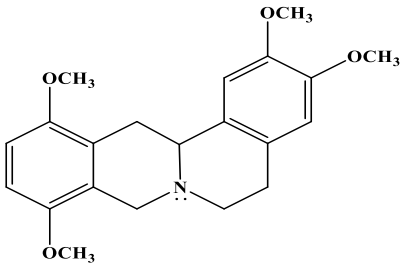
STRUCTURAL ELUCIDATION OF PALMATINE COMPOUND AND ITS ANTIMICROBIAL ACTIVITY ISOLATED FROM THE TUBER OF *STEPHANIA GLABRA* (ROXB.) MIERS

Khup Lam Tuang[1], Hla Myoe Min[2], Myint Myint Sein[3]

Abstract

The aim of this research was to extract, isolate, determine the structure of isolated compound and its antimicrobial activity from the ethanolic tuber extract of *Stephania glabra* (Roxb.) Miers (Menispermaceae family). The tuber of *Stephania glabra* (Roxb.) Miers, Taung-Kya (Myanmar name) was selected for this present work. It was rinsed with tap water, chopped in to small pieces and dried in air. The prepared dry sample was extracted with 95% of ethanol for one month. After doing filtration and evaporation, the remaining extract mass was further extracted with ethyl acetate and ran by different solvents ratios using thin layer sheet (spot test on TLC plate). A biologically active pure compound (colourless needle shaped crystals) could be isolated from n-hexane and ethyl acetate solvent ratio (n-hex 1:1EtOAc, 400 mg, and R_f value 0.5) by passing through the prepared column chromatogram. The molecular formula of an unidentified palmatine compound was assigned as $C_{21}H_{25}NO_4$ (assumed as glabrine) by using advanced spectroscopic techniques such as FT IR, 1D NMR (^1H, ^{13}C and DEPT), 2D NMR (HSQC, DQF-COSY, HMBC, NOESY) and DART MS spectral evidences. The antimicrobial activity of palmatine compound was examined by agar disc diffusion method against six selected organisms. This compound responds medium inhibition zone (15mm to 18mm) on all tested microorganisms, namely, *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (NCPC 6371), *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (NCIB 8982), *Candida albicans* and *Escherichia coli* (NCIB 8134). The IUPAC name of palmatine compound was named as (*R*)- 2, 3, 9, 12- tetramethoxy- 5, 8, 13, 13a- tetrahydro-*6H*- isoquinolino [2,1- b] isoquinoline.



Keywords: *Stephania glabra* (Roxb.) Miers, extraction, isolation, thin layer and column chromatogramphy, palmatine compound, spectroscopic techniques, antimicrobial activity

Introduction

Stephania glabra (Roxb.) Miers (*S. glabra*), Myanmar name Taung-Kya is a species under the genus of climbers belonging to family Menispermaceae. It is a large climbering shrub with greenish yellowish flowers and large tubers (Hemraj *et al.*, 2012). The plant is found in many countries depending on tropical and subtropical regions (Titova *et al*., 2012). The tuber part of *Stephania glabra* (Roxb.) Miers was collected from Kalay Township, Sagaing Region, Myanmar. This plant was identified by Deputy Director General, Dr Soe Myint Aye, Department of Higher Education, Ministry of Education, Naypidaw, Myanmar. The various parts of this plant are also used to treat diabetes, edema, pain, stomach disorders, helminthiasis, malaria, hepatitis, tuberculosis and hypertension (Jahan *et al*., 2010). The plant is extensively used in folk medicine in Asian countries, especially for diabetes (Semwal, D.K and Semwal, R.B. 2015). A dried powder

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of the root pulp with tea is used to treat puerperal fever in women (Rahmatullah *et al*., 2014). Until now, *S. glabra* mainly contains over 30 alkaloids classes such as bisbenzylisoquinolines, hasubanalactams, berberines and aporphines have been isolated from its tuber. The palmatine compound has been widely used in pharmaceutical fields such as in pharmacology, toxicity and pharmacokinetics studies (Semwal, D.K. and Semwal, R.B. 2015). Tetrahydropalmatine a class of palmatine compound derived from the tubers of *S. glabra* produces remarkable sedation of the central nervous system (CNS), and also exerts significant effects against dopamine receptors localised in the brain (Semwal, D.K. and Semwal, R.B. 2015). In traditional medicine, *S. glabra* has been used for the treatment of cancer, but no scientific evidence is not yet available (Semwal, D.K. and Semwal, R.B. 2015). The presence of bisbenzylisoquinoline alkaloids in the *S. glabra* plant, which are well known for their anticancer activity (Kuroda *et al*., 1976).

In recent years, the isoquinoline types of a palmatine compound (named as stephanine, $C_{21}H_{25}O_4N$) was elucidated from the rhizome of *Stephania rotunda* lour in Myanmar (Myint Myint Mar. 2007). The molecular formula and structure of an unidentified palmatine compound from the tuber of Myanmar plant *S. glabra* was assigned as $C_{21}H_{25}NO_4$ (assumed as glabrine) by using advanced spectroscopic techniques such as FT IR (Fourier Transform Infrared), ^{1}H NMR (Proton Nuclear Magnetic Resonance), ^{13}C NMR (Carbon Nuclear Magnetic Resonance), DEPT (Distortion Enhancement by Polarization Transfer), HSQC (Heteronuclear Single Quantum Coherence), DART (Direct Analysis of Real Time Mass Spectroscopy) DQF-COSY (Double Quantum Filtered Correlation Spectroscopy), HMBC (Heteronuclear Multiple Bond Coherence) NOESY (Nuclear Overhauser Effect Spectroscopy) spectral evidences. This compound is firstly reported from *S. glabra* tuber in Myanmar, until 2020 year. At present, this isolated palmatine compound (assumed as glabrine), separated from the tuber of *S. glabra* showed the remarkable sedation of the central nervous system (CNS) (Khup Lam Tuang. 2019). Currently, the bioactivity of this compound is determining with the inhibition zone (mm) by agar disc diffusion method against on selected microorganisms.

Figure 1 The Tuber of *Stephania glabra* (Roxb.) Miers (Source by Researcher)

Materials and Mehods

General Experimental Procedure

Commercial grade solvents and analytical grade reagents were utilized throughout this research work. The solvents were purified by distillation method before they were used in experiment. Thin-Layer Chromatographic separation was performed by using aluminum coated sheets silica gel (Merck Co.Inc., Kiesel gel 60 F_{256}) was run for solvents and silica gel (Merck Co.Inc., Kiesel gel 70-230 mesh ASTM) was used for column chromatographic separation. I_2 vapour and UV detector (Lambda-40, Perkin-Elmer Co., England) were used to identify the color of constituent compounds as spot on TLC sheets. The melting point was recorded on a Gallenkamp melting point apparatus (England). Common laboratory tools were used for extraction, isolation and purification of bioactive compound. Shimadzu electronic balance (Japan) was used to determine the weights of substances. The FT IR spectrum was measured by using SHIMADZU

spectrometer. The 1DNMR spectra for ^{1}H NMR (600 MHz), $^{13}CNMR$ (150 MHz), DEPT (150 MHz), DART MS were measured with BRUKER model spectrometry. The ^{2}D NMR spectra for DQF COSY (600 MHz), HSQC, HMBC, NOESY (600 MHz) spectra were also recorded with BRUKER model. All NMR spectra were measured in $CDCl_3$ solvent. Chemical shift scales were expressed in delta (δ/ppm) and solvent peaks were used as internal standards for both ^{1}H (7.28 ppm) and ^{13}C (70.7 ppm) down field from TMS internal reference.

Sample Collection and Preparation

The tuber of *Stephania glabra* (Roxb.) Miers to be analyzed was collected in October, 2015 in Kalay Townships, near Lett-Sey-Kan leak in Myanmar. After washing and cleaning, the tuber was chopped in to small pieces and dried under room temperature using electric fan. The dried tuber was stored in a well stopper bottle for experiment.

Extraction and Isolation

The air dried sample (650 g) was macerated and occasionally percolated with 1000 mL ethanol (95%) for one month. After doing filtration and evaporation, the obtained ethanolic extract (8.5 g) was further extracted with ethyl acetate to isolate pure compound by using column chromatogram over SiO_2 (70-230 mesh) eluting with n-hexane: ethyl acetate in 1:1 ratio. This ratio obtained the colourless crystals (400 mg, R_f = 0.5) having needle shapes. It was showed a yellow spot on TLC and a black spot on UV respectively.

Molecular Formula Determination of Pure Compound

The molecular formula of pure compound (colourless and needle- shaped crystal) could be isolated by column chromatographic method and its structure was elucidated in accordance with ^{1}D and ^{2}D NMR spectral evidence (Silverstein *et al*.,2005).

Antimicrobial Activity of Palmatine Compound

Antimicrobial activity of palmatine compound was tested against microorganisms in ethyl acetate solvent using agar well diffusion method. Agar plug method was adopted for the antimicrobial screening on six selected microorganisms (five bacteria and one fungi) namely, *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (NCPC 6371), *Pseudomonas aeruginosa* (6749), *Bacillus pumilus* (NCIB 8982), *Candida albicans* and *Escherichia coli* (NCIB 8134). 2 mL of the test organisms was aseptically injected into the sterilized plate. 10 mL of the sterilized nutrient agar were poured on top of the test organisms after cooling the disc. Sterilized cork borer of 10 mm diameter was used and made 2 wells on each solidified agar sheet into which 0.5 mL of the prepared palmatine compound were aseptically introduced by the use of sterilized clinical syringe. The plates were incubated at 37°C for 24 hours. Zone of inhibition (mm) were observed around each well after 24 hours (Adewole *et al.*, 2013).

Results and Discussion

Molecular Formula Determination of Pure Compound

According to FT IR spectrum (Figure 2), pure compound consists of sp^3 hydrocarbon carbons (2931.90, 2839.31, 2798.80 and 2752.51 cm^{-1}), sp^2 hydrocarbons (3005.20 cm^{-1}), aromatic benzene ring (1612.54 and 1506.46 cm^{-1}), aromatic amine group (1338.64cm^{-1}), allylic hydrocarbons (1458.23 cm^{-1}), ether group (1139.97, 1078.24, 1033.88, 1273.06 and 1224.84cm^{-1}) and cis or trans alkenic (864.14 and 781.20 cm^{-1}) functional groups respectively (Silverstein *et al*.,2005).

Table 1 ^{1}H NMR (600MHz) Spectrum of Pure Compound in $CDCl_3$

No.	^{1}H (δ/ppm)	Splitting Pattern	J values (Hz)	No. of Proton	Proton Assignment
1	2.65	dd	15.87, 10.23	1	sp^2 methine proton
2	2.68	dd	14.54, 11.33	1	sp^2 methine proton
3	2.83	dd	15.87, 3.86	1	sp^2 methine proton
4	3.15	dd	14.54, 10.23	1	sp^2 methine proton
5	3.20	dd	15.87, 11.33	1	sp^2 methine proton
6	3.27	dd	15.87, 4.25	1	sp^2 methine proton
7	3.53	d	15.87	1	sp^2 methine proton
8	3.55	dd	4.25, 3.86	1	sp^2 methine proton
9	3.85	s	s	3	sp^3 methyl proton
10	3.86	s	s	3	sp^3 methyl proton
11	3.87	s	s	3	sp^3 methyl proton
12	3.89	s	s	3	sp^3 methyl proton
13	4.24	d	15.87	1	sp^2 methylene proton
14	6.62	s	s	1	sp^2 methine proton
15	6.73	s	s	1	sp^2 methine proton
16	6.79	d	8.21	1	sp^2 methine proton
17	6.88	d	8.21	1	sp^2 methine proton
	Total number of protons			=	25

Table 2 ^{1}H-^{13}C NMR (HSQC) and DEPT (150 MHz) Spectral Data of Pure Compound in $CDCl_3$

No	^{1}H (δ/ppm)	^{13}C NMR	DEPT
1	2.65	29.10	sp^2CH
2	2.68	36.33	sp^2CH
3	2.83	51.49	sp^2CH
4	3.15	53.99	sp^2CH
5	3.20	55.86	sp^2CH
6	3.27	55.95	sp^2CH
7	3.53	56.14	sp^2CH
8	3.55	59.30	sp^2CH
9	3.85	60.12	sp^3CH_3
10	3.86	108.66	sp^3CH_3
11	3.87	110.96	sp^3CH_3
12	3.89	111.37	sp^3CH_3
13	4.24	123.80	sp^2CH
14	6.62	126.81	sp^2CH
15	6.73	127.76	sp^2CH
16	6.79	128.69	sp^2CH
17	6.88	129.74	sp^2CH
18	-	145.09	sp^2C
19	-	147.44	sp^2C
20	-	147.49	sp^2C
21	-	150.25	sp^2C
	Total number of carbons		= 21

According to FT IR, ^{1}H NMR, ^{13}C NMR and DEPT spectral data, the complete molecular formula of compound could be deduced (Table 3).

Table 3 Molecular Formula Determination of Pure Compound

Assignments	No. of Carbon	No. of Proton	No. of Oxygen	No. of Nitrogen
From ^{1}HNMR, ^{13}CNMR and DEPT data				
- Four sp^3 methyl	4	12	-	-
- Four sp^2 methylene	4	8	-	-
- Four sp^2 methine	4	4	-	-
- One sp^2 methine	1	1	-	-
- Four methoxy groups	-	-	4	-
- Eight sp^2 quaternary	8	-	-	-
From FT IR data				
- One amine group (tertiary amine)	-	-	-	1
Complete molecular formula	C_{21}	H_{25}	O_4	N

DART MS mass spectrum (Figure 6) of pure compound represented the molecular ion peak at m/z = 356.1844 Da. However, the evaluated mass of molecular formula, $C_{21}H_{25}NO_4$ is 355. Therefore, the actual mass could be match of when only subtracting one unit proton from m/z 356.1844. Hence, the molecular mass of compound is now assigned as 355.1764 Da. This is agreed with the actual mass 355. The molecular mass is also consistent with "Nitrogen rule".

Hydrogen Deficiency Index (HDI) $= 21 - \frac{25}{2} + \frac{1}{2} + 1 = 10$

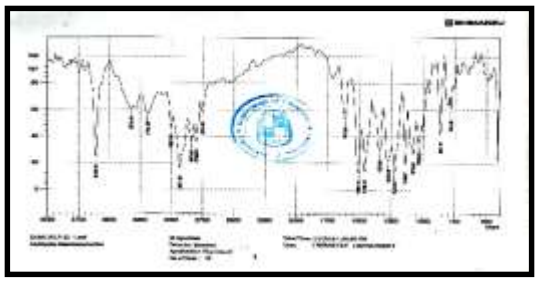


Figure 2 FT IR Spectrum of Pure Compound

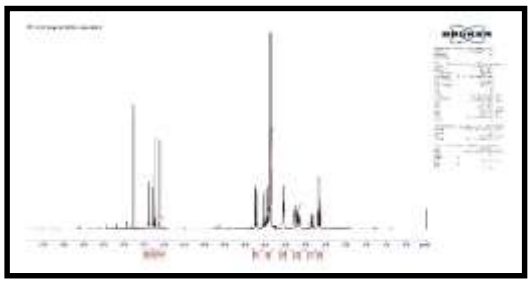


Figure 3 ^{1}H NMR (600MHz) Spectrum of Pure Compound

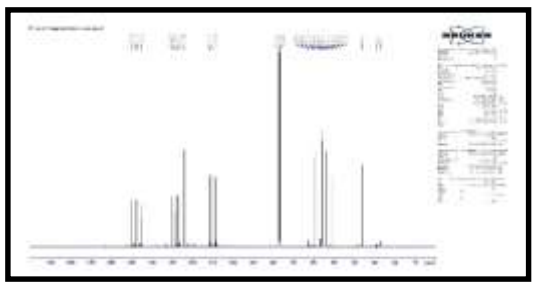


Figure 4 ^{13}C NMR (150MHz) Spectrum of Pure Compound

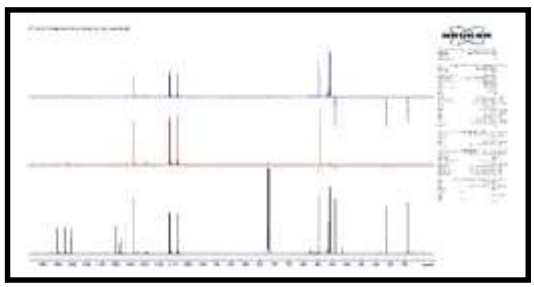


Figure 5 DEPT (150MHz) Spectrum of Pure Compound

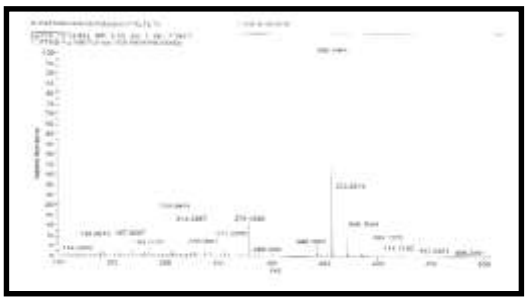


Figure 6 DART MS Spectrum of Pure Compound

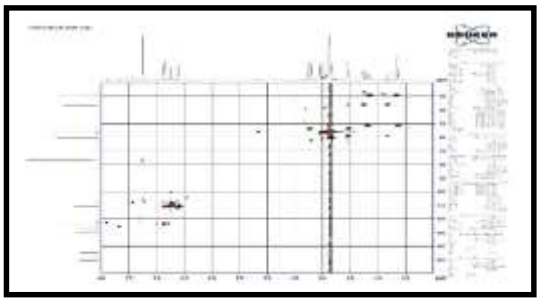
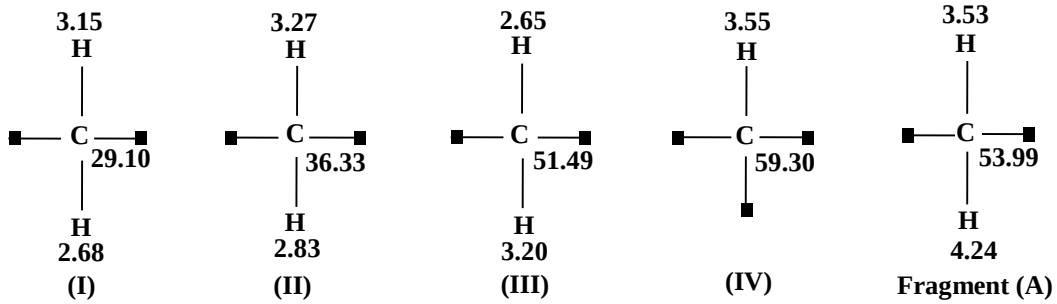


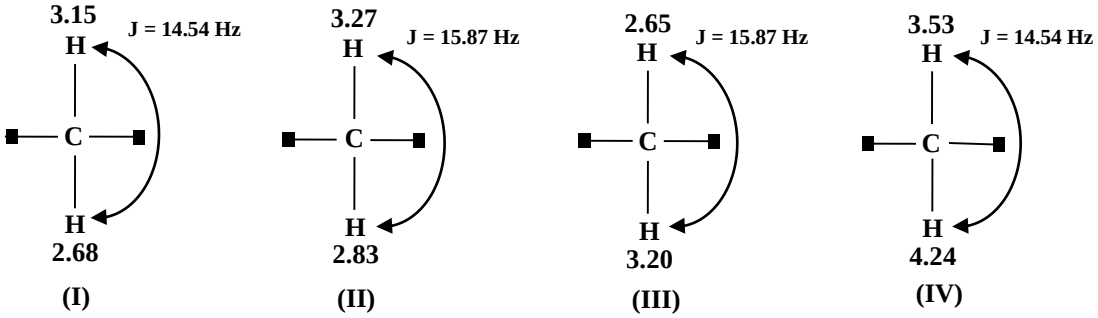
Figure 7 HSQC Spectrum of Pure Compound

Structural Elucidation of Pure Compound

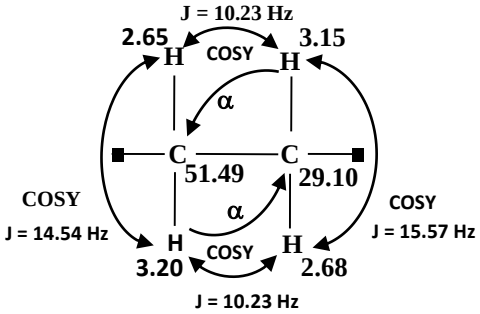
The following fragments (I) to (IV) and fragment (A) could be confirmed by downward appearance of four sp^3 methylene carbons, upward appearance of one sp^3 methine carbon and one sp^3 methylene carbons in DEPT spectrum (Figure 5). Hence, their proton-carbon direct correlation could be observed in HSQC spectrum (Figure 7).



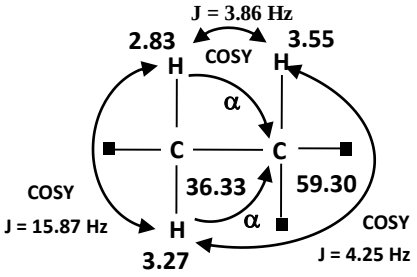
The four set of geminal methylene protons and their spin-spin coupling fragments (I, II, III and IV) in DQF COSY spectrum (Figure 8) are assigned as follow.



DQF COSY (Figure 8), ^{1}H NMR (Figure 3) and HMBC (Figure 9) spectra show the present of the following fragments (B) and (C).

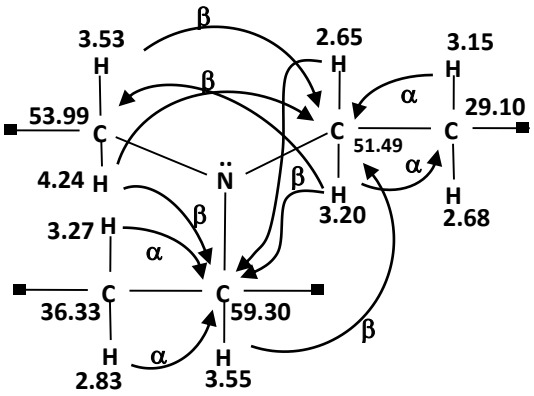


Fragment (B)

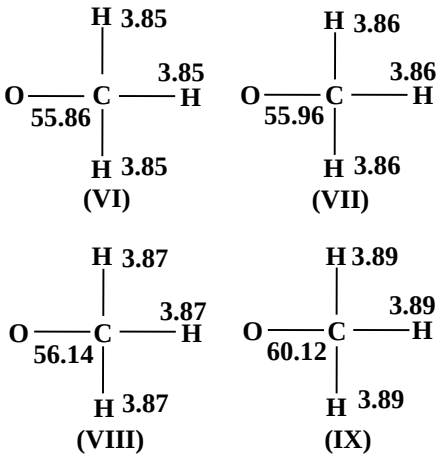


Fragment (C)

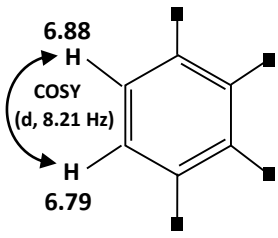
In HMBC spectrum (Figure 9), there are proton-carbon long range coupling around methylene and methine groups in fragments (A), (B) and (C) are considered to be connected with N atom. All proton-carbon long range correlation signals are shown in following fragment (D). In accordance with HSQC (Figure 7), ^{1}H NMR (Figure 3) and DEPT (Figure 5) data the four methoxy groups are assigned as below.



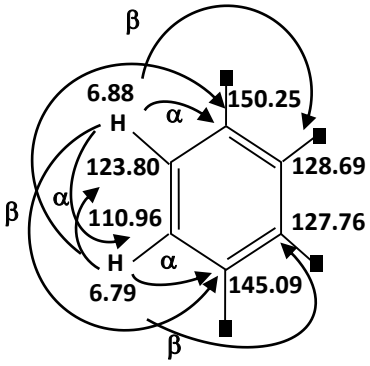
Fragment (D)



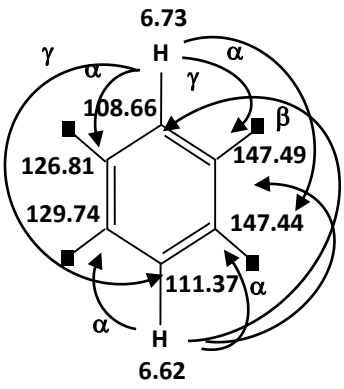
In the ^{1}H NMR spectrum (Figure 3), both of the two aromatic protons of δ 6.88 ppm and δ 6.79 ppm have the splitting patterns and coupling constants (d, J = 8.21 Hz). Hence, the fragments (E) and (F) could be assigned by the observation of proton-carbon direct attachment in HSQC spectrum (Figure 7) and ^{1}H-C long range coupling with both of sp^{2} quaternary carbons in HMBC spectrum (Figure 9).



Fragment (E)

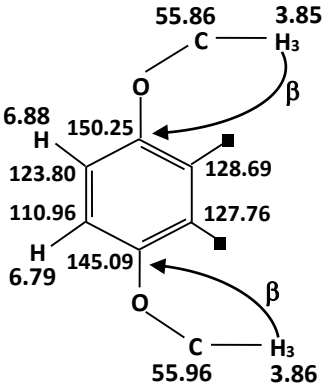


Fragment (E)

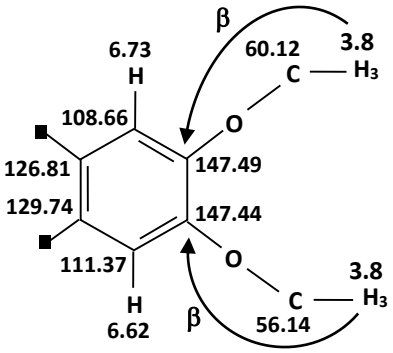


Fragment (F)

In fragments (E) and (F), lower field chemical shift values of sp^2 carbons (150.25 145.09 ppm and 108.66, 111.37 ppm) are considered to be connected with methoxy groups (δ 3.85, 3.86 ppm and δ 3.87, 3.89 ppm) (Figure 9). Methoxy protons (δ 3.85, 3.86 ppm) shows β-correlation cross peak to δ 150.25, 145.09 ppm carbons and similarly, other methoxy protons (δ 3.87, 3.89 ppm) shows β-correlation to 147.44, 147.99 ppm carbons (Figure 9). Therefore, these attachments lead to the following fragments bearing two methoxy groups on benzene ring.

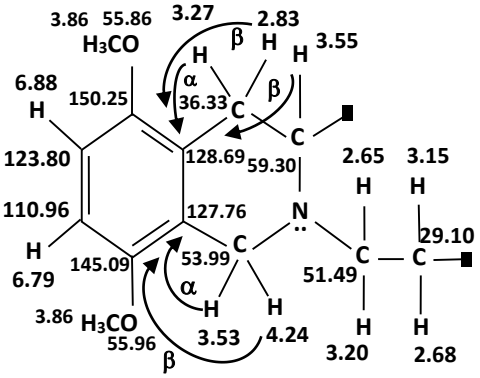


Fragment (E)

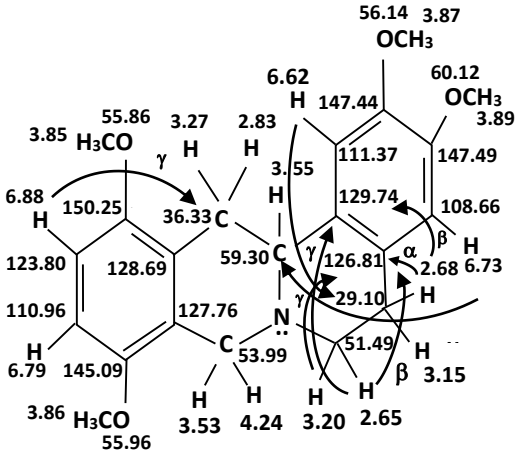


Fragment (F)

Fragments C_5H_9N(D), $C_8H_8O_2$ (E) and $C_8H_8O_2$ (F) could be connected ^{1}H-C long range cross peaks in HMBC spectrum (Figure 9). This correlation gives a good confirmation to be assigned the following fragment (G). Next, the connection between the fragments (G) and (F) could be confirmed by HMBC spectrum (Figure 9). Thus, HMBC correlation confirms the complete structure of pure compound as described below.

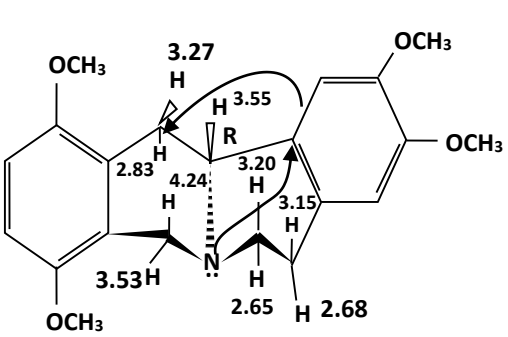


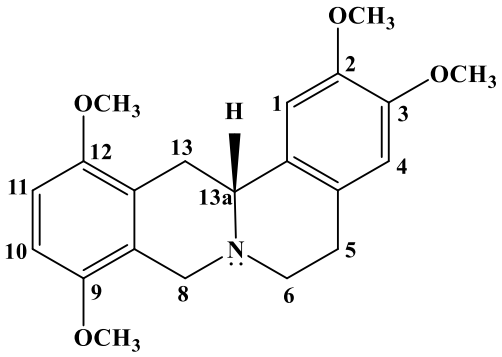
Fragment (G), $C_{13}H_{17}O_2N$



Complete Structure of Pure Compound ($C_{21}H_{25}O_4N$)

The above elucidated structure is also agreed with hydrogen deficiency index (10) calculated from its molecular $C_{21}H_{25}NO_4$ compound having four cyclic ring as describe as follows. In accordance with NOESY spectrum and model analysis (Figure 10), the relative conformation of chiral carbon can be assigned as 'R' configuration.





The IUPAC name of palmatine compound is (*R*)- 2, 3, 9, 12- tetramethoxy- 5, 8, 13, 13a-tetrahydro-*6H*- isoquinolino [2,1- b] isoquinoline.

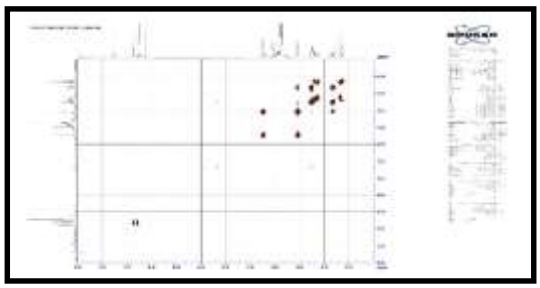


Figure 8 DQF COSY (600MHz) Spectrum of Pure Compound

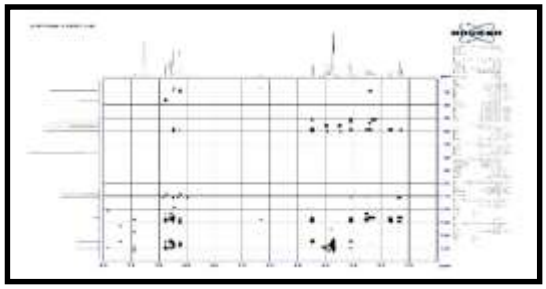


Figure 9 HMBC Spectrum of Pure Compound

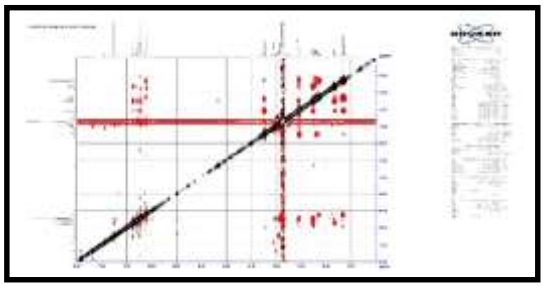


Figure10 NOESY (600MHz) Spectrum of Pure Compound

Antimicrobial Activity of Palmatine Compound

In this study, the palmatine compound preparing with ethyl acetate solvent was tested against microorganisms. The antimicrobial activity of palmatine compound showed medium inhibition zone (mm) on all tested organisms (Table 4).

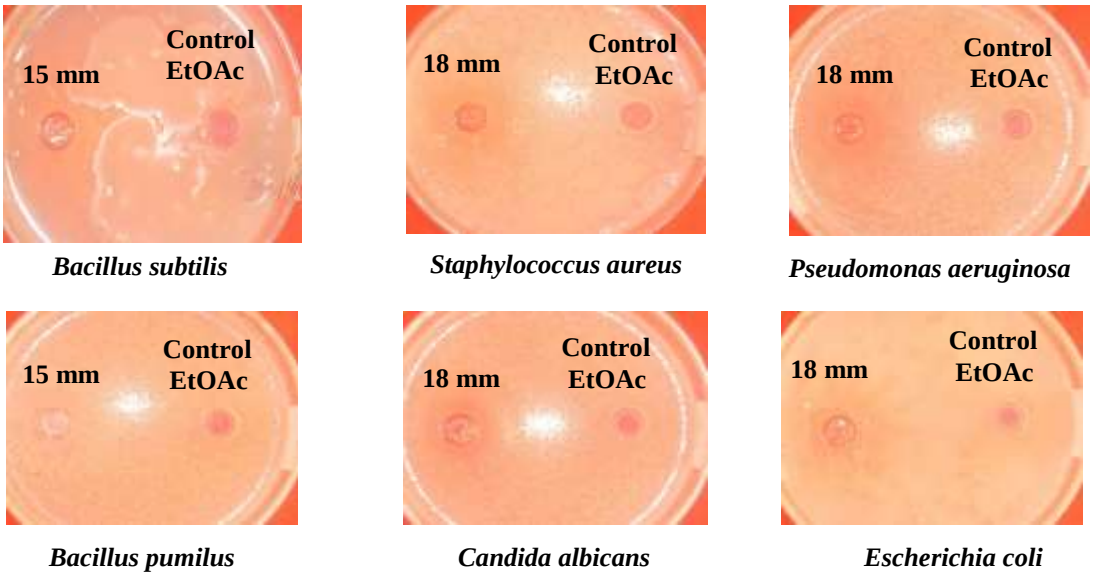
Table 4 Results of Antimicrobial Activity of Palmatine Compound

Compound	Inhibition Zone (mm)					
	A	B	C	D	E	F
Palmatine	15 (++)	18 (++)	18 (++)	15 (++)	18 (++)	18 (++)

Organisms:

A = *Bacillus subtilis* (NCTC 8236)
B = *Staphylococcus aureus* (NCPC 6371)
C = *Pseudomonas aeruginosa* (6749)
D = *Bacillus pumilus* (NCIB 8982)
E = *Candida albicans*
F = *E.coli* (NCIB 8134)

Inhibition zone diameter of standard Agar Well – 10 mm
(++) Inhibition zone diameter ranging 15mm ~ 19 mm (medium activity)



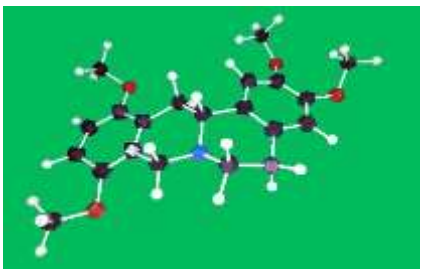
Bacillus subtilis ***Staphylococcus aureus*** ***Pseudomonas aeruginosa***

Bacillus pumilus ***Candida albicans*** ***Escherichia coli***

Figure 11 Antimicrobial Activity of Palmatine Compound by Agar Disc Diffusion Method

Conclusion

A separation method, column chromatography was applied to isolate the palmatine compound depending on different solvent ratios from *S. glabra* tuber. This paper reported the extraction, isolation, structure elucidation, and antimicrobial activity of isoquinoline alkaloid class; an unidentified palmatine, $C_{21}H_{25}NO_4$ compound (named as glabrine). The structure of this compound was done by interpretation of the 1D and 2D NMR and DART MS spectra. Palmatine compound has a wide spectrum of pharmacological effects, including anti-cancer, anti-oxidation, anti-inflammatory, neuroprotection, anti-bacterial, anti-viral and regulating blood lipids. However, palmatine has obvious DNA toxicity, and has a complex effect on metabolic enzymes in the liver (Long *et al* 2019). The present results support that the bioactive palmatine compound isolated from the tuber of *S. glabra* has potent activity against the tested microorganisms. According to the available knowledge about *S. glabra* containing over 30 alkaloids, it has a great potential source of natural health products for pharmaceutical studies.



(R)- 2, 3, 9, 12- tetramethoxy- 5, 8, 13, 13a- tetrahydro- 6H- isoquinolino [2,1- b] isoquinoline

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