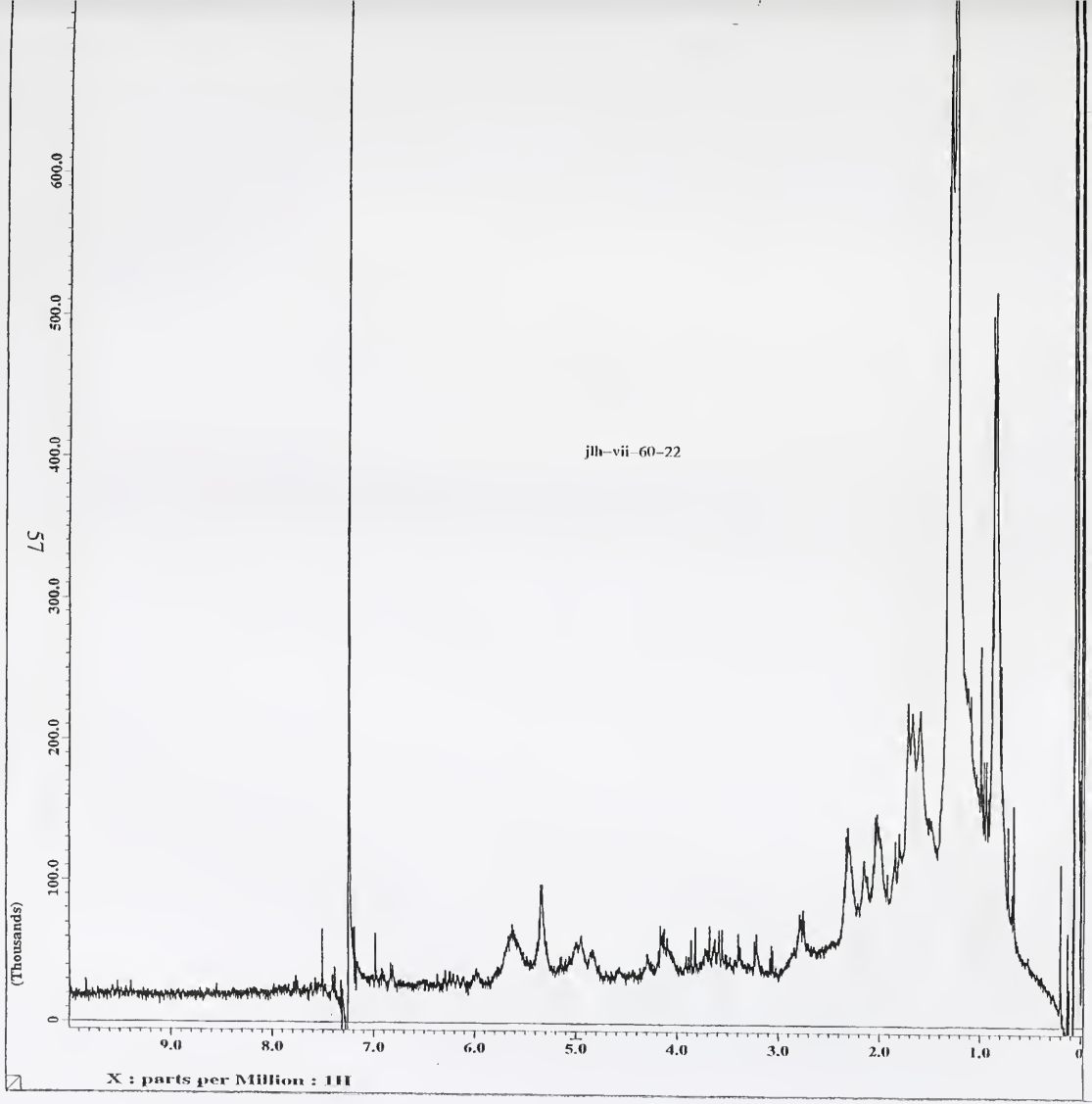
```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-21 1h.1
File Name          = jlh-vii-60-21 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-60-21
Content            = LG Hex. Fracs 108-118
Creation Data      = 10-FEB-2004 15:42:42

Revision Date      = 10-FEB-2004 15:47:55
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D COMPLEX
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```



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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-60-22 1h.1
File Name          = jlh-vii-60-22 1h.3
Author             = SSC Researchers
Sample ID          = jlh-vii-60-22
Content            = LG Hex. Fracs 119-160
Creation Date      = 10-FEB-2004 15:52:08

Revision Date      = 10-FEB-2004 16:01:30
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D COMPLEX
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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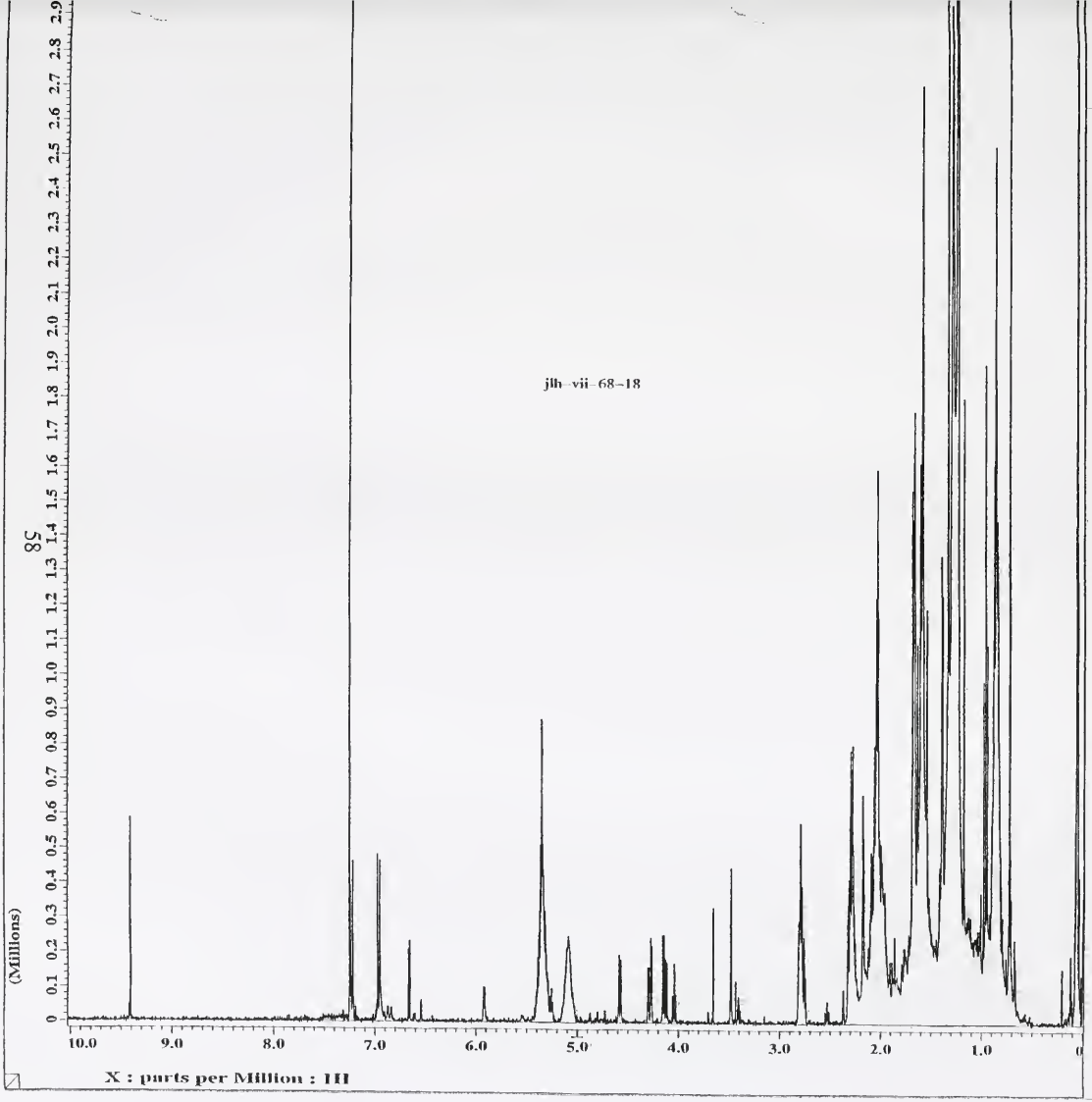


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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-18 1h.3
File Name          = jlh-vii-68-18 1h.5
Author             = SBC Researchers
Sample ID          = jlh-vii-68-18
Content            = LG hex. frace.13-21
Creation Date      = 29-JAN-2004 13:58:39

Revision Date      =  5-FEB-2004 17:14:39
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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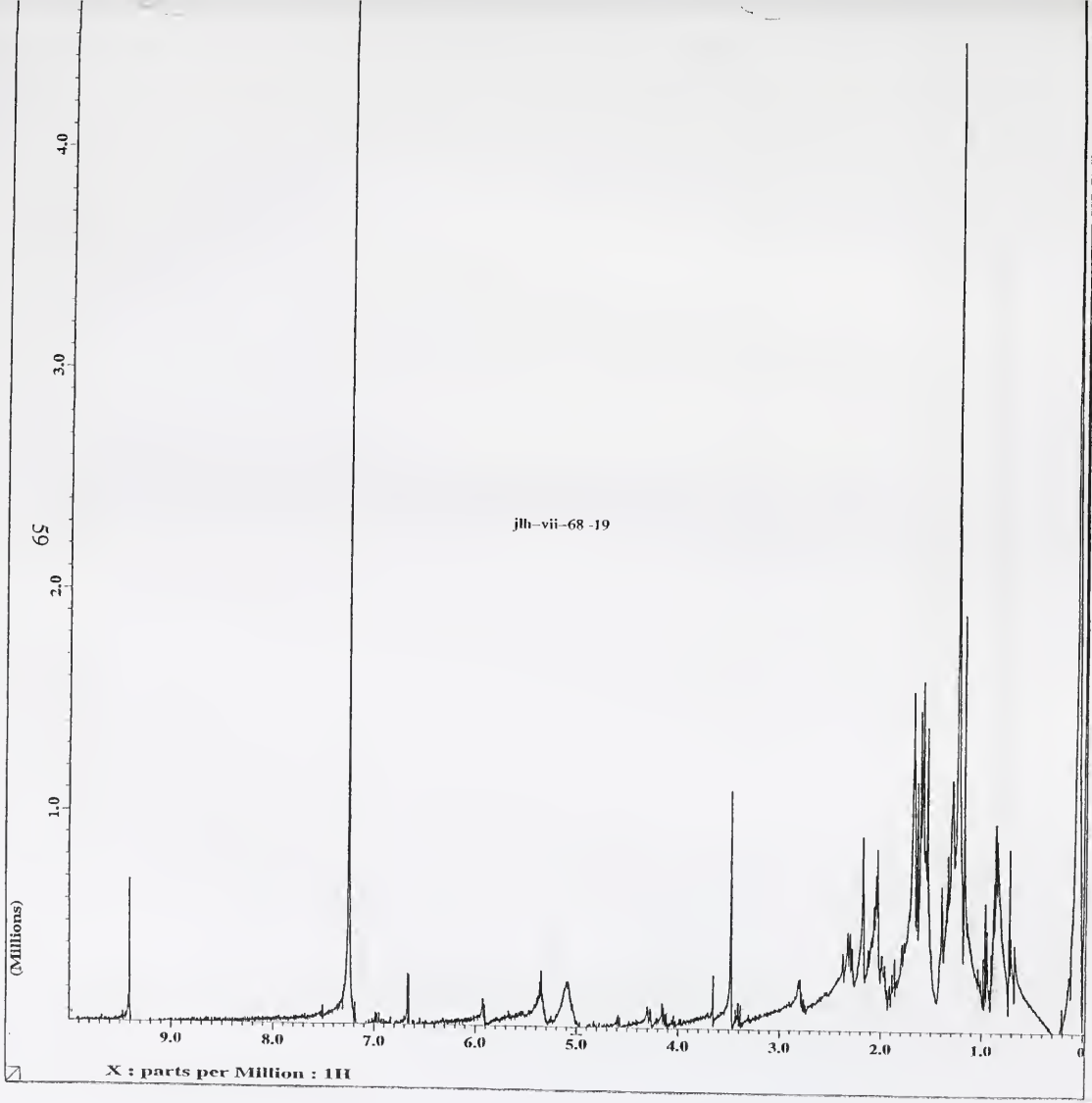

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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-19 1h .1
File Name          = jlh-vii-68-19 1h .3
Author             = SBC Researchers
Sample ID          = jlh-vii-68-19
Content            = LC hex. fracs.22-25
Creation Date      = 29-JAN-2004 14:33:01

Revision Date      =  5-FEB-2004 17:11:42
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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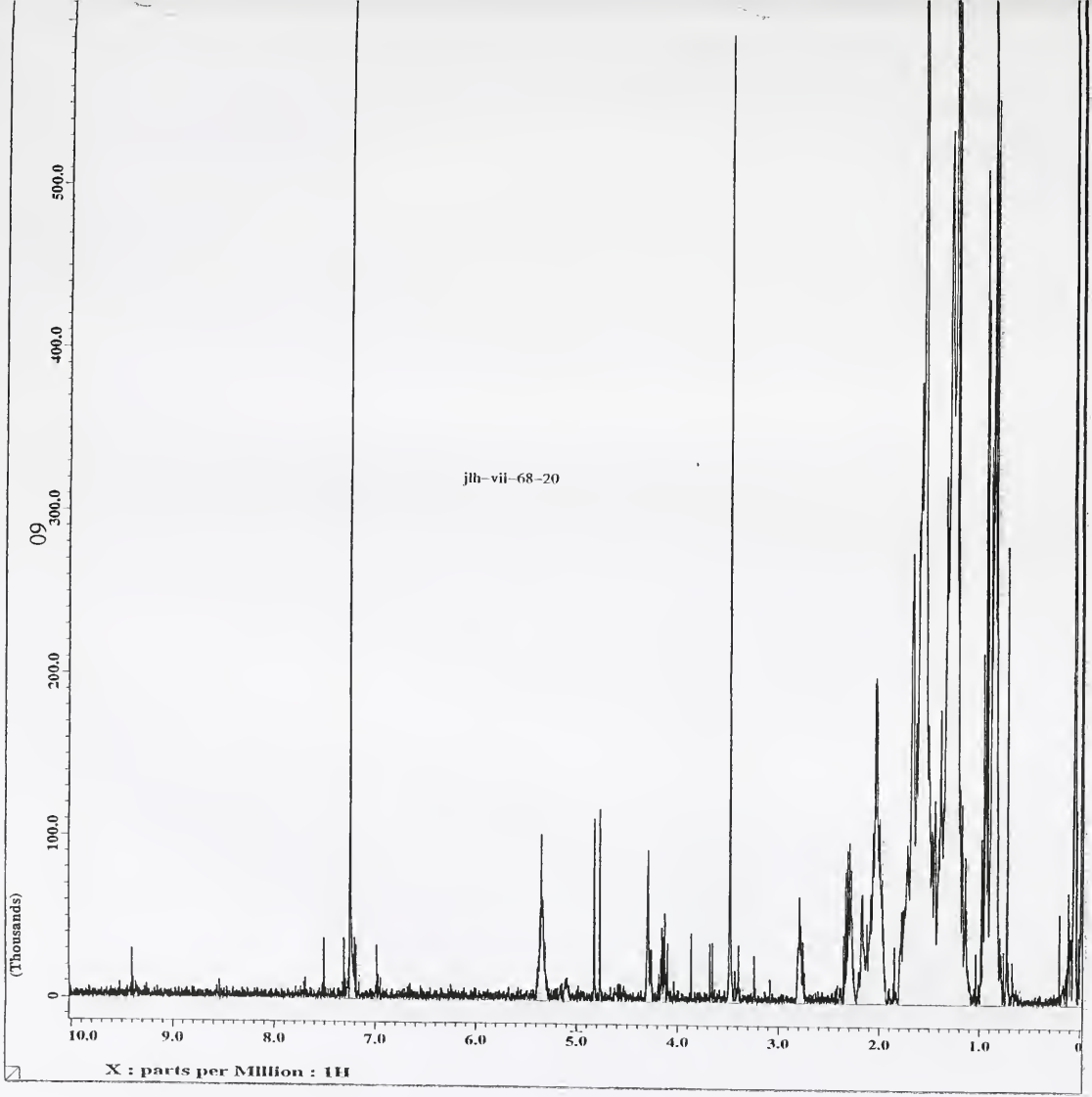

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---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-20 1h.3
File Name          = jlh-vii-68-20 1h.5
Author             = SBC Researchers
Sample ID          = jlh-vii-68-18
Content            = LG hex. fracs.26-28
Creation Date      = 29-JAN-2004 13:40:01

Revision Date      =  5-FEB-2004 17:10:39
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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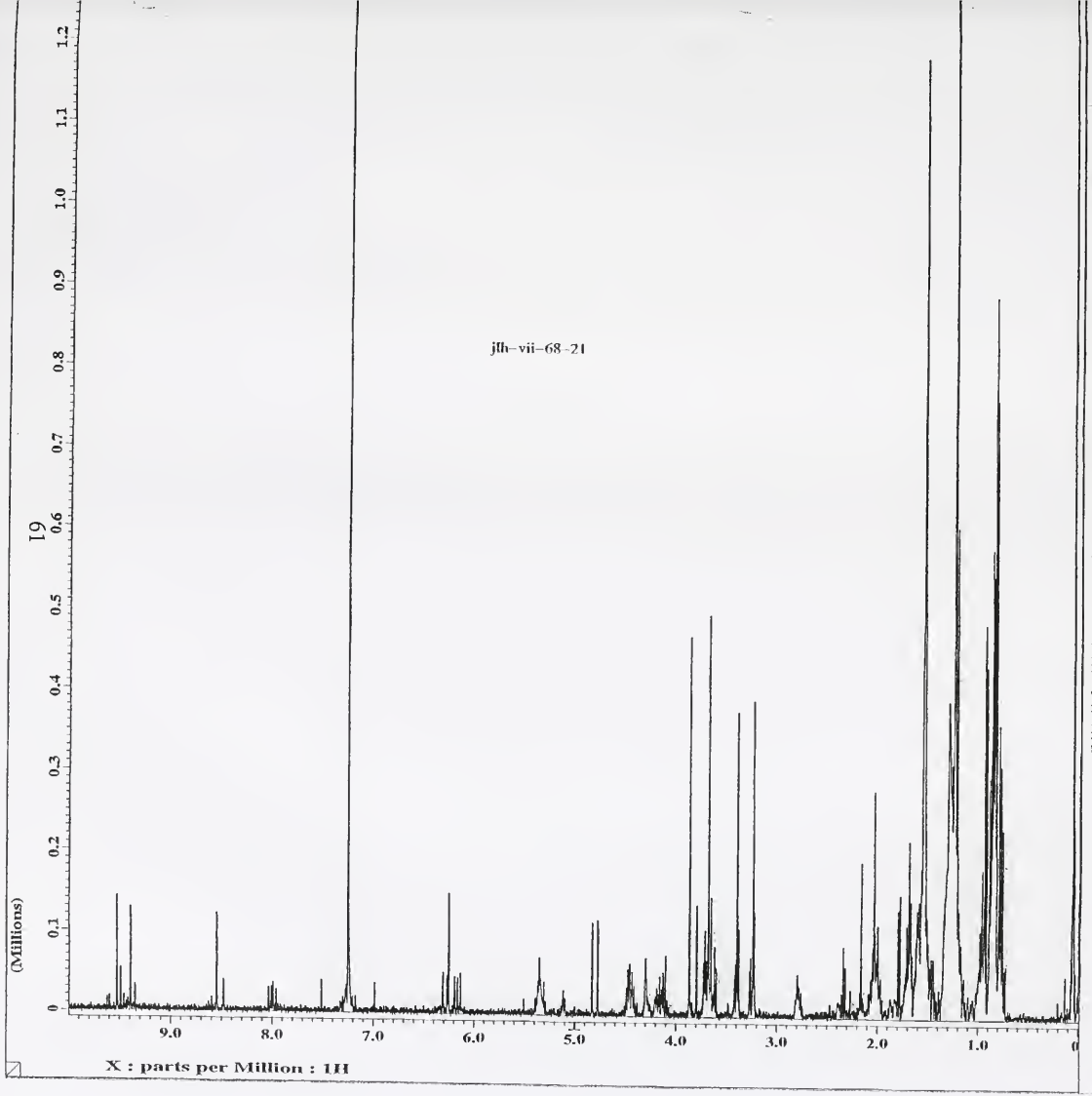

Natural Products Chemistry
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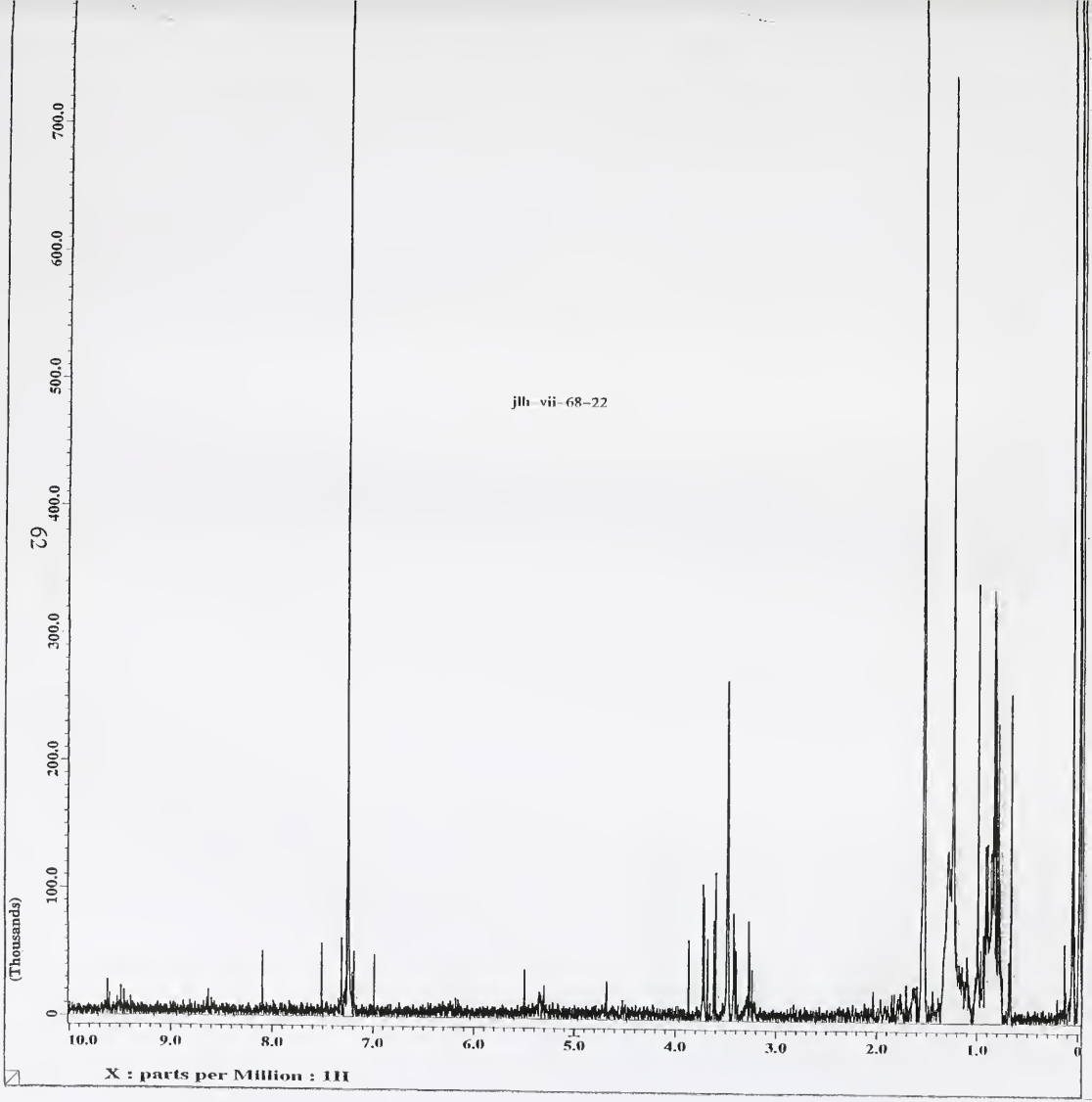
```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-21  1h.3
File Name          = jlh-vii-68-21  1h.5
Author             = SSC Researchers
Sample ID          = jlh-vii-68-21
Content            = LG hex. frace.29-45
Creation Date      = 29-JAN-2004 14:52:17

Revision Date      =  5-FEB-2004 17:09:42
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```



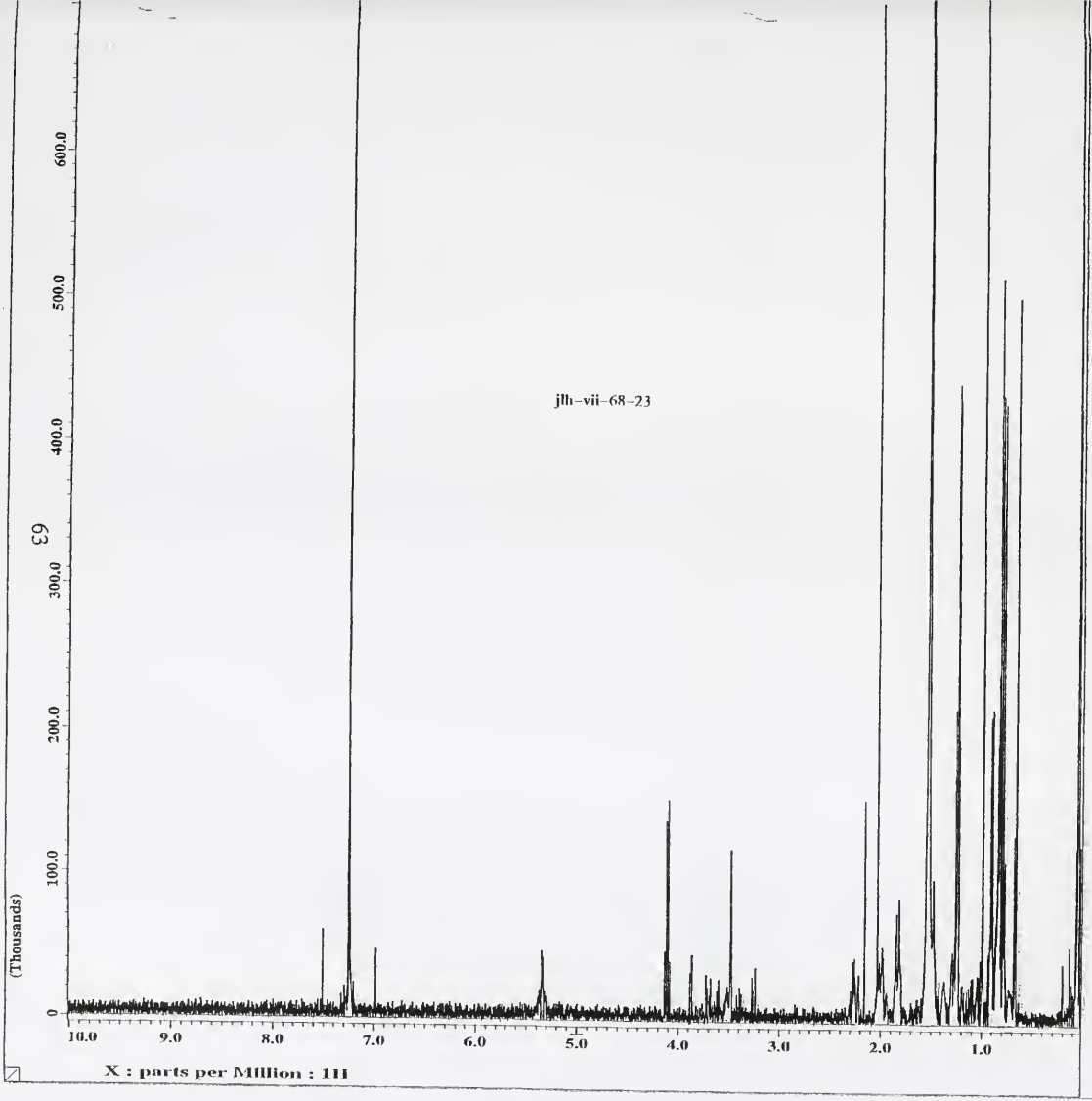
Natural Products Chemistry at Sweet Briar College

```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-22  1h.1
File Name          = jlh-vii-68-22  1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-68-22
Content            = LG hex. fracs.46-55
Creation Date      = 29-JAN-2004 15:02:31

Revision Date      =  5-FEB-2004 17:08:02
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```



Natural Products Chemistry at Sweet Briar College

```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-23 1h.1
File Name          = jlh-vii-68-23 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-68-23
Content            = LG hex. fracs.56-64
Creation Date      = 29-JAN-2004 14:42:54

Revision Date      =  5-FEB-2004 17:05:31
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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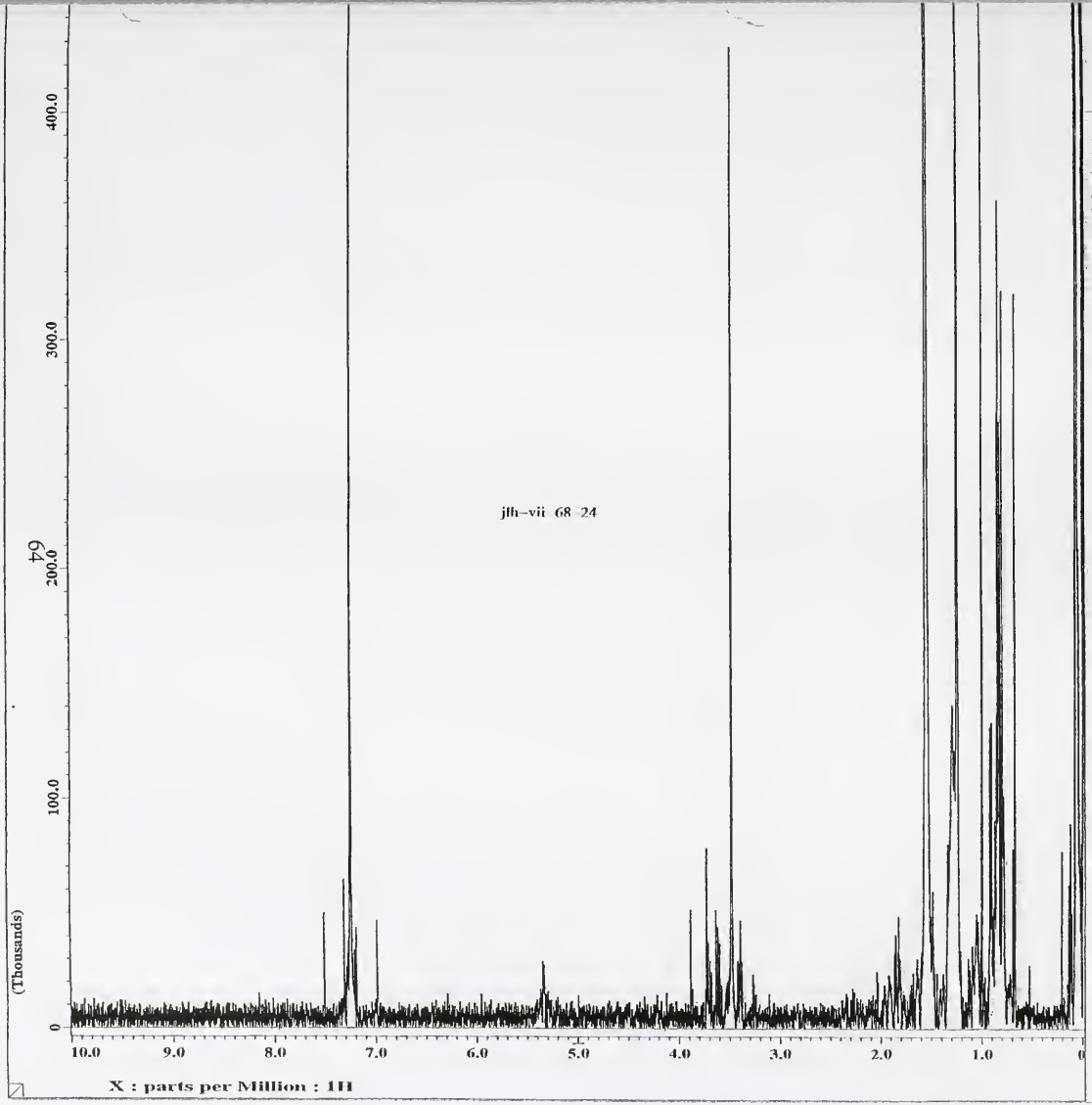

Natural Products Chemistry at Sweet Briar College

```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-24 1h.1
File Name          = jlh-vii-68-24 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-68-24
Content            = LG hex. fraca.65-71
Creation Date      = 29-JAN-2004 14:26:01

Revision Date      =  5-FEB-2004 17:02:28
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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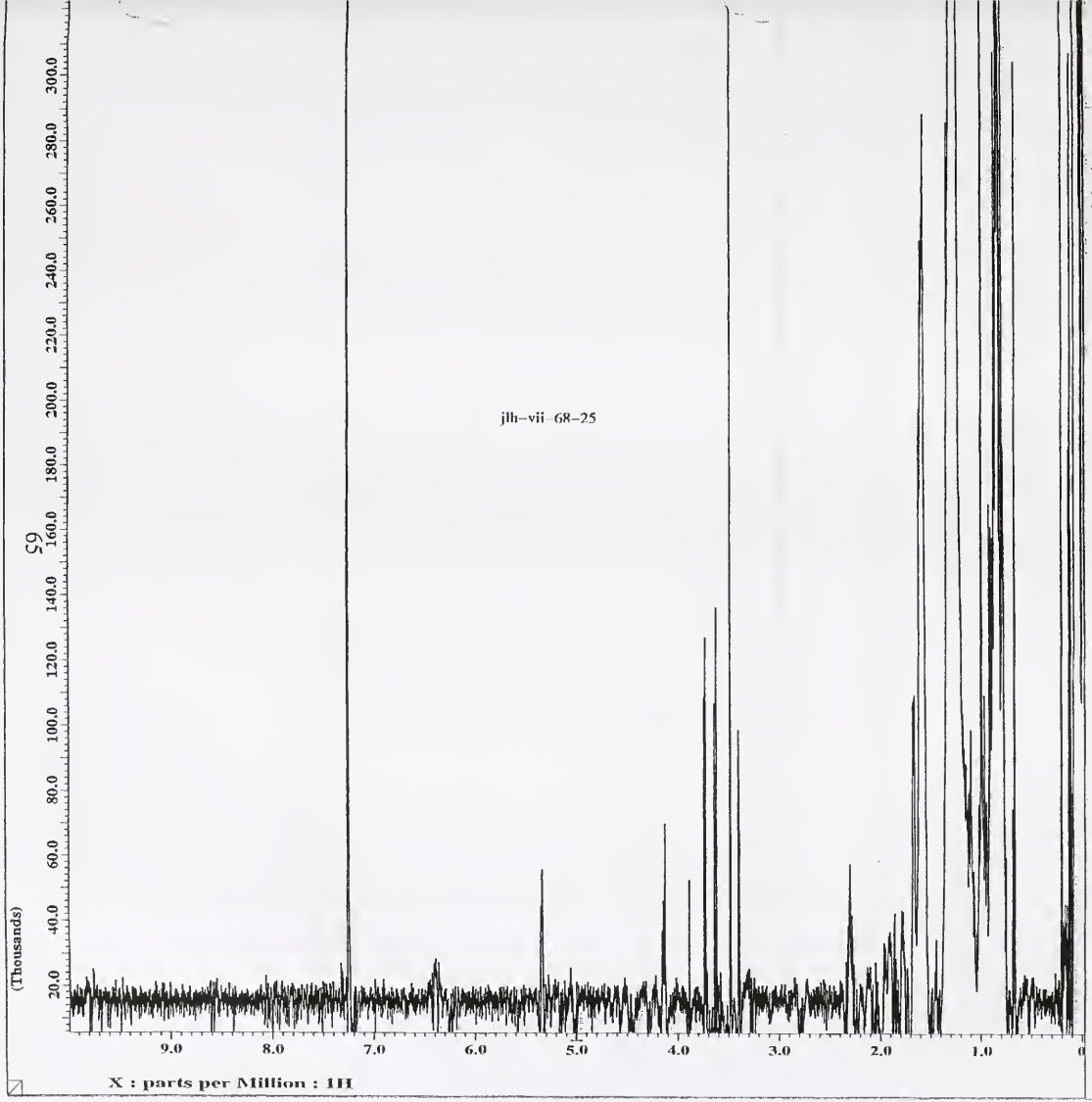


```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-68-25  1h.1
File Name          = jlh-vii-68-25  1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-68-25
Content            = LG hex. fracs.72-97
Creation Data      = 29-JAN-2004 15:11:05

Revision Date      =  5-FEB-2004 16:59:14
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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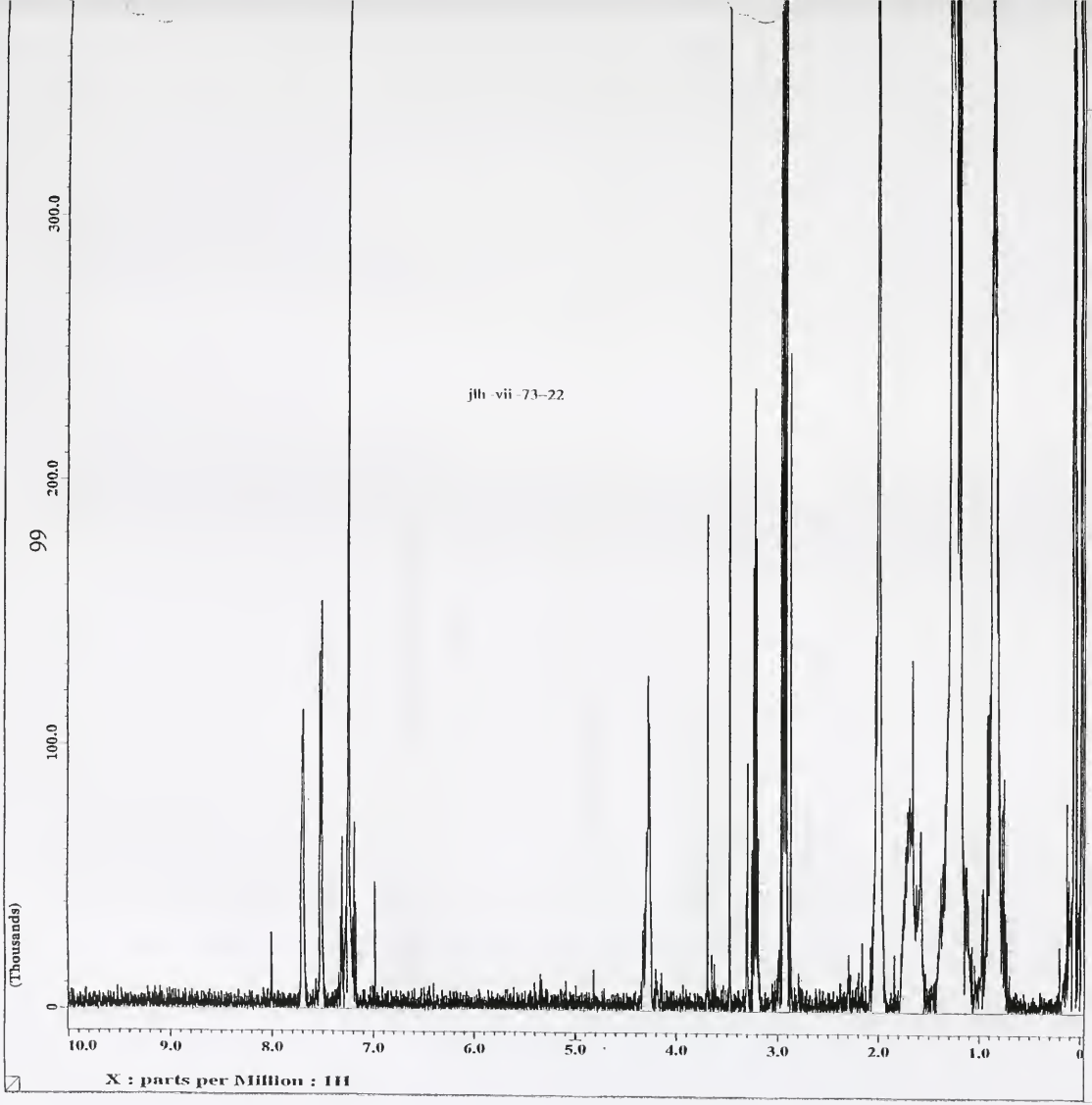

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```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-73-22 1h.1
File Name          = jlh-vii-73-22 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-73-22
Content            = column remnants
Creation Date      = 29-JAN-2004 15:38:48

Revision Date      =  5-FEB-2004 16:57:45
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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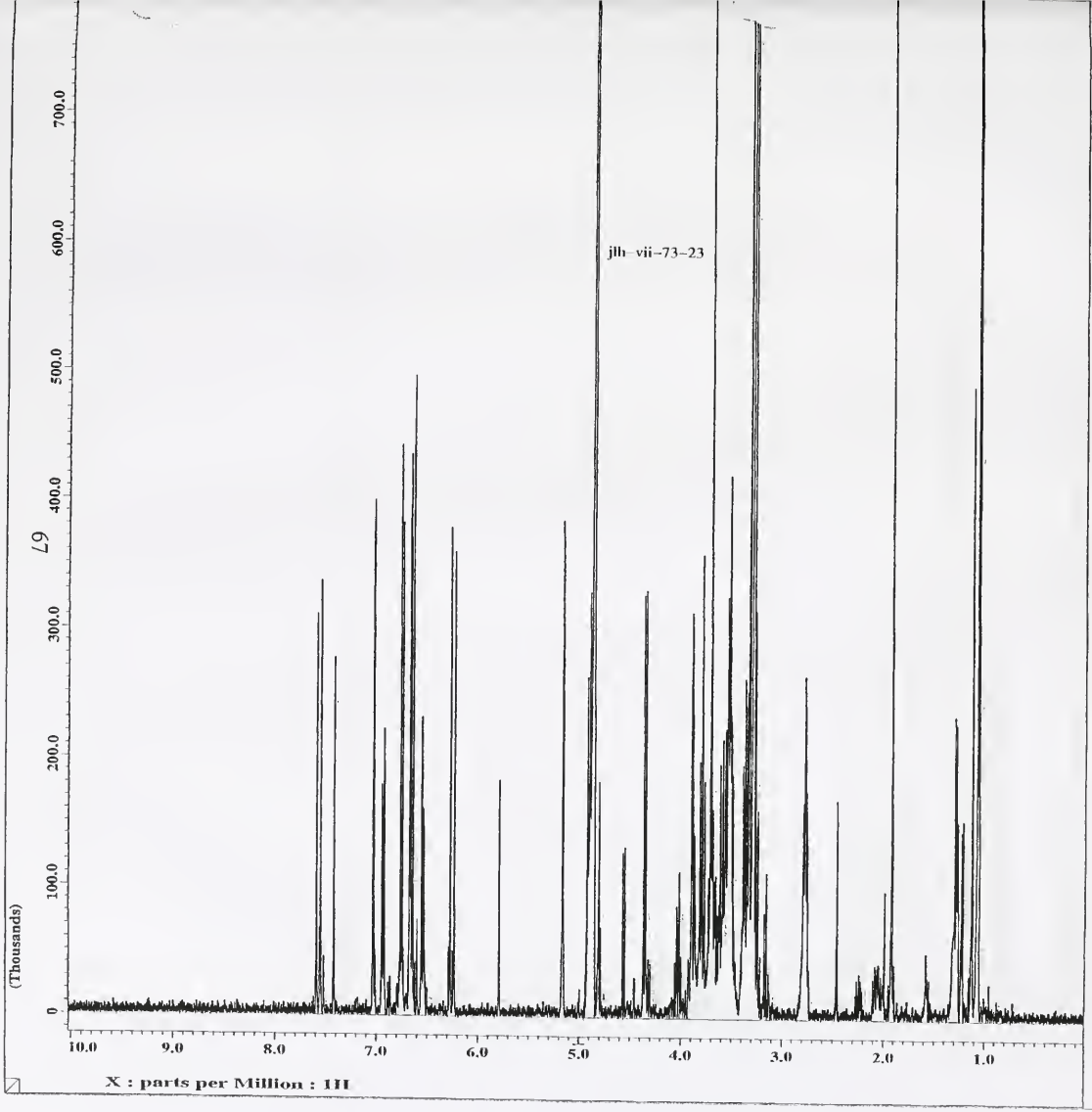
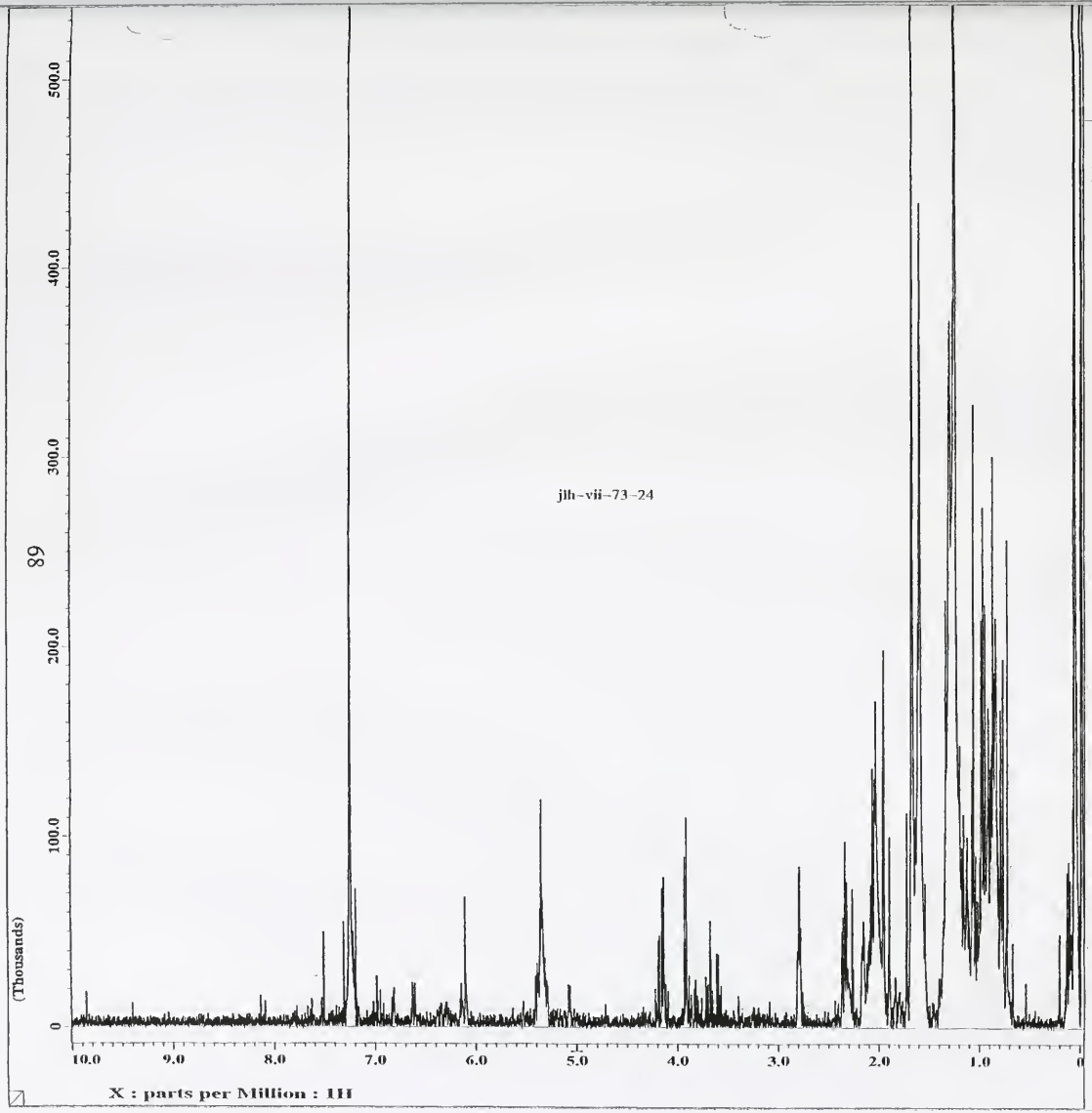



```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-73-23 1h.2
File Name           = jlh-vii-73-23 1h.4
Author              = SBC Reasearchere
Sample ID           = jlh-vii-73-23
Content             = LG EtOAc fracs.10-23
Creation Date       =  5-FEB-2004 14:44:54

Revision Date       =  5-FEB-2004 16:56:43
Spec Site           = ECX 400

Spec Type           = DELTA2_NMR
Date Format         = 1D REAL
Dimensions          = X
Dim Title           = 1H
Dim Size            = 13107
Dim Units           = [ppm]
Field_strength      = 9.389766[T] (400[MHz]
X_acq_duration      = 2.18365952[s]
X_domain            = 1H
X_freq              = 399.78219838[MHz]
X_offset            = 5[ppm]
X_points            = 16384
X_prescans          = 1
X_resolution        = 0.45794685[Hz]
X_sweep             = 7.5030012[kHz]
Irr_domain          = 1H
Irr_freq            = 399.78219838[MHz]
Irr_offset          = 5[ppm]
Tri_domain          = 1H
Tri_freq            = 399.78219838[MHz]
Tri_offset          = 5[ppm]
Mod_return          = 1
Scans               = 8
Total_scans         = 8

X_90_width          = 13.7205[us]
X_acq_time          = 2.18365952[s]
X_angle             = 45[deg]
X_atn               = 3.00032[dB]
X_pulse             = 6.86025[us]
Irr_mode            = Off
Tri_mode            = Off
Dante_presat        = FALSE
Initial_wait        = 1[s]
Relaxation_delay    = 5[s]
Repetition_time     = 7.18365952[s]
```



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```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-73-24 1h.1
File Name          = jlh-vii-73-24 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-73-24
Content            = LG EtOAc frac.24-31
Creation Date      = 29-JAN-2004 16:00:08

Revision Date      =  5-FEB-2004 16:55:22
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dnute_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
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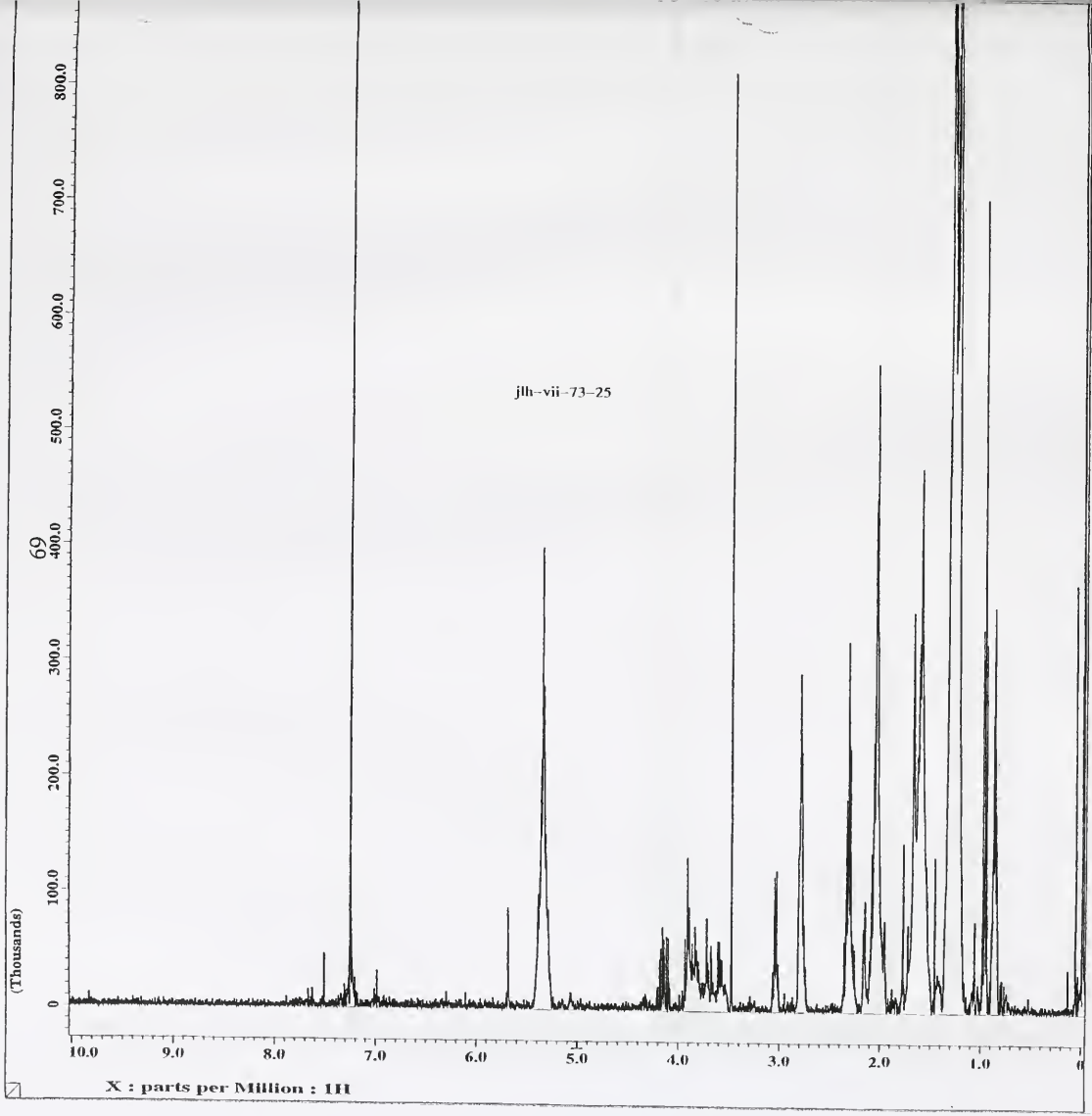

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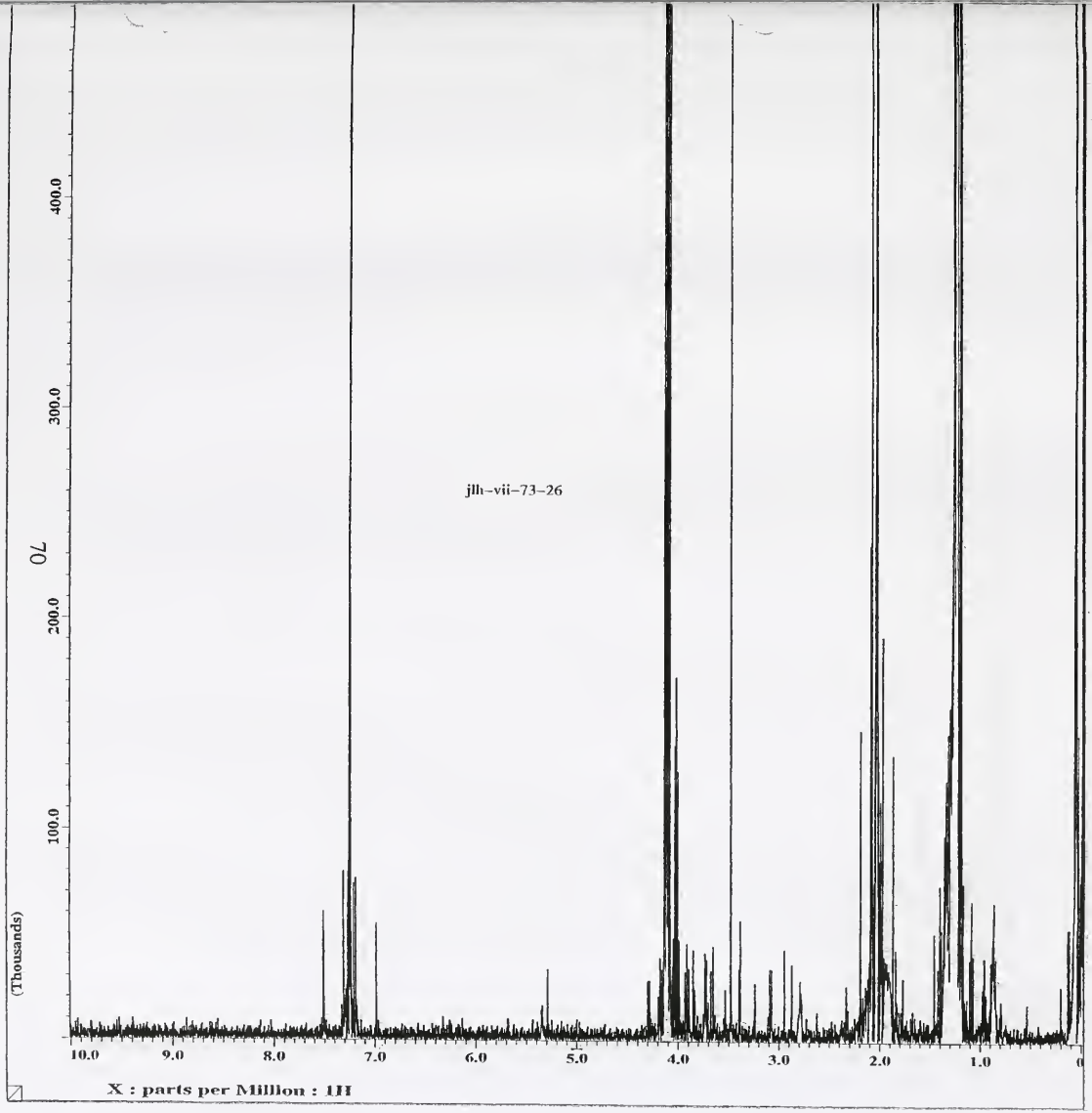
```
----- ACQUISITION PARAMETERS -----
Derived from: jlh-vii-73-25 1h.1
File Name          = jlh-vii-73-25 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-73-25
Content            = LG EtOAc frac.32-39
Creation Date      = 29-JAN-2004 17:09:25

Revision Date      =  5-FEB-2004 16:54:23
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```



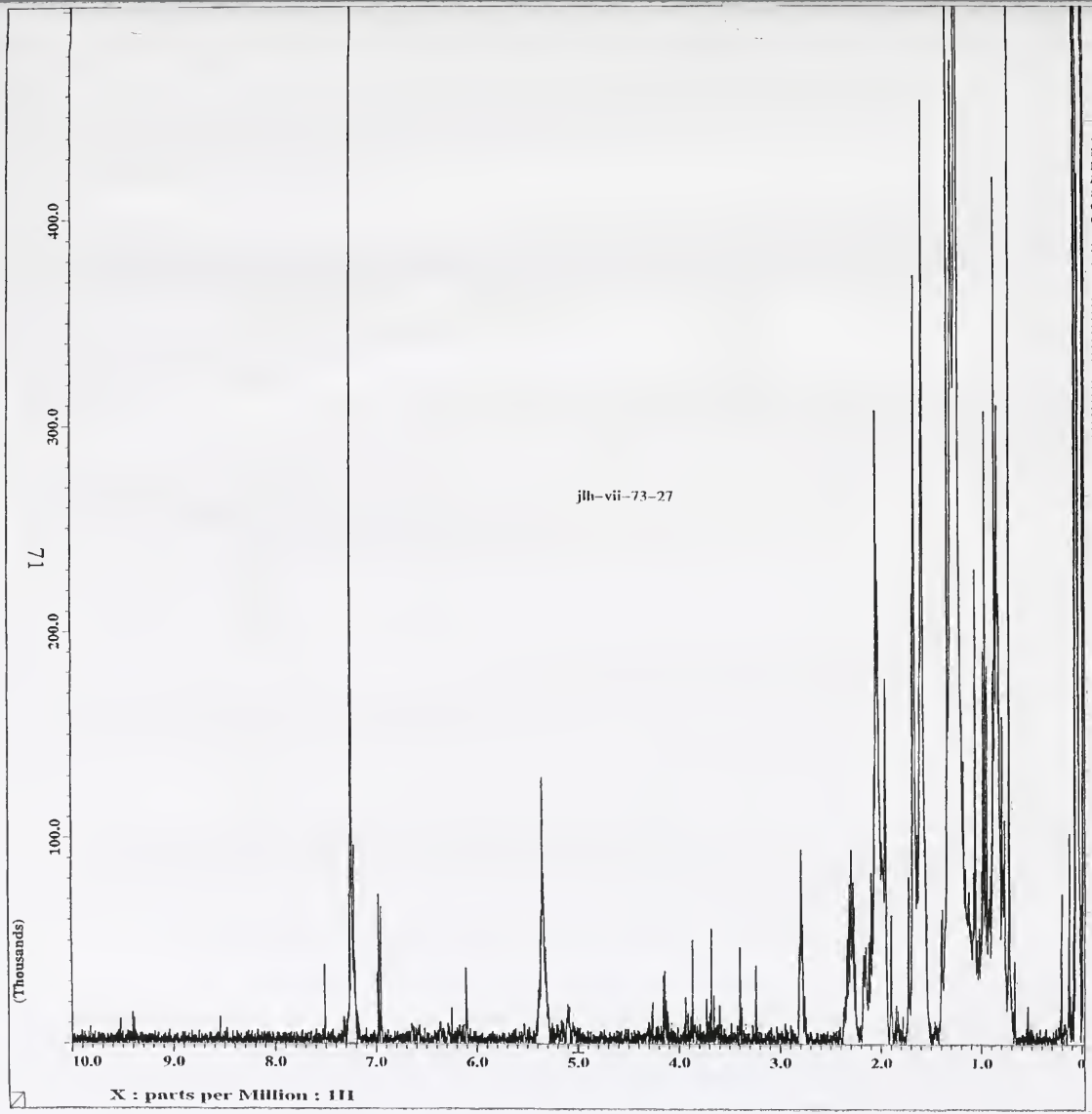
Natural Products Chemistry at Sweet Briar College

```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-73-26 1h.1
File Name          = jlh-vii-73-26 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-73-26
Content            = LG EtOAc fracs.40-56
Creation Date      =  5-FEB-2004 14:28:39

Revision Date      =  5-FEB-2004 16:52:35
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dante_preset       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```



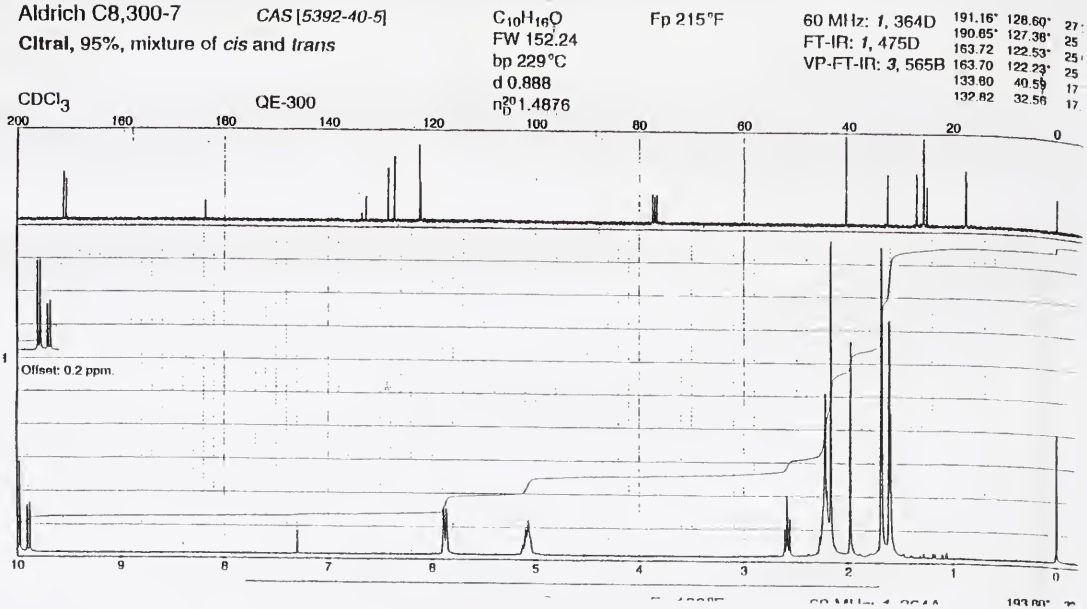
Natural Products Chemistry at Sweet Briar College

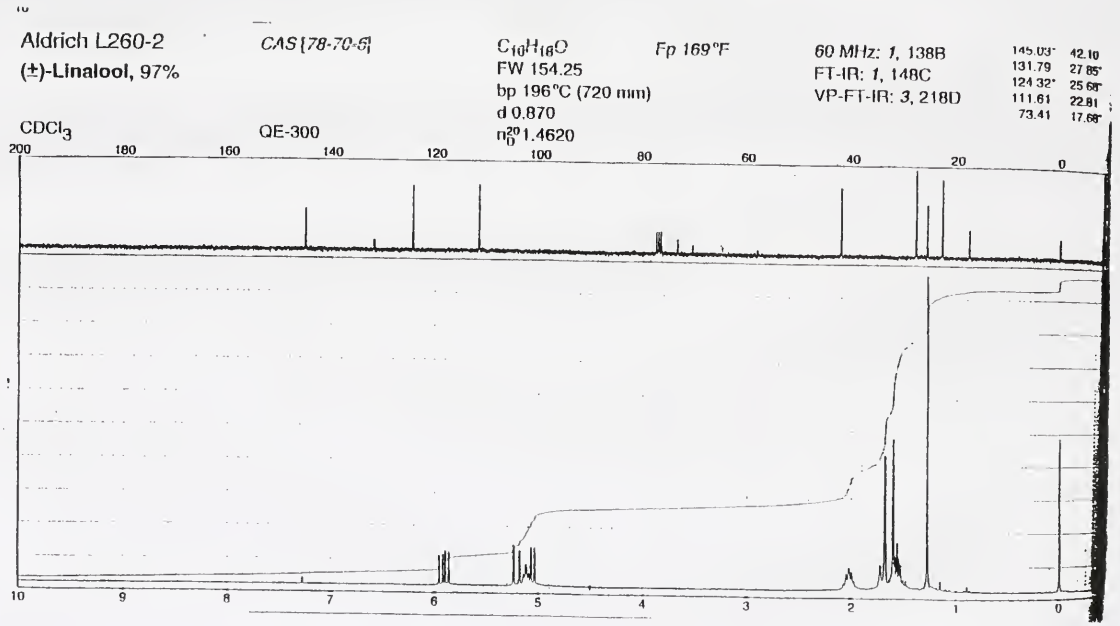
```
---- ACQUISITION PARAMETERS ----
Derived from: jlh-vii-73-27 1h.1
File Name          = jlh-vii-73-27 1h.3
Author             = SBC Researchers
Sample ID          = jlh-vii-73-27
Content            = LG EtOAc frac.57-113
Creation Date      = 29-JAN-2004 16:11:57

Revision Date      =  5-FEB-2004 16:48:22
Spec Site          = ECX 400

Spec Type          = DELTA2_NMR
Data Format        = 1D REAL
Dimensions         = X
Dim Title          = 1H
Dim Size           = 13107
Dim Units          = [ppm]
Field_strength     = 9.389766[T] (400[MHz]
X_acq_duration     = 2.18365952[s]
X_domain           = 1H
X_freq             = 399.78219838[MHz]
X_offset           = 5[ppm]
X_points           = 16384
X_prescans         = 1
X_resolution       = 0.45794685[Hz]
X_sweep            = 7.5030012[kHz]
Irr_domain         = 1H
Irr_freq           = 399.78219838[MHz]
Irr_offset         = 5[ppm]
Tri_domain         = 1H
Tri_freq           = 399.78219838[MHz]
Tri_offset         = 5[ppm]
Mod_return         = 1
Scans              = 8
Total_scans        = 8

X_90_width         = 13.7205[us]
X_acq_time         = 2.18365952[s]
X_angle            = 45[deg]
X_atn              = 3.00032[dB]
X_pulse            = 6.86025[us]
Irr_mode           = Off
Tri_mode           = Off
Dauto_presat       = FALSE
Initial_wait       = 1[s]
Relaxation_delay   = 5[s]
Repetition_time    = 7.18365952[s]
```


Aldrich C8,300-7
CAS [5392-40-5]
Citral, 95%, mixture of cis and trans
C10H16O
FW 152.24
bp 229°C
d 0.888
n20D 1.4876
Fp 215°F
60 MHz: 1, 364D
FT-IR: 1, 475D
VP-FT-IR: 3, 565B
191.16 128.60
190.65 127.38
163.72 122.53
163.70 122.23
133.60 40.59
132.82 32.56
CDCl3
QE-300
Offset: 0.2 ppm.


Aldrich L260-2
(±)-Linalool, 97%
CAS [78-70-6]
$C_{10}H_{18}O$
FW 154.25
bp 196°C (720 mm)
d 0.870
n_D^{20} 1.4620
Fp 169°F
60 MHz: 1, 138B
FT-IR: 1, 148C
VP-FT-IR: 3, 218D
145.03 42.10
131.79 27.85
124.32 25.68
111.61 22.81
73.41 17.68
CDCl3
QE-300
200 180 160 140 120 100 80 60 40 20 0
10 9 8 7 6 5 4 3 2 1 0

Aldrich 21,834-0 *CAS [1139-30-6]*

Caryophyllene oxide, 97%

$C_{15}H_{24}O$
FW 220.36
mp 62°C

60 MHz: *1*, 199B
FT-IR: *1*, 237C

151.75	48.71*	29.88*
112.67	39.78	29.86
03.64*	39.18	27.22
59.88	33.98	21.63*
50.83*	30.18	16.99*

$CDCl_3$ QE-300