Isolation and Structural Elucidation of an Unknown Bioactive Organic Compound from the Root of *Litsea cubeba* Pers.

Win Win Nwe[1], Aung Kyaw Moe[2], Thinn Myat Nwe[3], Myint Myint Sein[4]
[1]Dr, Lecturer, Department of Chemistry, Meiktila University
[2]U, Lecturer, Department of Chemistry, Meiktila University
[3]Dr, Professor, Department of Chemistry, Kalay University
[4]Dr, Professor and Head (Retired), Department of Chemisty, University of Mandalay

Abstract

In this research work, preliminary phytochemical screening of one Myanmar indigenous medicinal plant, *Litsea cubeba* Pers. (Say-ta-lone) collected from Mogok, Mandalay Region was done. This test gives rise to positive for alkaloid, flavonoid, terpene, glycoside and polyphenol. Moreover, antimicrobial activities of crude extract of this compound were performed by agar well diffusion method. A pure bioactive organic compound was separated as yellow grains by modern separation methods such as thin layer and column chromatograms from the roots of Say-ta-lone. The yield percent is found to be (68 mg, 2.2 %) based upon the crude extract. The molecular formula of this compound was determined as $C_{17}H_{17}NO_5$ using some sophisticated spectroscopic methods such as, FT-IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMQC, DEPT, DQF-COSY, HMBC and EI-mass spectrum respectively. Finally, this pure compound was tested for its MIC (Minimal Inhibitory Concentration) by microplate dilution method.

Key words: Phytochemical, antimicrobial, structural elucidation, thin layer chromatography, Fourier Transform Infrared

Introduction

Traditional medicine includes diverse health practices, approaches, knowledge and beliefs incorporating plant, animal and/or mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness.

Traditional medicine and complementary and alternative medicine become more popular within the context of health care provision and health sector reform. Many factors are contributing to widespread use of traditional medicine and complementary and alternative medicine (WHO, 2002). In this research work, one Myanmar Traditional indigenous medicinal plant *Litsea cubeba* Pers. Local name (Say-ta-lone) was selected. The antifungal activity of the root of *Litsea cubeba* Pers. (Say-ta-lone), was evaluated by agar plate assay. *Litsea cubeba* Pers. was selected for chemical analysis.

Botanical Description of *Litsea cubeba* Pers.

Botanical name – *Litsea cubeba* Pers.
Family – Lauraceae
Local name – Say-ta-lone
Medicinal uses – Antiphlogistic, Expectorant Treatment for cutaneous

Figure 1. The flower of *Litsea cubeba* Pers.

Figure 2 .The root of *Litsea cubeba* Pers.

Materials and Methods

Sample Collection

The root of *Litsea cubeba* Pers. (Say-ta-lone) was collected from Mogok Mandalay Division. This plant material was screened and identified by authorized botanist from Botany Department, University of Mandalay. The collected sample was chopped into small pieces, allowed to dry well and stored in a well-stopped bottle and used throughout the experiment.

Preliminary Phytochemical Screening of Plant Extracts

The preliminary phytochemical screening alkaloid, flavonoid, sterol, terpene, glycoside and polyphenol in the plant extract was carried out to determine the presence or absence of chemical constituents in it (Harbone, 1982).

Antimicrobial Activities of one Myanmar Indigenous Medicinal Plant (Say-ta-lone)

The antimicrobial activities of plant extracts of selected plant in various solvent system n-hexane, chloroform, acetone, ethyl acetate and ethanol were tested by Agar well diffusion method at Development Center for Pharmaceutical Technology), Insein, Yangon.

Extraction and Isolation of Compound

The dried root sample (900 g) was percolated with ethanol (2120 mL) for two months. The ethanol extract was evaporated and extracted with ethyl acetate (300 mL). When ethyl acetate extract was concentrated, the crude sample (3.1 g) was obtained. It was chromatographed on silica gel column, eluting with n-hexane and ethyl acetate with various ratio from non-polar to polar. Each and every fractions were checked by TLC, and the same R_f value fractions were combined. Nine-combined fractions (A-I) were obtained and combined fraction D is the main combined fraction. This combined fraction D gave yellow grain compound. Finally, the fraction D which showed only one spot on TLC was recrystallized and the pure yellow grain compound (68 mg) was obtained. The total yield percent of this compound is found to be (2.2 %) based upon the crude extract of ethyl acetate. The melting point of this pure compound is observed as 136-137 °C without decomposition.

Antimicrobial Activities of an Unknown Pure Organic Compound by Agar Well Diffusion Method

Antimicrobial activities of the pure organic compound on six tested organisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albican* and *Mycobacterium* species were tested by using Agar well diffusion method.

Structural Elucidation of the Pure Organic Compound

The structural elucidation of the pure organic compound was done by using sophisticated spectroscopic methods such as FTIR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMQC, DEPT, DQF-COSY, HMBC and EI Mass spectrum respectively.

Results and Discussion

Preliminary Phytochemical Screening of Plant Extracts

The preliminary phytochemical screening of the plant extracts of *Litsea cubeba* Pers. (Say-ta-lone) showed the presence of alkaloid, flavonoid, terpene, glycoside and polyphenol. Many chemical constituents, except steroid were presented in the root of Say-ta-lone.

Antimicrobial Activities of one Myanmar Indigenous Medicinal Plant (Say-ta-lone)

Antimicrobial activities of the plant extracts were tested by Agar well diffusion method in various solvents on six selected organisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albican* and *Mycobacterium* species were tested by using Agar well diffusion method.

The results showed that n-hexane and chloroform extracts of Say-ta-lone gave rise to the high activities on all tested organisms. But, there are medium activities on ***Mycobacterium*** species with acetone, ethyl acetate and ethanol extracts. Moreover, ethyl acetate extract gave no activity on ***Candida albican***. Then, the phytochemical screening of the root of Say-ta-lone responds positive for variety of constituents. Hence, this
plant was used for detailed chemical analysis.

Antimicrobial Activities of an Unknown Pure Organic Compound by Agar Well Diffusion Method

The antimicrobial activities of pure compound was rechecked by using agar well diffusion method with the six tested organisms. The measurable zone diameter shows the degree of antimicrobial activities.

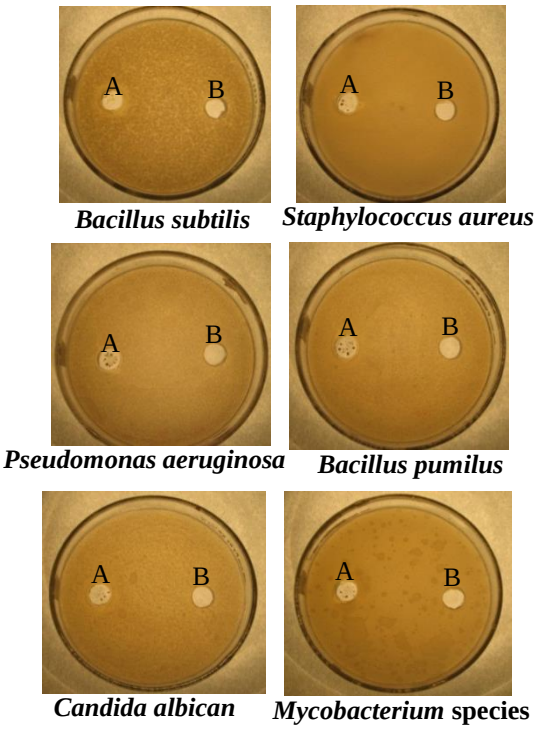


Figure 3. Antimicrobial activities of an unknown pure compound

Table 1. Antimicrobial activities of an unknown pure organic compound

No.	Microorganisms	Remarks
1	*Bacillus subtilis*	+
2	*Staphylococcus aureus*	+
3	*Pseudomonas aeruginosa*	+
4	*Bacillus pumilus*	+
5	*Candida albican*	+
6	*Mycobacterium* species	+

Agar Well – 10 mm
10 mm ~ 14 mm (+) low activity
15 mm ~ 19 mm (++) medium activity
20 mm above (+++) high activity

According to these data pure unknown compound responds the low activity on all tested organisms.

Molecular Formula Determination of compound

The molecular formula of compound could be determined by applying FT-IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT and EI-MS spectral data respectively.

Infrared Spectrum of Compound

The FT-IR spectrum represents the prominent functional groups of an unknown compound (Dolphan, 1997). It was measured at the Department of Chemistry, University of Mandalay. This spectrum was described in Figure 4.

This spectrum represents the existence of N–H stretching vibration of amine group could be determined at 3363.6 cm^{-1}. The peak at 3078.2 cm^{-1} shows the sp^2 C–H stretching frequency of alkenic group. The two peaks which appears at 2954.7 cm^{-1} and 2839.0 cm^{-1} are due to C–H stretching vibration of asymmetrical and symmetrical sp^3 hydrocarbons. Moreover, the occurrence of C=O stretching vibration at 1666.4 cm^{-1} implies the detection of carbonyl functional group in this compound. The C=C stretching vibration of alkenic group could be observed at 1620.1 cm^{-1}. In addition, C –N stretching vibration of amine could be detected at 1573.8 cm^{-1}. The bands which appear at 1465.8 cm^{-1} and 1442.7 cm^{-1} may be in plane bending vibration of allylic hydrocarbons. The peak at 1319.2 cm^{-1} must be C–H in plane bending vibration of methyl group. The –C–O–C stretching vibration of ether group was occurred at 1080.1 cm^{-1}. The bands which appear at 972.1 cm^{-1} and 933.5 cm^{-1} are attributed to C – H out of plane bending vibration of E or trans alkene. Finally the bands at 840.9 cm^{-1} and 756.0 cm^{-1} signify C – H out of plane bending vibration of Z or cis alkene.

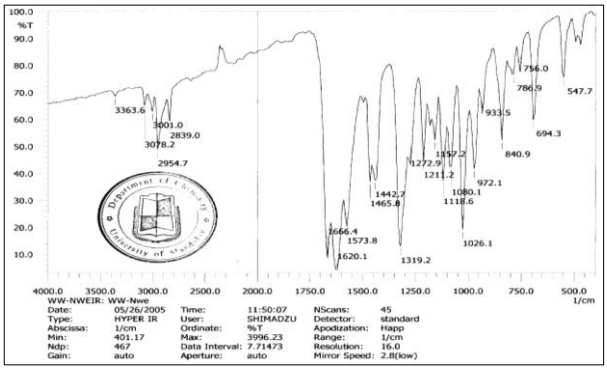


Figure 4. FT-IR spectrum of compound

^{1}H NMR Spectrum of Compound

The ^{1}H NMR (500 MHz) spectrum was described in Figure 5. According to (Eberharnd, 2002), this compound contains 16 protons and their data are shown in Table 2.

Table 2. ^{1}H NMR spectral data of compound

Protons Assigned	δ/ppm	Splitting Patterns	J values (Hz)
—CH_3	4.10	singlet	–
—CH_3	4.19	singlet	–
—CH_3	4.20	singlet	–
H | C	7.37	Triple triplet	7.37, 1.4
H | C	7.38	double doublet	7.37, 6.5
H | C	7.38	double doublet	7.37, 6.5
H | C	7.51	doublet	15.8
H | C	7.60	double doublet	6.5, 1.4
H | C	7.60	double doublet	6.5, 1.4
H | C	7.95	doublet	15.8

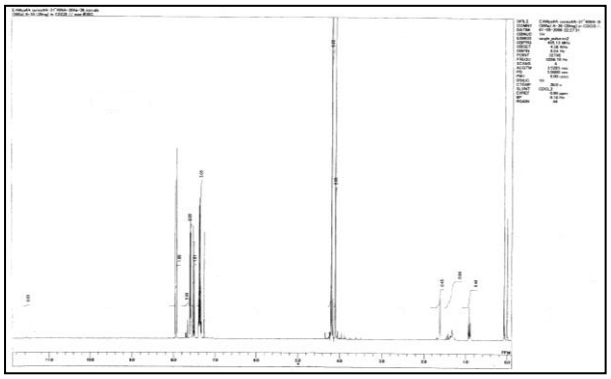


Figure 5. ^{1}HNMR (500 MHz) spectrum of compound

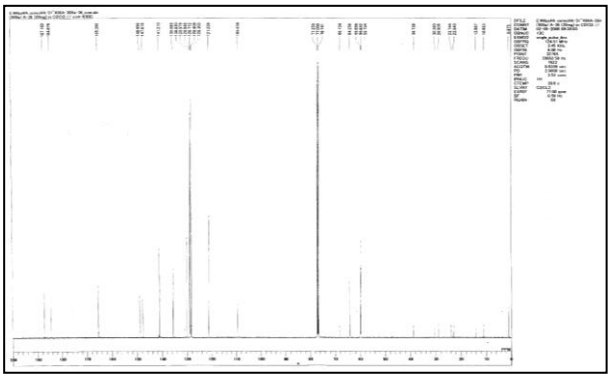


Figure 6. ^{13}CNMR (125 MHz) spectrum of compound

^{13}C NMR Spectrum of Compound

From ^{13}C NMR spectrum Figure 6, 17 carbon atoms could be assigned (John, 2003). The chemical shift values δ/ppm and their assignments are tabulated in Table 3.

Table 3. ^{13}C NMR spectral data of Compound

No	δ/ppm	Type of carbons
1.	187.16	sp^2 quaternary carbonyl carbon
2.	184.67	sp^2 quaternary carbonyl carbon
3.	165.34	sp^2 quaternary carbon
4.	148.96	sp^2 quaternary carbon
5.	147.82	sp^2 quaternary carbon
6.	141.21	sp^2 methine carbon
7.	135.59	sp^2 quaternary carbon
8.	129.98	sp^2 methine carbon
9.	128.85	sp^2 methine carbon
10.	128.85	sp^2 methine carbon
11.	128.30	sp^2 methine carbon
12.	128.30	sp^2 methine carbon
13.	121.24	sp^2 methine carbon
14.	109.43	sp^2 quaternary carbon
15.	64.26	methoxy methyl carbon
16.	59.89	methoxy methyl carbon
17.	59.84	methoxy methyl carbon

HMQC Spectrum of Compound

The HMQC spectrum was assigned (Silverstein, 1998) and the resulting compound was shown in Figure 7, which gives rise to the information of proton-carbon direct correlation.

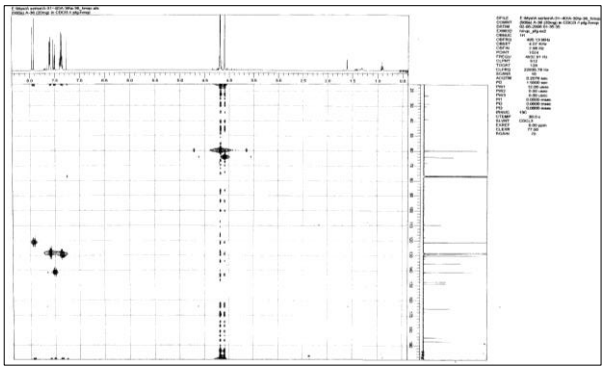


Figure 7. HMQC spectrum of compound

DEPT Spectrum of Compound

According to (Manfred, 1997), the DEPT spectrum Figure 8 also confirms the number of protons, carbons and the kinds of carbons.

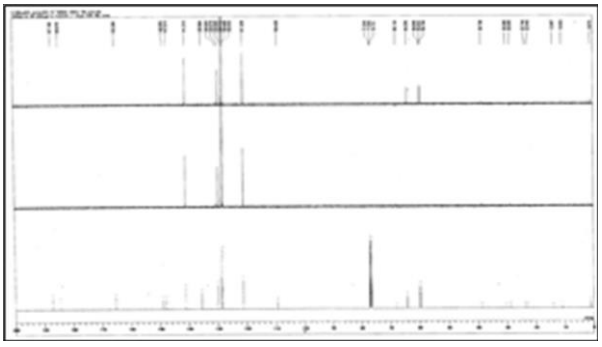


Figure 8. DEPT spectrum of compound

Electron Impact Mass Spectrum

EI mass spectrum of this compound was shown in Figure 9. Determination of FT-IR, ^{1}H NMR and ^{13}C NMR spectral data, two carbonyl carbons appeared at down field chemical shifts (δ 187.16 ppm, δ 184.67 ppm) and three methoxy methyl carbons observed at

(δ 64.26 ppm, δ 59.89 ppm, δ 59.84 ppm) were assigned (Silverstein, 1998). Hence, the partial molecular formula of compound could be assigned as $C_{17}H_{16}O_5$.

Therefore, the partial molecular mass = 300

From EI Mass spectrum, Figure.9, the molecular ion peak (m/z 315) verifies the molecular mass of this compound.

Therefore, the remaining molecular mass is 15. So, it may be one –NH group.

Moreover as described in FT-IR spectrum, Figure.4, the existence of one –NH group could be observed at (3363.6 cm^{-1}). Therefore, the remaining molecular mass (15) must be one –NH group. Finally, the real molecular formula could be determined as $C_{17}H_{17}NO_5$, which agrees with the Nitrogen Rule.

$$\text{Hydrogen deficiency index} = 17 - \frac{7}{2} + \frac{1}{2} + 1$$
$$= 10$$

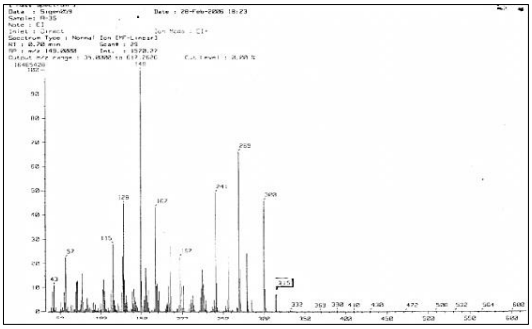


Figure 9. EI mass spectrum of compound

Confirmation of Molecular Formula of Compound

According to FT-IR spectrum Figure 4 and DEPT spectrum Figure 8, the kinds of carbon, proton and oxygen were confirmed. They are tabulated in Table 4.

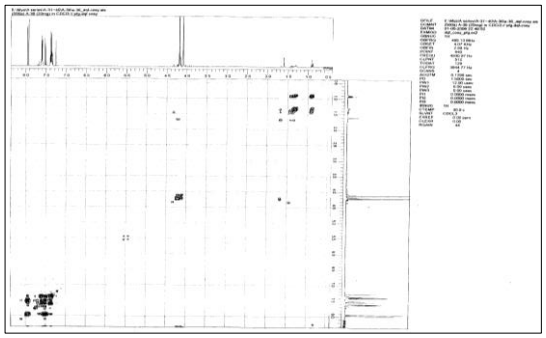


Figure 10. DQF-COSY spectrum of compound

Table 4. Confirmation of molecular formula

Assignments	no: of carbon	no: of proton	no: of oxygen	no: of nitrogen
seven sp^2 quaternary carbons	7			
seven sp^2 methine carbons	7	7		
Three sp^3 methoxy methyl carbon	3	9	3	
two carbonyl groups			2	
one-NH group		1		1
Total no: of carbon, proton, oxygen, and nitrogen	17	17	5	1
Complete molecular formula	$C_{17}H_{17}NO_5$			

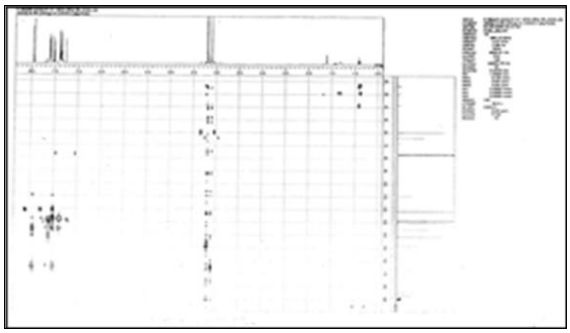
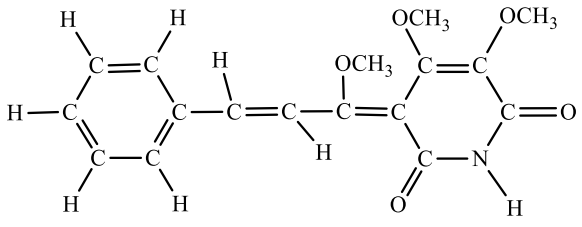


Figure 11. HMBC spectrum of compound

Structure Elucidation of Pure Unknown Bioactive Organic Compound

The structure of isolated pure compound was elucidated by DQF-COSY spectrum Figure 10 and HMBC spectrum Figure 11. This compound was named as (Z).4,5dimethoxy-3-(E-1-methoxy-3phenylallylidene)

pyridine-2,6 (1*H*, 3*H*)-dione, according to (Phillip, *et al.*, 1998), the elucidated structure of compound could be described as follows.



Conclusion

An interesting one Myanmar Indigenous Medicinal Plant, namely (Say-ta-lone) was chosen for chemical investigations. The crude extract of this plant consists of some chemical constituents, such as alkaloid, flavonoid, terpene, glycoside and polyphenol applying by phytochemical screening technique. Furthermore, ethyl acetate extract responds high activities on four tested organisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa* and, *Bacillus pumilus.* Ethyl acetate extract of *Litsea cubeba* Pers gives rise to medium activity on *Mycobacterium* species and no activity on *Candida albican*.

In addition a pure compound as yellow grains were isolated from ethyl acetate extract by Thin-layer and Column separation techniques. The yield percent of pure compound is found to be (68.0 mg, 22%) based upon the ethyl acetate crude extract and its melting point could be measured as (136-137°C).

On the other hand, antimicrobial activities of this unknown organic compound on six selected organisms were tested by using by agar well diffusion method. This compound responds low activities on all tested organisms. Furthermore, the molecular formula and conformational structure of this isolated compound could be determined by applying sophisticated spectroscopic methods such as FT-IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), HMQC, DEPT, DQF-COSY, HMBC and EI mass spectrometric methods.

Acknowledgements

We would like to express our deepest thanks to Dr Ba Han, Rector Meiktila University and Dr Tin Tun Aung, Pro-Rector, Meiktila University for their kind permission and research facilities to carry out this paper. I would like to express our deepest thanks to Dr Ni Ni Aung, Professor and Head, Department of Chemistry, Meiktila University, for her kind permission and research facilities to carry out this paper.

References

Dolphan, D. *et a*l., (1997) "Tabulation of Infrared Spectral Data." John Wiley and sons, Ltd. New York.

Eberharnd, Breitmaier. (2002) "Structure Elucidation by NMR in Organic Chemistry." A Practical Guide, 3rd Edition, University of Bonn, Germany, John Wiley &Sons, Ltd.

Harbonne, J. B. (1982) "Phytochemical Methods : A Guide to Modern Techniques of Plant Analysis," Chapman and Hall Ltd, USA.

John, and H. Nelson, (2003) “Nuclear Magnetic Resonance Spectroscopy", Pearson Education, Inc.

Manfred Hesse, *et al.*, (1997) "Spectrometric Methods in Organic Chemistry": Georg. Thiene Verlag Stuttgart. New York.

Phillip Crews, Jaime Rodriguez, and Mareel Jaspar, (1998) “Organic Structure Analysis" Oxford University Press.

Silverstein, R.M. I. (1998) "Spectrometric Identification of Organic Compound : 6th Edition, John Wiley & Sons, Inc, New York.

WHO Traditional Medicine Strategy (2002-2005).