MOLECULAR FORMULAR DETERMINATION OF AN UNKNOWN BIOACTIVE ORGANIC COMPOUND ISOLATED FROM MYANMAR INDIGENOUS MEDICINAL PLANT *Ellipeia ferruginea* Hook. f. & Thoms. (Ingyin-sint)

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ABSTRACT

An indigenous medicinal plant; *Ellipeia ferruginea*, Hook. f & Thoms Ingyin-sint in Myanmar, was selected to investigate phytochemical screening and antimicrobial activitiy . Ethyl acetate and ethanol extracts of Ingyin-sint give high activities on all selected organisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus pumalis*, *Pseudomonas aeruginosa*, *Candida albican* and *Mycobacterium* species. A pure unknown organic compound (95 mg, 2.7%) was isolated from *Ellipeia ferruginea*, (except leaves), by applying advanced chromatographic techniques such as Thin Layer and Column Chromatography. The melting point of this pure compound is 164-165°C. The antimicrobial activities of this pure unknown compound were rechecked by using agar-well diffusion method on above six organisms. It gives medium activities on *Staphylococcus aureus*, *Bacillus pumalis* and *Candida albican*. In addition, the molecular formula of this pure bioactive organic compound could be determined as $C_{23}H_{23}O_4N$ applying some modern spectroscopic methods such as FT-IR, ^{1}H NMR, ^{13}C NMR, HMQC, DEPT and EI mass spectra.

INTRODUCTION

In many countries of the world and especially in the developing countries, traditional medicine is an important part of health care system. In countries of South East Asia Region, although modern medicines are now increasingly available throughout different levels of health care, traditional medicine has maintained its popularity since it has been used for generations in the past.[1] Internationally, herbal medicine is already assumed its important role in providing health care services for the global community.[2]

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Medicinal plants are a valuable resource of new drugs as well as for economic development.[3] They are used as raw materials for the extraction of active constituents in pure form. Moreover plants are not only the major source of energy-rich foods in most societies but are also an indispensable source of vitamins and other substances promoting healthy growth. Many kinds of aromatic plants are traditionally used medicinally, usually prepared as teas. The World Health Organization has compiled a list of more than 21000 plant species used globally in medicine. It is estimated that 2000-3000 species are used for medicinal purposes in South-East Asia.[4]

Not only plant constituents are used directly as therapecutic agents, but also as starting materials for the synthesis of drugs together with international cooperation and coordination for their conservation so as to ensure their availability for the future.[5]

It is well known that the Union of Myanmar is a country with a rich source of traditional indigenous medicinal plants. Traditional use of medicinal plants has flourished over many centuries. In recent years, research and scientific development of the practice of traditional medicine have been handed from generation to generation. In this respect *Ellipeia ferruginea* Hook. f. & Thoms, known as Ingyin-sint, in Myanmar, was selected for chemical analysis. This plant is one of the species belonged to the Annonaceae family. It is distributed in open fields of lowland forest in Myanmar[6]. This plant is used for treatment of fever[7].

Aim of the Present Research Work

- To strengthen and promote the rational use of herbal medicines.
- To provide research criteria for evaluating the safety and efficacy of herbal medicines
- To study the chemical constituents and antimicrobial activity of Myanmar indigenous medicinal plant
- To isolate the pure bioactive organic compound from Myanmar indigenous medicinal plant

Botanical Description[6]

Figure : *Ellipeia ferruginea* Hook. f. & Thoms. (Ingyin-sint)

Family	-	Annonaceae
Botanical name	-	*Ellipeia ferruginea* Hook. f. & Thoms.
Genus	-	Ellipeia
Species	-	ferruginea
Local name	-	Ingyin-sint

Traditional Use of Ingyin-sint[7]

The whole plant of Ingyin-sint (except leaves) is used for medical purpose. It is used for treatment of fever.

EXPERIMENTAL

Sample Collection

The indigenous medicinal plant, *Ellipeia ferruginea* which is called Ingyin-sint in Myanmar was collected from near Ka Phyu village in Kyauk-padaung township, Mandalay Division. The whole plant (except leaves) was chopped into tiny pieces and allowed to dry well in air. It was stored in a well-stoppered bottle and used throughout the experiment..

The plant material was screened and identified by authorized botanist from Botany Department, University of Mandalay.

Preliminary Phytochemical Tests for the Selected Plant[8,9]

Phytochemical constituents of the selected medicinal plant species were flavonoid, steroid, polyphenol and phenolic.

Determination of the Antimicrobial Activity of the Crude Extract of The Selected Plant

The antimicrobial activitiy of the crude extract of the selected plant was tested in various solvent systems by agar-well diffusion method in DCPT (Development Center for Pharmaceutical Technology), Insein, Yangon. The applying organisms are *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumalis*, *Candida albican* and *Mycobacterium* species. The results are recorded in table.

Table: Antimicrobial Activity of Selected Plant

		Organisms					
Samples	Solvents	I	II	III	IV	V	VI
Ingyin-sint	n-hexane	–	++	–	–	–	++
	Chloroform	++	+	++	++	+	++
	Acetone	+++	+++	+++	+++	+++	+++
	EtOAc	+++	++	+++	+++	+++	+++
	EtOH	+++	+++	+++	+++	+++	+++

Organisms Agar-Well (10mm)

I = *Bacillus subtilis* IV = *Bacillus pumalis* 10 mm ~ 14 mm (+)

II = *Staphylococcus aureus* V = *Candida albican* 15 mm ~ 19 mm (++)

III = *Pseudomonas aeruginosa* VI = *Mycobacterium* species 20 mm above (+++)

no activity (–)

Extraction and Isolation of a pure Unknown Organic Compound from Ingyin-sint

(450 g) of the air dried sample (Ingyin-sint) was percolated with (1000 ml) of 98% ethanol for three months. This percolated solution was filtered and concentrated at room temperature. The residue was re-extracted with (500 ml) of ethyl acetate. When the ethyl acetate extracted solution was concentrated, (3.55 g) of crude extract was obtained.

Then the extract (3.55 g) was fractionated by column chromatography over SiO_2 (50 g) eluting with n-hexane and ethyl acetate with various ratios from nonpolar to polar to produce (135) fractions. Each fraction was checked by TLC with suitable solvent system and then the fractions of same R_f values were combined; (12) combined fractions were produced. The combined fraction 'H' has found to be main portion. It shows one spot on TLC and it is UV active. Then this portion was purified by recrystallization with 30% ethyl acetate in n-hexane. After recrystallization, pale yellow crystal (95 mg) was obtained. The yield percent of this pure compound is (2.7%) on the basic of ethyl acetate crude extract.

Determination of Melting Point of the Pure Unknown Organic Compound [8]

Melting point of the pure compound was determined by capillary method. It was found to be 164-165°C without decomposition.

Phytochemical Screening of the Pure Unknown Organic Compound [8,9]

The pure compound gives positive for phenolic with 10% $FeCl_3$ solution. Blue colour indicates the presence of phenolic compound.

Determination of Antimicrobial Activities of the Pure Unknown Organic Compound

The antimicrobial activity of the pure compound was rechecked by using agar-well diffusion method on six organisms. The results are shown in table and in figure.

Table: Antimicrobial Activities of the Pure Unknown Organic Compound

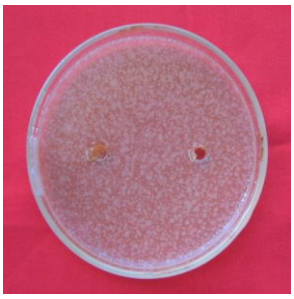
		Organisms					
Sample	Solvent	I	II	III	IV	V	VI
Ingyin-sint	EtOAc	–	(++)	–	(++)	(++)	–
		–	–	–	–	–	–

Agar-Well – 5 mm Organisms

5 mm ~ 9 mm (+) I = *Bacillus substilis* IV = Bacillus pumalis

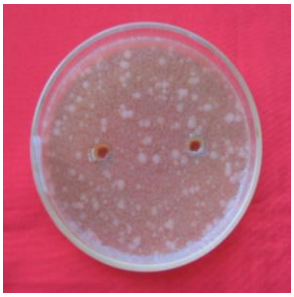
10 mm ~ 14 mm (++) II = *Staphylococcus aureus* V = *Candida albican*

15 mm above (+++) III = *Pseudomonas aeruginosa* VI=*Mycobacterium species*



Bacillus subtilis

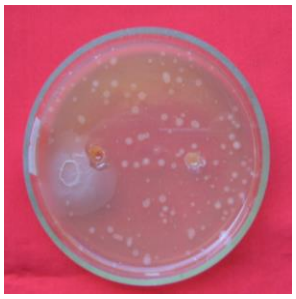
Staphylococcus aureus



Pseudomonas aeruginosa

Bacillus pumalis

Candida albican



***Mycobacterium* species**

Figure: Antimicrobial Activities of the Pure Unknown Organic Compound

Spectroscopic Studies of the Pure Unknown Bioactive Organic Compound

The pure compound was subjected to analyze by Infrared Spectrophotometer (Hyper-IR, SHIMADZU). ^{13}C Nuclear Magnetic Resonance ,(^{13}C NMR , 125 MHz), Distortionless Enhancement by Polarization Transfer (DEPT), Proton Nuclear Magnetic Resonance (^{1}H NMR, 500 MHz), Heteronuclear Multiple Quantum Coherence (HMQC), and EI Mass spectral data were measured.

RESULTS AND DISCUSSIONS

Molecular Formula Determination of the Unknown Bioactive Organic Compound

The molecular formula of the pure bioactive organic compound could be determined by some modern spectroscopic methods such as FT-IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, HMQC and EI Mass spectral data respectively.

FT-IR Spectroscopic Determination of the Unknown Bioactive Organic Compound [10,11,12]

The FT-IR spectrum which was measured at the Department of Chemistry, University of Mandalay is shown in figure. The functional groups observed in FT-IR spectrum are –OH stretching vibration, N–H stretching vibration, =CH stretching vibration of sp^2 hydrocarbon, unsymmetrical and symmetrical stretching vibration of sp^3 hydrocarbons, C=O stretching vibration of carbonyl group, C⎓C ring skeletal stretching vibration of aromatic ring, –CH in plane bending vibration of allylic hydrocarbon,,C–N stretching vibration, O–H out of plane bending vibration of alcohol,

C– C –C stretching vibration, C–C–O stretching vibration of alcohol group, C–O–C stretching vibration of ether group and C–H out of plane bending vibration of trans or E and cis or Z alkene.

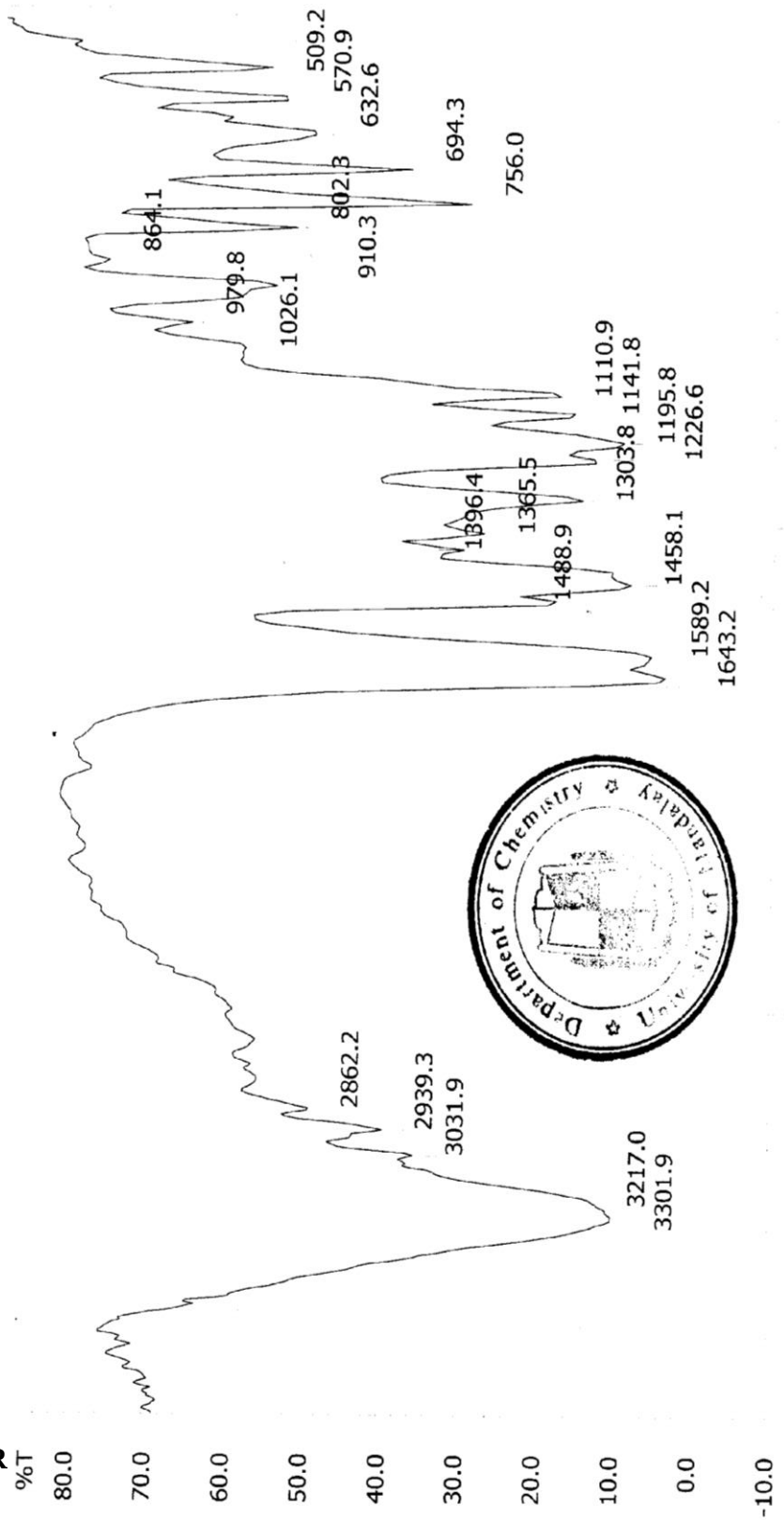


Figure: FT-IR Spectrum of the Unknown Bioactive Organic Compound

^{1}H NMR

The ^{1}H NMR spectrum (500 MHz) is shown in figure. In this spectrum, totally 19 protons can be observed.

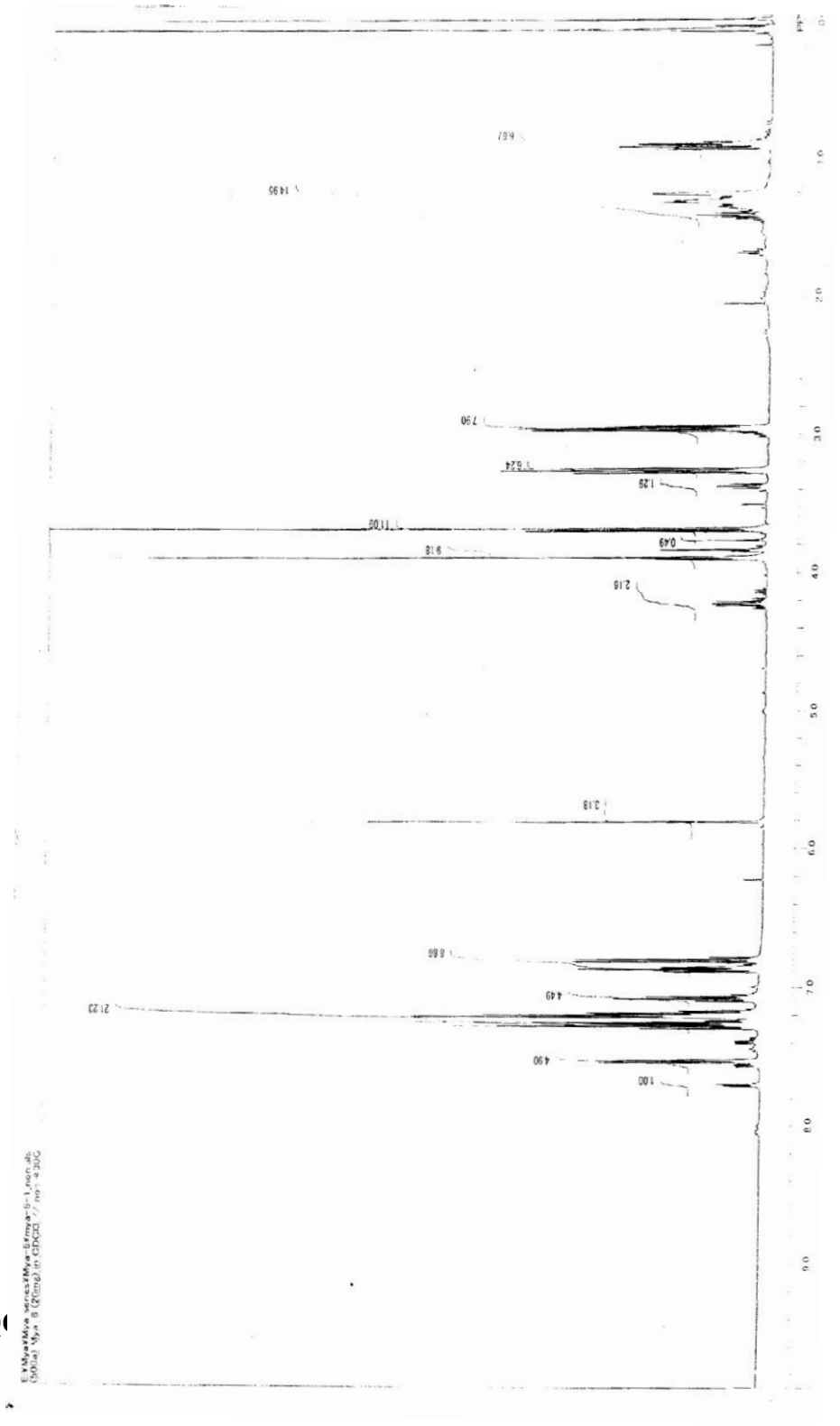


Figure : ^{1}H NMR Spectrum of the Unknown Bioactive Organic Compound

HMQ

The HMQC spectrum of organic compound is shown in figure. The direct proton carbon attachment of the compound could be observed in this spectrum. According to this spectrum, the compound contains nine sp^2 quaternary carbons, ten sp^2 methine carbons, three sp^3 methylene carbons and one sp^3 methyl carbon.

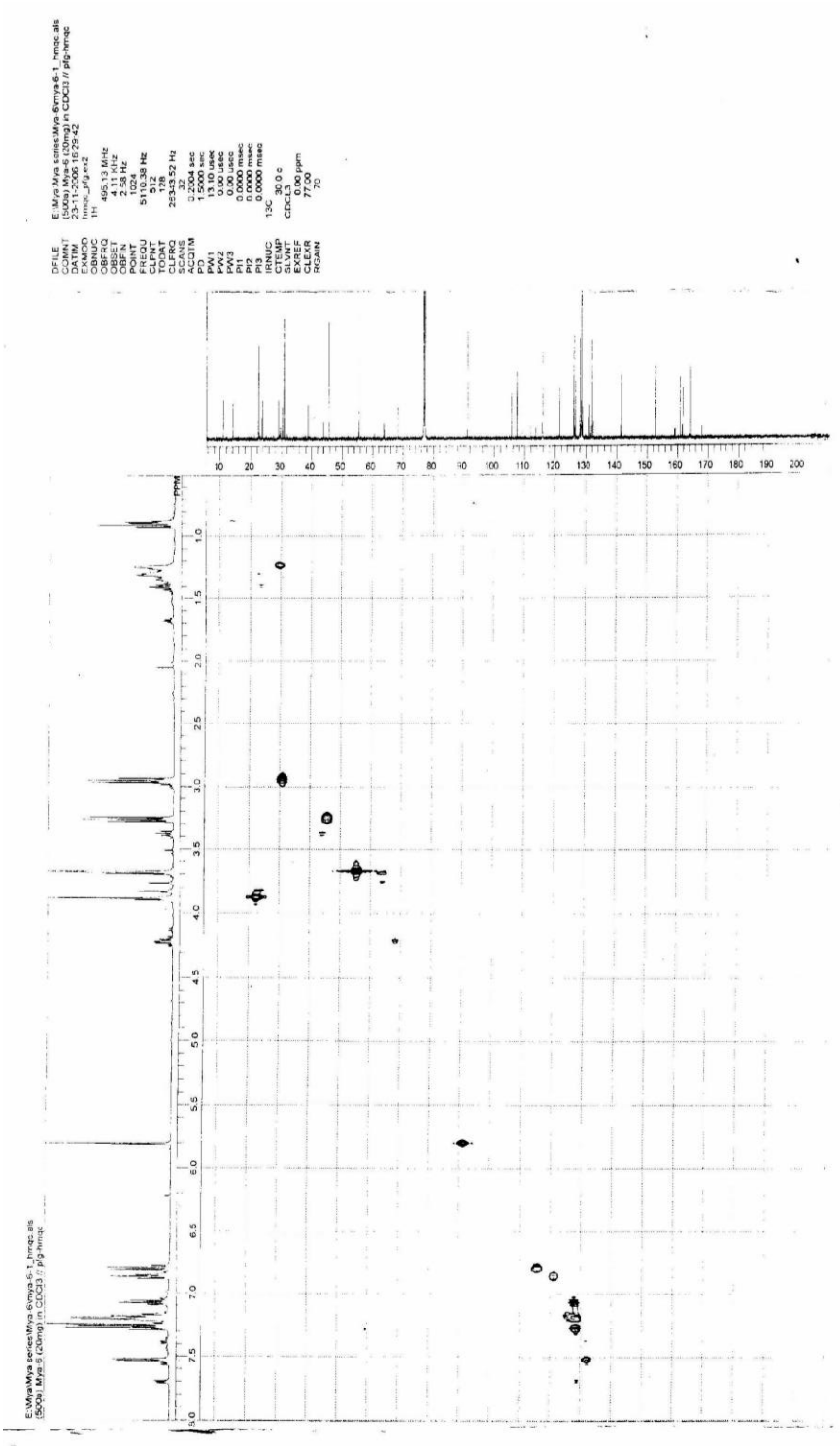


Figure : HMQC Spectrum of the Unknown Bioactive Organic Compound

DEPT Spectrum of the Unknown Bioactive Organic Compound [10,13]

The DEPT spectrum, figure, also confirms the number of protons, carbons and kinds of carbons.

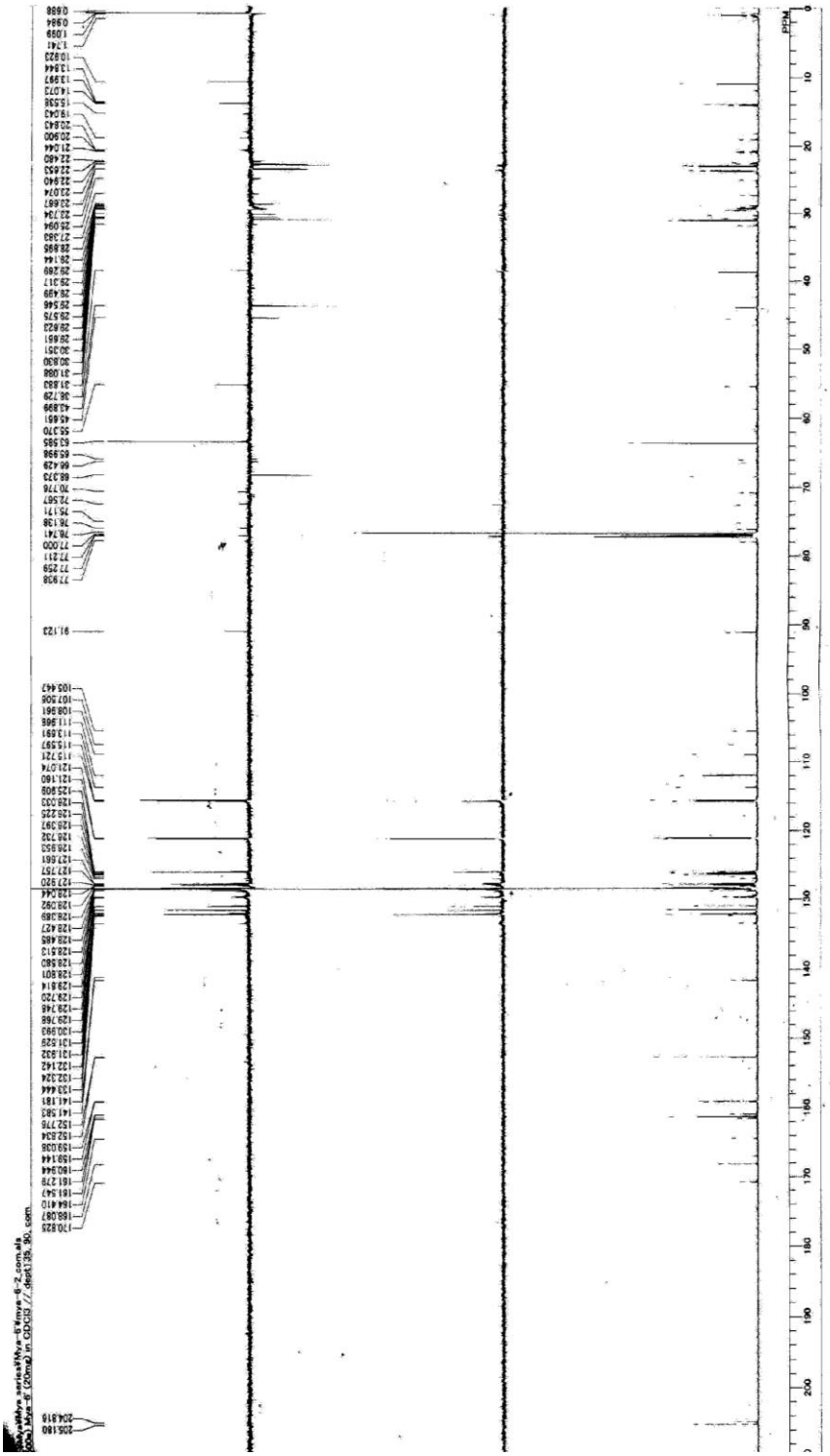


Figure : DEPT Spectrum of the Unknown Bioactive Organic Compound

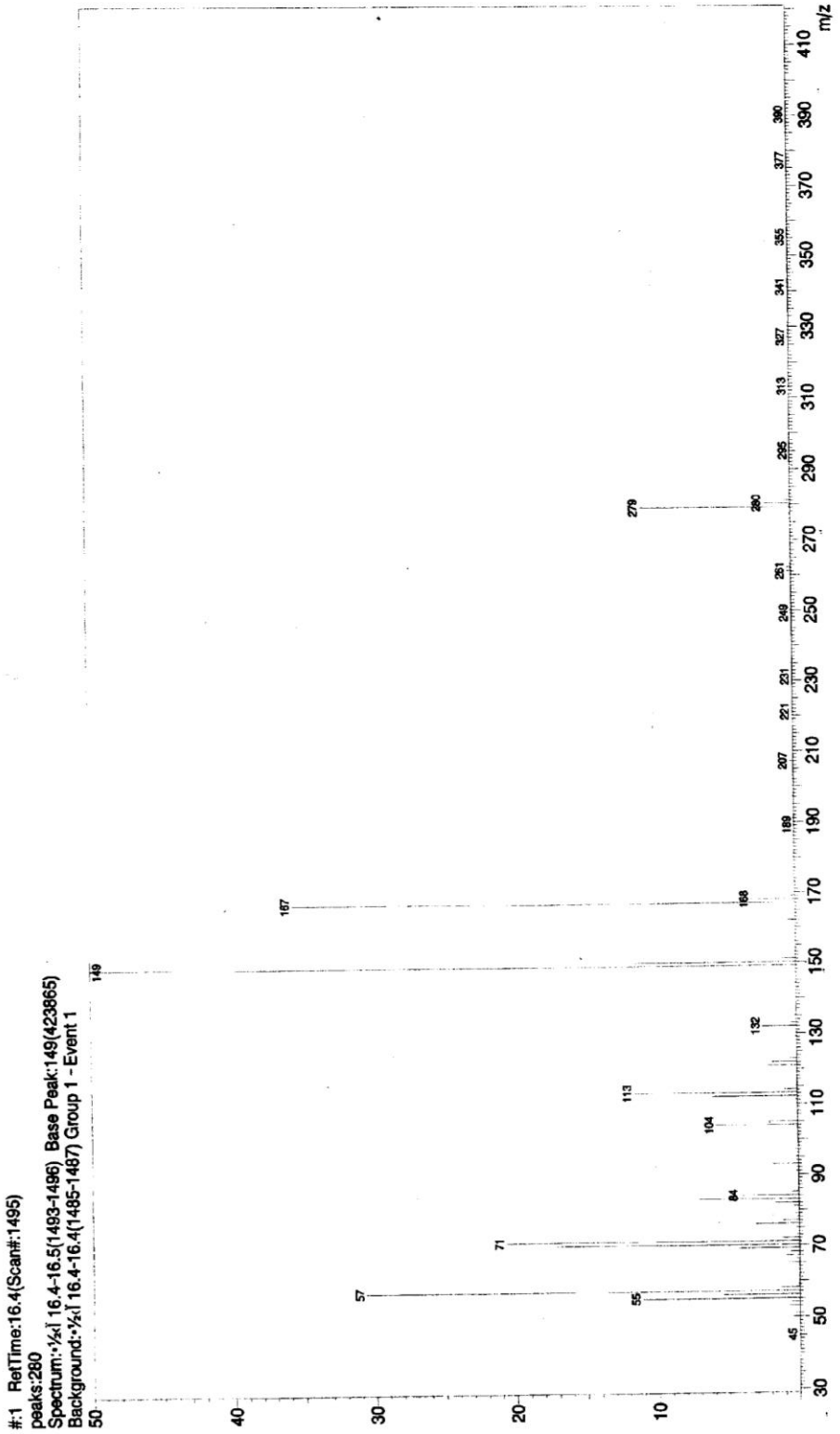


Figure: EI-Mass Spectrum of the Unknown Bioactive Organic Compound

According to ^{1}H NMR spectrum, and ^{13}C NMR spectrum, the partial molecular formula is $C_{23}H_{19}$ and its molecular mass is 295. The FT-IR spectrum shows at least one –OH functional group, one ether functional group, one carbonyl group and one NH_2 group. Therefore the extended partial molecular formula is $C_{23}H_{22}O_3N$ and its molecular mass is 360. However, in EI mass spectrum, the molecular ion peak (m/z = 377) represents the molecular mass of this compound. Therefore the remaining molecular mass is 17. It must be one phenolic –OH functional group because of the appearance of broad band of alcohol at 3301.9 cm^{-1} in FT-IR spectrum. Consequently, the real molecular formula of pure compound could be assigned as $C_{23}H_{23}O_4N$ which agrees with "Nitrogen Rule."Therefore Hydrogen Deficiency Index of this pure compound is as follow.

$$\text{HDI} = 23 - \frac{23}{2} + \frac{1}{2} + 1 = 13$$

Confirmation of Molecular Formula of the Unknown Bioactive Organic Compound by DEPT and FT-IR Spectra

According to DEPT spectrum, the number and kinds of carbon as well as protons in this compound could be assigned. FT-IR spectrum, represents the existence of the functional groups. In accordance with FT-IR spectrum, and DEPT spectrum, the number and kinds f carbon, proton, oxygen and nitrogen could be confirmed and they are tabulated.

Table : The Results given by DEPT Spectrum and FT-IR Spectrum

No.	Assignments	no. of Carbons	no. of Hydrogens	no. of Oxygens	no. of Nitrogens
1	One sp^3 methyl carbon	1	3	–	–
2	Three sp^3 methylene carbons	3	6	–	–
3	Ten sp^2 methine carbons	10	10	–	–
4	Eight quaternary carbons	8	–	–	–
5	One carbonyl group	1	–	1	–
6	One –OH group	–	1	1	–
7	One ether group	–	–	1	–
8	One NH_2 group	–	2	–	1
	Number of carbons, protons, oxygens and nitrogen	23	22	3	1

$\therefore$ The partial molecular formula = $C_{23}H_{22}O_3N$

The partial molecular mass = 360

$\therefore$ The remaining partial mass = 377 – 360

= 17

It must be one phenolic –OH group.

$\therefore$ The real molecular formula of this unknown compound is $C_{23}H_{23}O_4N$.

CONCLUSION

In this research work, the phytochemical screening and antimicrobial activity of the slected plant sample were performed. Ethyl acetate and ethanol extracts of Ingyin-sint respond high activity on all tested organisms; *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albican*, *Bacillus pumalis* and *Mycobacterium* species by using agar-well diffusion method.

Moreover, a pure unknown bioactive organic compound could be isolated from Ingyin-sint by Column Chromatography and checked by TLC. The isolated pure compound was rechecked by phytochemical test. It gives positive for phenolic test. The antimicrobial activity of this pure unknown organic compound was also examined. This pure unknown compound shows medium activity on *Staphylococcus aureus*, *Bacillus pumalis* and *Candida albican* by using agar-well diffusion method.

Furthermore, the molecular formula of this pure bioactive organic compound could be determined as $C_{23}H_{23}O_4N$ applying some modern spectroscopic methods such as FT-IR, ^1H NMR (500 MHz), ^{13}C NMR (125 MHz), HMQC, DEPT and EI mass spectra.

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