ISOLATION AND STRUCTURAL ELUCIDATION OF PURE ORGANIC COMPOUND ISOLATED FROM THE ROOT AND STEM OF *VANDA COERULEA* GRIFF.

Nwet Nwet Win[1], Myat Lay Nwe[2],Thida Win[3]

Abstract

In this research work, *Vanda coerulea* Griff. (local name: Vanda Thit-Kwa) was selected for the chemical analysis. The root and stem of this plant contains alkaloids, glycosides, flavonoids, polyphenols, sugars, lipophilic, terpene, saponins and phenolic compounds respectively. The antimicrobial activities of the plant extracts and the pure isolated organic compound were tested by agar well diffusion method using six organisms. The pure organic compound (NNW-1) was isolated from the root and stem of Vanda Thit-Kwa by thin layer and column chromatographic separation techniques. The pure organic compound was obtained as colourless amorphous. The melting point of this compound was found to be 180-182 °C. The yields percent of pure compound is 1.408 % (35.2 mg) based upon the EtOAc crude extract. The molecular formula was done by using FT IR, ^{1}HNMR, ^{13}CNMR, DEPT, HMQC spectroscopy and Mass spectrometry and its formula is $C_{24}H_{28}O_5$ and hydrogen deficiency index is 11. In addition, the complete structure of the organic compound (NNW-1) was elucidated by applying 1D and 2D NMR spectroscopy as well as EI-Mass spectrometry. The name of the isolated compound is 3-[(5-(3-hydroxypropyl)-2–propenyl-phenyl]-2-(4-methoxy-phenoxymethyl) acrylic acid methyl ester.

Keywords: *Vanda coerulea* Griff., Vanda Thit-Kwa, chromatographic separation techniques, antimicrobial activities

Introduction

Plants have played an important role in traditional medicine in Myanmar since ancient times. Some plants are used as diet. Nowadays, plants have been used as medicines all over the world and medicinal plants are great economic importance for their medicinal values.

Orchid family is the second largest family of flowering plants with approximately 20,000 species with more than 850 genera. *Vanda coerulea* Griffis one of the important plants that can be used as antioxidant biomarker, anti-pyretic, anticonsumption and antidiarrheal effects, and for the treatment of alcoholic gastritis. A wide range of chemical compounds are presented including alkaloids, bibenzyl derivatives, flavonoid, phenanthrene and terpenoid which have been isolated recently from this species.

In this research work, the isolation of pure organic compound (NNW-1) from the root and stem of Vanda-Thit-Kwa (Figure 1) was done by using column and thin layer chromatography. The complete structure of the isolated compound (NNW-1) could be assigned by using modern sophisticated methods such as ^{1}HNMR, ^{13}CNMR, DEPT, DQF-COSY, HMQC and HMBC respectively.

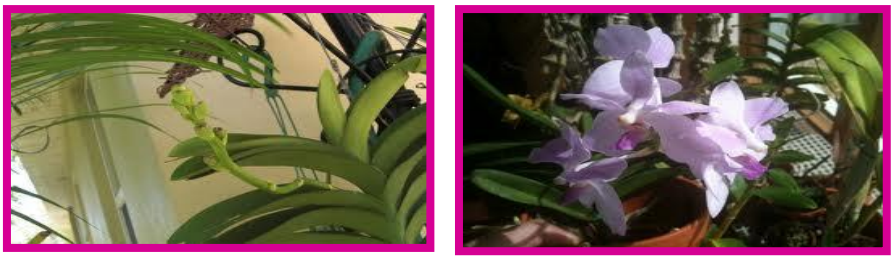

Figure 1 The Whole Plant of Vanda Thit-Kwa

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Materials and Methods

The advanced instruments used in the characterization of samples and elucidation of pure compounds were UV lamp, Lambda - 40 Perkin-Elmer Co. England, FT IR spectrometer (Shimadsu, Japan), NMR spectrometer (500 MHz for 1H, 125 MHz for ^{13}C), EI-Mass spectrometer (JEOL, Japan), Melting Point Apparatus, SMP30 and UV spectrometer (Perkin Elmer (Lambda 25) UV/VIS spectrometer). Commercial grade reagents and solvents were used without further purification. Analytical thin layer Chromatography was performed by using precoated silica gel (Merck. Co. Inc, Kiesel gel 60 F256).

Sample Collection

In this research work, the sample, the root and stem of Vanda Thit-Kwa were collected from Loikaw Township, Kayah State. The root and stem were cut into small pieces and allowed to dry in good ventilation place. The dried sample was stored in stoppered bottle and used throughout the experiment.

Preliminary Phytochemical Test on Sample

Preliminary detection of phytochemical compounds present in plant was carried out according to the general methods mentioned in phytochemical methods (Harborne, 1993). Preliminary phytochemical analysis was performed in order to know different types of chemical constituents present in the plant sample.

Examination of Antimicrobial Activity of Plant Extracts

For the examination of antimicrobial activity of extracts from the root and stem of Vanda Thit-Kwa *in-vitro*, agar well diffusion method was used because of its simplicity, speed of performance and economy (Finegold, 1978).

Extraction and Isolation of Pure Compound (NNW-1)

450 g of air dried sample was percolated in 3 L of ethanol for two months. The ethanol extract was filtered and dried at room temperature. The ethanol crude extract was again extracted with ethyl acetate and concentrated under normal condition. The ethyl acetate crude sample (2.5 g) was obtained. It was separated by using column chromatography. Silica gel (70-230 mesh) was used as adsorbent and n-hexane: ethyl acetate mixture was used as eluent with various solvent ratios from non-polar to polar. Totally (65) fractions were obtained. Each and every fraction was checked on TLC using iodine as visualizing agent. The fractions with same R_f values were combined and nine combined fractions were obtained. Among them, fraction (IX) was rechromatographed by using same adsorbent and same eluent as mentioned in the previous column. Pure colourless amorphous was obtained and checked on TLC for purity. It gave one spot on TLC (R_f= 0.5) with n-hexane: ethyl acetate (1:1) (v/v). The weight of the isolated compound (NNW-1) was 35.2 mg and its yield percent was found to be (1.408 %) based on the ethyl acetate crude extract.

Determination of Melting Point and Phytochemical Screening of Pure Compound (NNW-1)

A few of pure organic compound (NNW-1) was inserted into the capillary tube and the melting point was determined by the aid of the electric melting point apparatus at Department of Chemistry, Monywa University. The phytochemical tests were carried out to identify the class of pure compound (NNW-1).

Examination of Antimicrobial Activity of Pure Organic Compound

Antimicrobial activity of pure compound (NNW-1) was tested by agar well diffusion method at Pharmaceutical Research Department (PRD).

Identification of the Isolated Compound (NNW-1)

Thin Layer Chromatography

Thin layer Chromatography (TLC) was conducted on 0.25 mm precoated silica gel (60 F254 Merck). It was cut into small plates (1×5 cm in size). The chromatogram was developed in the specified solvent systems for pure compound (NNW-1) (Stable, 1965).

Identification of the Pure Isolated Compound (NNW-1) by Fourier Transform Infrared (FT IR) Spectroscopic Study

The infrared spectrum of isolated compound was recorded by using Shimadzu Fourier Transform Infrared Spectrophotometer, at the Department of Chemistry, University of Mandalay. The resulted IR spectrum was applied for the identification of functional groups of the pure organic compound (NNW-1).

Identification of the Pure Isolated Compound (NNW-1) by Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopic Study

The ^{1}H NMR spectrum of isolated compound was recorded by means of a 500 MHz spectrometer at Meijo University, Tempaku, Nagoya, Japan. The spectrum was recorded for the DMSO-d6 solution of sample. By means of ^{1}HNMR spectrum, the number of protons of the pure compound could be estimated.

Identification of the Pure Isolated Compound (NNW-1) by Carbon Nuclear Magnetic Resonance (^{13}C NMR) Spectroscopic Study

The ^{13}C NMR spectrum of isolated compound was recorded by using a 500 MHz spectrometer (125 MHz for ^{13}C) at Meijo University, Tempaku, Nagoya, Japan. The spectrum was recorded for the DMSO-d6 solution of sample. According to ^{13}C NMR spectrum, the number of carbons present in the pure compound (NNW-1) could be determined.

Identification of the Pure Isolated Compound (NNW-1) by Distortionless Enhancement by Polarization Transfer (DEPT) Spectroscopic Study

The DEPT spectrum of isolated compound was recorded by using a 500 MHz spectrometer (125 MHz for ^{13}C) at Meijo University, Tempaku, Nagoya, Japan. The spectrum was recorded for the DMSO-d6 solution of sample. This spectrum showed the presence of quaternary carbons, methane carbons, methylene carbons and methyl carbons.

Identification of the Pure Isolated Compound (NNW-1) by Electron Impact Mass Spectrometry (EI-MS) Study

Electron impact ionization mass spectrum (EI - mass) of the isolated compound was recorded by using mass spectrometer (JEOL model, Japan) at Meijo University, Tempaku, Nagoya, Japan. The molecular mass of the pure compound (NNW-1) could be determined by using EI mass spectral data.

Results and Discussion

Preliminary Phytochemical Tests for the Root and Stem of Vanda Thit-Kwa

The results of the phytochemical tests for the root and stem of Vanda Thit-Kwa are shown in Table 1.

According to the results shown in Table 1, the root and stem of Vanda-Thit-kwa contains chemical constituents tested except steroid.

Table 1 Results of Preliminary Phytochemical Test on Vanda Thit-Kwa

No.	Tests	Reagents	Observation	Results
1.	Alkaloid	Dragendorff's reagent	Orange ppt	+
		Wagner's reagent	Reddish brown ppt	+
2.	Flavonoid	conc: HCl, Mg tunning	Red colour solution	+
3.	Terpene	Acetic anhydride , conc: H_2SO_4, $CHCl_3$	Pink colour solution	+
4.	Steroid	Acetic anhydride, conc: H_2SO_4	No reaction	-
5.	Glycoside	10 % lead acetate	Yellow ppt	+
6.	Reducing Sugar	Benedict solution	Red ppt	+
7.	Polyphenol	10 % $FeCl_3$	Green Blue colour solution	+
8.	Saponin	Distilled water	Frothing	+
9.	Lipophilic	0.5N KOH+NaOH	Deep colour solution	+

(+) = Presence of constituents (-) = Absence of constituents

Antimicrobial Activities of the Root and Stem of Vanda-Thit-kwa

From Table 2, the ethyl acetate crude extract of the root and stem of VandaThit-kwa responds highest activities on selected microorganisms such as *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli*. The n-hexane exctract shows no activity on all selected microorganisms. Hence, the ethyl acetate crude extract was selected for the isolation of pure organic compound. The ethyl acetate extract of the pure compound responds medium activities on selected microorganisms except *Bacillus subtilis*.

Table 2 Antimicrobial Activities of the Extracts of Vanda Thit-kwa and the Pure Compound (NNW-1)

Organisms	Inhibition Zone (mm)			
	Vanda Thit-kwa			Pure Compound
	***n*-hexane**	**EtOAc**	**EtOH**	**EtOAc**
Bacillus subtilis	-	-	-	-
Staphylococcus aureus	-	23 (+++)	18 (++)	15 (++)
Pseudomonas aeruginosa	-	23 (+++)	18 (++)	15 (++)
Bacillus pumilus	-	25 (+++)	19 (++)	-
Candida albicans	-	20 (+++)	17 (++)	-
Escherichia coli	-	25 (+++)	18 (++)	-

Agar well – 10 mm

(+) = Low activity (10 mm ~ 14mm)
(++) = Medium activity (15 mm ~ 19 mm)
(+ + +) = High activity (20 mm above)

Determination of Physicochemical Properties of the Pure Organic Compound (NNW-1)

One of the physical properties, i.e melting point of compound (NNW-1), was determined. The melting point was found to be 180-182 °C.

The FT IR spectrum (Figure 2 a) of the pure organic compound (NNW-1) was measured at the Department of Chemistry, University of Mandalay. The spectral data are described in Table 3.

Table 3 FT IR Assignments of Pure Organic Compound (NNW-1)

No.	Frequency (cm^{-1})	Assignments
1	3390	OH stretching vibration band
2	3045	sp^2 CH stretching vibration band
3	2941, 2887	sp^3 CH asymmetric and symmetric stretching vibration band
4	1722	C=O stretching vibration band
5	1606,1514, 1458	C=C ring skeletal stretching vibration of aromatic hydrocarbon
6	1269, 1155	C-C-O stretching vibration band
7	1118	C-O-C stretching vibration of ether group
8	1035	C-O stretching vibration of primary alcohol
9	977	-OH out of plane bending vibration band

The isolated pure organic compound (NNW-1) contains ether, alcohol, sp^2 hydrocarbon, sp^3 hydrocarbon and carbonyl groups, respectively, by applying the FT IR spectrum.

^{1}H NMR Spectral Data of the Pure Organic Compound (NNW-1)

The ^{1}H NMR spectrum (500 MHz) was described in (Figure 2 b). In accordance with this spectrum, the number of protons could be calculated as (27) in pure organic compound and their chemical shift values were shown in Table 4.

Table 4 ^{1}H NMR Spectral Data of the Pure Compound (NNW-1)

No.	Chemical Shift (ppm)	No. of protons	Proton Assignment
1	1.81	2	sp^3 CH_2
2	2.44	2	sp^3 CH_2
3	2.52	3	sp^3 CH_3
4	3.98	2	sp^3 CH_2
5	3.72	3	sp^3 -OCH_3
6	3.73	3	sp^3 -OCH_3
7	4.98	2	sp^3 CH_2
8	6.15	1	sp^2 CH
9	6.25	1	sp^2 CH
10	6.53	1	sp^2 CH
11	6.61	1	sp^2 CH
12	6.64	1	sp^2 CH
13	6.64	1	sp^2 CH
14	6.66	1	sp^2 CH
15	6.72	1	sp^2 CH
16	6.99	1	sp^2 CH
17	6.99	1	sp^2 CH
	Total	**27**	**$C_{17}H_{27}$**

^{13}C NMR and DEPT Spectral Data of the Compound (NNW-1)

The ^{13}C NMR (125 MHz) spectrum (Figure 2 c) indicated the number of carbons to be (24) in this compound. Also DEPT spectrum (Figure 2 d) gave information of variety of hydrocarbons. In accordance with these spectral data (Table 5), six quatrenary carbons, one carbonyl carbon, ten methine carbons, four methylene carbons and three methyl carbons could be detected.

Table 5 Variety of Carbons from ^{13}CNMR and DEPT Spectrum

No.	Chemical Shift (ppm)	Variety of carbon	No. of carbons	No. of protons
1	30.71	sp^3 CH_2	1	2
2	31.23	sp^3 CH_2	1	2
3	35.45	sp^3 CH_3	1	3
4	55.22	sp^3 -OCH_3	1	3
5	55.79	sp^3 -OCH_3	1	3
6	63.11	sp^3 CH_2	1	2
7	67.4	sp^3 CH_2	1	2
8	109.72	sp^2 CH	1	1
9	113.81	sp^2 CH	1	1
10	114.13	sp^2 CH	1	1
11	114.32	sp^2 CH	1	1
12	115.01	quaternary carbon	1	-
13	115.07	sp^2 CH	1	1
14	115.28	sp^2 CH	1	1

No.	Chemical Shift (ppm)	Variety of carbon	No. of carbons	No. of protons
15	115.28	sp^2 CH	1	1
16	117.28	quaternary carbon	1	-
17	121.19	sp^2 CH	1	1
18	129.0	sp^2 CH	1	1
19	129.0	sp^2 CH	1	1
20	130.49	quaternary carbon	1	-
21	131.8	quaternary carbon	1	-
22	154.9	quaternary carbon	1	-
23	156.2	quaternary carbon	1	-
24	172.0	sp^2 carbonyl carbon	1	-
		Total	**24**	**27**

HMQC Spectrum of the Pure Organic Compound (NNW-1)

The HMQC spectrum of this compound is shown in Figure 2 e. The directly attached proton-carbon coupling could be observed in this spectrum. The chemical shift value of protons and their related carbons were shown in Table 6.

Table 6 ^{1}H-^{13}C Correlation in HMQC Spectrum of the Compound (NNW-1)

No.	Chemical Shift carbon (δppm)	Chemical Shift Proton (δppm)	Assignments
1	30.71	1.81	sp^3 methylene carbon
2	31.23	2.44	sp^3 methylene carbon
3	35.45	2.52	sp^3 methyl carbon
4	55.22	3.72	sp^3 methoxy carbon
5	55.79	3.73	sp^3 methoxy carbon
6	63.11	3.98	sp^3 methylene carbon
7	67.4	4.98	sp^3 methylene carbon
8	109.72	6.25	sp^2 methine carbon
9	113.81	6.72	sp^2 methine carbon
10	114.13	6.15	sp^2 methine carbon
11	114.32	6.53	sp^2 methine carbon
12	115.01	-	quaternary carbon
13	115.07	6.66	sp^2 methine carbon
14	115.28	6.64	sp^2 methine carbon
15	115.28	6.64	sp^2 methine carbon
16	117.28	-	quaternary carbon
17	121.19	6.61	sp^2 methine carbon
18	129.0	6.99	sp^2 methine carbon
19	129.0	6.99	sp^2 methine carbon
20	130.49	-	quaternary carbon
21	131.8	-	quaternary carbon
22	154.9	-	quaternary carbon
23	156.2	-	quaternary carbon
24	172.0	-	carbonyl carbon

According to ^{1}H NMR spectrum (Figure 2 b), there are 27 protons in this compound. There are 24 carbons in the isolated compound according to ^{13}C NMR spectrum (Figure 2 e).

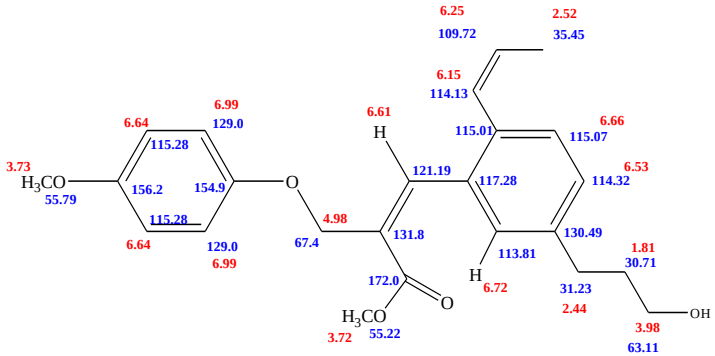
From DEPT spectrum of compound (NNW-1) 6 quaternary carbons, 1 carbonyl carbon, 10 methine carbons, 4 methylene carbons and 3 methyl carbons could be observed. So the partial molecular formula of compound (NNW-1) is $C_{24}H_{27}O$ and partial mass is 331 Da according to DEPT spectrum (Figure 2 d) and HMQC spectrum (Figure 2 e).

According to EI-MS spectrum (Figure 2 f), the molecular mass of pure organic compound (NNW-1) is 396 Da. So the remaining molecular mass is 65 Da. The FT IR spectrum of the isolated compound (Figure 2 a) shows that this compound contains the hydroxyl groups 3390 cm^{-1} and the ether groups 1118 cm^{-1}. The chemical shifts of carbons in the ^{13}C NMR spectrum show that the compound should contain one hydroxyl group and three ether oxygen atoms. So, the molecular formula of the pure isolated organic compound (NNW-1) is $C_{24}H_{28}O_5$.

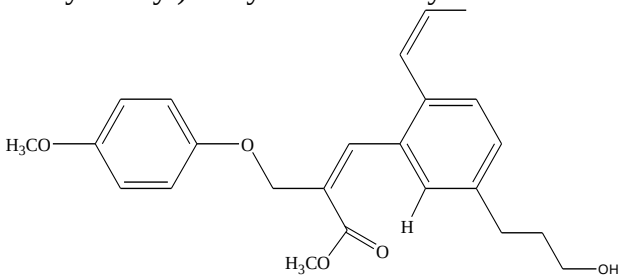
Its hydrogen deficiency index is 11. The calculated molecular mass of the isolated pure organic compound is in agreement with the measured molecular mass of that compound (m/z = 396 Da).

Structural Elucidation of the Compound (NNW-1)

The structure elucidation of a pure bioactive organic compound could be done by applying ^{1}HNMR, splitting patterns and coupling constant (J values) of some prominent protons, FT IR, DEPT, DQF-COSY (Figure 2 g), HMQC, and HMBC (Figure 2 h) spectral data, respectively. The complete structure of the compound NNW-1 with the value of chemical shift of carbons and protons is as follows:



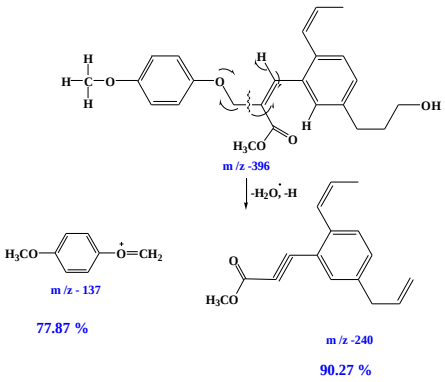
The IUPAC name of the compound NNW-1 is 3-(5- (3-hydroxypropyl)-2-propenyl-phenyl)-2-(4-methoxyphenoxy methyl)- acrylic acid methyl ester.



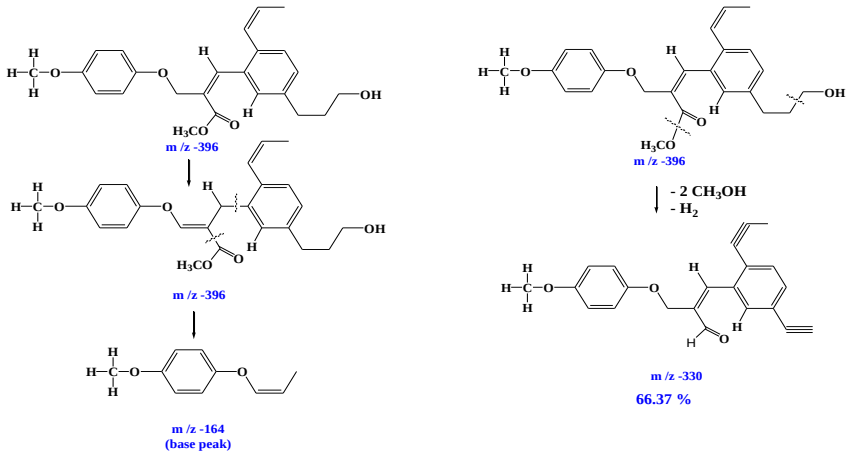
3-[5-(3-Hydroxy-propyl)-2-propenyl-phenyl]-2-(4-methoxy-phenoxymethyl)-acrylic acid methyl ester

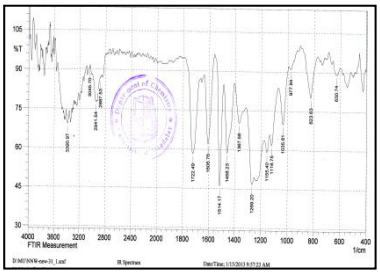
EI -MS Fragmentation Behaviour of the Compound NNW-1

The removal of a water molecule and hydrogen radical from the pure compound NNW-1 produce the two fragment ions peaks at m/z 137 (77.87%) and at m/z 240 (90.27%) . The existence of this two fragments in EI-Mass spectrum is the good evidence for the real structure of the compound NNW-1.



The proposed mechanisms for the formation of the base peak m/z 164 (100%) and the fragment ion peak at m/z 330 (66.37%).





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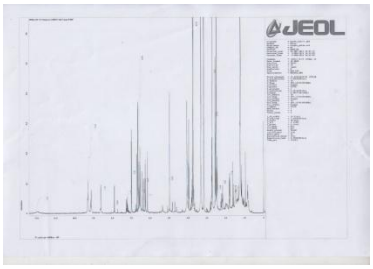

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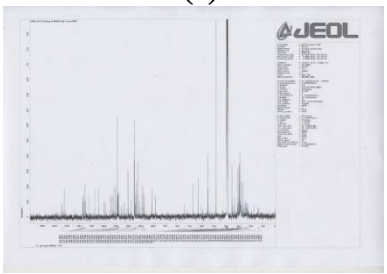

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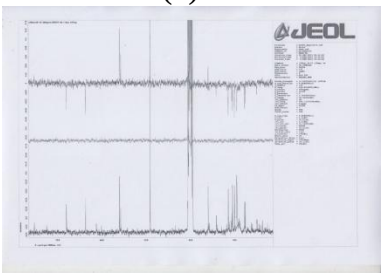

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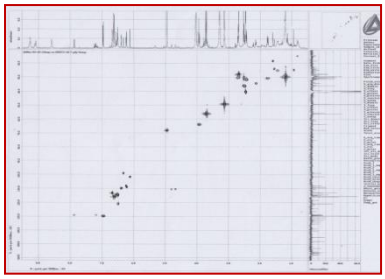

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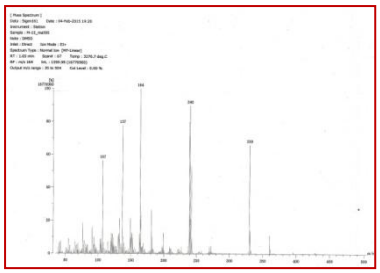

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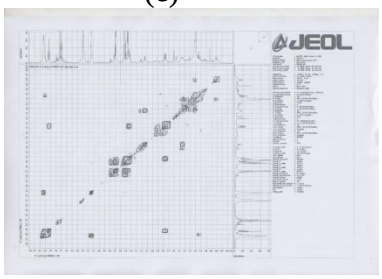

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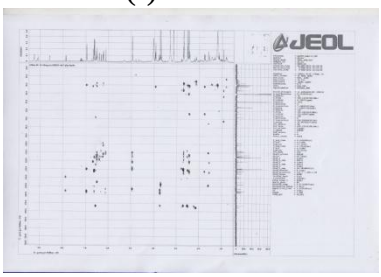


(h)

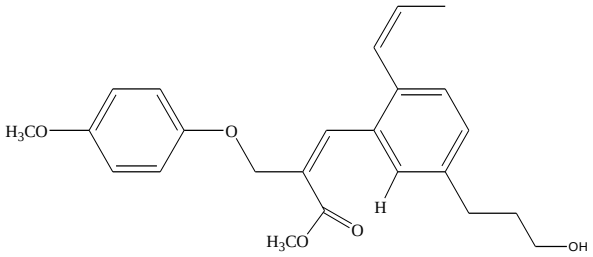
Figure 2 (a) FT IR Spectrum (b) ^{1}H NMR Spectrum (c) ^{13}C NMR Spectrum (d) DEPT Spectrum (e) HMQC Spectrum (f) EI-MS Spectrum (g) DQF-COSY Spectrum (h) HMBC Spectrum of the Pure Organic Compound (NNW-1)

Conclusion

The root and stem of *Vanda coerulea* Griff. was selected to determine the phytochemical constituents, to isolate the pure organic compound and to test the bioactivity of plant extract amd the pure organic compound. The root and stem of this plant contains alkaloids, glycosides, flavonoids, polyphenols, sugars, lipophilic,terpene, saponins and phenolic compounds respectively.

The pure organic compound (NNW-1) was isolated from the root and stem of Vanda Thit-Kwa by thin layer and column chromatographic separation techniques. The pure organic compound was obtained as amorphous. The melting point of this compound was found to be 180-182 °C. The yields percent of pure compound was 1.408 % (35.2 mg) based upon the EtOAc crude extract. The antimicrobial activities of the plant extracts and the pure isolated organic compound were tested by agar well diffusion method using six organisms. Although the EtOAc extract of plant was shown to possess high activity, the pure compound (NNW-1) was low activity on all selected organisms by agar well diffusion method.

The FT IR spectrum indicated that hydroxyl group (3390 cm^{-1}), the carbonyl group (1722 cm^{-1}), the ether group (1118 cm^{-1}) were presented in this compound. The mass spectroscopy displayed (M^{+}) at m/z 396 (corresponding to $C_{24}H_{28}O_5$) and significant peak at m/z 164 showed from the cleavage of the double bond exchange to more stable compound. The structure of compound NNW-1 could be confirmed by the combination these two fragments at m/z 137 and at m/z 240. The molecular formula determination was done by using FT IR, ^{1}HNMR, ^{13}CNMR, DEPT, HMQC spectroscopy and Mass spectrometry and its formula is $C_{24}H_{28}O_5$ and hydrogen deficiency index is 11. In addition, the complete structure of the organic compound (NNW-1) was elucidated by applying 1D and 2D NMR spectroscopy as well as EI-Mass spectrometry. The name of the isolated compoundis 3- (5- (3- hydroxypropyl)- 2- propenyl- phenyl)- 2- (4-methoxy phenoxy methyl)-acrylic acid methyl ester.



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