ISOLATION AND STRUCTURE ELUCIDATION OF PHENYLNAPHTHALENE DERIVATIVE FROM TUBEROUS ROOTS OF *ORTHOSIPHON RUBICUNDUS* (D.DON) BENTH. AND STUDY ON ITS ANTIOXIDANT PROPERTY

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Abstract

The present study was conducted to investigate bioactive chemical constituent from Myanmar medicinal plant, *Orthosiphon rubicundus* (D.Don) Benth. Firstly, phytochemical screening of the selected medicinal plant was performed according to standard procedure. Then, the antimicrobial assay of various extracts was performed on six test microorganisms. Phenylnaphthalene derivative namely, 1-(4-hydroxy-3,5-dimethoxyphenyl)-2,3-dimethyl-5,6,7-trimethoxynaphthalene (1) was isolated from ethyl acetate extract by using separation techniques such as thin layer and column chromatographic methods. Moreover, the antioxidant activities of crude extract and isolated compound were evaluated using DPPH radical scavenging assay. Furthermore, structure elucidation of isolated compound was performed by spectroscopic techniques, particularly by 1D and 2D NMR spectroscopy.

Keywords: DPPH, phenylnaphthalene, spectroscopic

Introduction

Herbal therapies have played a vital role in the progress of human culture. Medicinal plants are resources of traditional medicines and many of the modern medicines are formed indirectly from plant life. According to WHO, about 80 percent of the world's population rely on traditional medicine and millions of people in the vast rural areas of developing countries use herbal medicines for their health care needs. Meanwhile, consumers in developed countries are becoming disillusioned with modern health care and are seeking alternatives (Hosseinzadeh *et al*., 2015). Although modern medicine may exist side-by-side with such traditional practice, herbal medicines have retained their popularity for historical and cultural reasons (Vishwakarma *et al*., 2013).

The *Orthosiphon* species have widely used in traditional medicines to cure various diseases such as diabetes, kidney stone, edema, rheumatism, hepatitis, hypertensive and jaundice. According to phytochemical investigation, *Orthosiphon* species contain phytoconstituents such as monoterpenes, diterpenes, triterpenes, saponins, organic acid and flavonoid compounds. Antidiabetic, anti-inflammatory, antioxidant, hepatoprotective, analgesic and nephroprotective activities have been reported in the plant extract and phytoconstituents of the *Orthosiphon* genus (Singh *et al*., 2015).

Also in Myanmar, there are many reputed traditional plants in pharmacology. Therefore, the study of traditional indigenous medicinal plants and their usages in therapy play a very important role. In the present work, one Myanmar medicinal plant, *Orthosiphon rubicundus* was selected due to its numerous medicinal properties. It is locally known as Nar-ga-ma. According to personal communication with traditional medicine practitioners, it is used to treat various types of cancer, venom, impetigo and eczema (interviewed with medicinal practitioner, Yesagyo, 2019).

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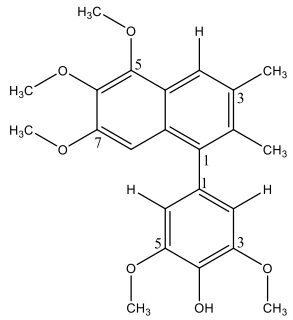


Figure 1 Structure of isolated compound (1)

Botanical Classification

Figure 2 Roots, plants and flowers of *Orthosiphon rubicundus*

Botanical name	-	*Orthosiphon rubicundus* (D.Don) Benth.
Local name	-	Nar-ga-ma
Family name	-	Lamiaceae
Common name	-	Red Java Tea
Part used	-	Tuberous root

Materials and Methods

General Experimental Procedures

^{1}H NMR spectra: Varian Unity 300 (300.542 MHz), Bruker AMX 300 (300.542 MHz), Varian Inova 500 (499.8 MHz). –^{13}C NMR spectra: Varian Unity 300 (75.5 MHz), Varian Inova 500 (125.7 MHz). Chemical shifts were measured relatively to tetramethylsilane as internal standard. - 2D NMR spectra: H, H COSY spectra (^{1}H,^{1}H-Correlated Spectroscopy), HMBC spectra (Heteronuclear Multiple Bond Connectivity) and HMQC spectra (Heteronuclear Multiple Quantum Coherence). Thin layer chromatography (TLC): DC-Folien Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co.). - Column chromatography (CC): MN silica gel 60: 0.05-0.2 mm, 70-270 mesh (Macherey-Nagel & Co). Sephadex LH-20 (Pharmacia) was used for size exclusion chromatography. Commercial grade reagents and solvents were purchased from Super Shell Co. Ltd, Yangon. Common laboratory apparatus were used. PerkinElmer C93927 was used for FT-IR spectra measurement. The antimicrobial activities of plant extracts were measured in Pharmaceutical Research Department, Insein, Yangon. The antioxidant activity of crude extract and pure compound was analyzed by DPPH radical scavenging method at the Department of Chemistry, Kyaukse University.

Plant Material

The tuberous roots of *Orthosiphon rubicundus* were collected from Shinmadaung, Yesagyo Township, Magwe Region and identified by Dr Khin Myo San, Associate Professor, Department of Botany, Yadanabon University. The root materials were cut into small pieces and dried at room temperature for about two weeks.

Preliminary Phytochemical Analysis

The preliminary phytochemical screening of tuberous roots of *Orthosiphon rubicundus* was determined according to the procedure of Harborne.

Antimicrobial Assays

The antimicrobial activities of crude extracts were tested by agar-well diffusion method on six microorganisms such as *Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus, Candida albicans* and *Escherichia coli*.

Determination of Antioxidant Activities

The antioxidant activities of the methanolic extract and pure compound were determined by DPPH radical scavenging assay. The control solution was prepared by mixing 2 mL of 24 μg/mL DPPH solution and 2 mL of 95% methanol with vortex mixer. Similarly, the blank solution was prepared by mixing 2 mL of test solution and 2 mL of 95% methanol with vortex mixer. The test sample solution was also prepared by mixing 2 mL of 24 μg/mL DPPH solution and 2 mL sample solution in various concentrations (11.25, 22.5, 45, 90, 180 μg/mL for methanolic extract and 25, 50, 100, 200, 400 μg/mL for compound 1). The resulting mixtures were thoroughly homogenized by using vortex mixer. The solutions were allowed to stand at room temperature for 30 mins. After 30 mins, measurements of absorbance at 517 nm were made for these solutions using UV-Vis spectrophotometer. The absorbance values obtained were applied to calculate percent inhibition by % Inhibition = $[A_{control}\text{-}A_{sample}/A_{control}]$ x 100, where, % inhibition = percent inhibition of test sample, $A_{control}$ = absorbance of control (DPPH) solution and A_{sample} = absorbance of test sample solution. The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using inhibition curve.

Extraction and Isolation of Pure Compound

The air dried samples (1000 g) were percolated with methanol for one month. The methanol crude extracts were filtered and evaporated the solvent. The residue was extracted with ethyl acetate to attain 7 g of ethyl acetate crude extracts. The obtained crude extracts were subjected to silica gel by using stepwise gradients of n-hexane and ethyl acetate. 1 (4-hydroxy-3,5-dimethoxyphenyl) -2,3-dimethyl-5,6,7-trimethoxy naphthalene (1) was isolated as white amorphous from selected combined fraction IV. It showed UV absorbing bands at 254 nm.

Results and Discussion

Phytochemical Analysis

According to preliminary phytochemical test, the crude extracts of *Orthosiphon rubicundus* contained alkaloids, flavonoids, glycosides, polyphenols, steroids, tannins, saponins and reducing sugars respectively.

Antimicrobial Activities

The antimicrobial activities of the roots of *Orthosiphon rubicundus* were tested in various solvent systems by using agar-well diffusion method. These results were shown in Table 1.

Table 1 Antimicrobial Activities of the Roots of *Orthosiphon rubicundus*

Sample	Solvent	Inhibition zone (mm)					
		I	II	III	IV	V	VI
Orthosiphon rubicundus	n-hexane	14	12	-	-	15	15
	MeOH	21	25	25	21	13	22
	EtOAc	15	25	20	-	24	25

Agar well – 8 mm
8 mm ~ 12 mm (+)
13 mm ~ 17 mm (++)
18 mm above (+++)

I = *Bacillus subtilis*
II = *Staphylococcus aureus*
III = *Pseudomonas aeruginosa*
IV = *Bacillus pumilus*
V = *Candida albicans*
VI = *Escherichia coli*

According to antimicrobial tests, the n-hexane extracts responded low activities on *Staphylococcus aureus* and medium activities on *Bacillus subtilis*, *Candida albicans* and *Escherichia coli*. The methanol extracts showed strong activities on all test microorganisms except *Candida albicans*. The ethyl acetate extracts exhibited medium activities on *Bacillus subtilis* and strong activities against other four test organisms except *Bacillus pumilus*.

Determination of Antioxidant Activities of Methanolic Extract and Isolated Compound

The radical scavenging activities of methanolic extract and pure compound (1) were determined by DPPH assay method. The methanolic extract and compound (1) showed the antioxidant activity on DPPH with IC_{50} values of 47.90 µg/ mL and 59.02 µg/mL respectively meanwhile IC_{50} values of standard ascorbic acid was 4.05 µg/mL.

Table 2 % Inhibition of Standard Ascorbic Acid in Different Concentrations and IC_{50} Value

Sample	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Standard Ascorbic Acid	1	7.322	4.05
	2	15.27	
	4	40.585	
	8	82.008	
	16	93.93	

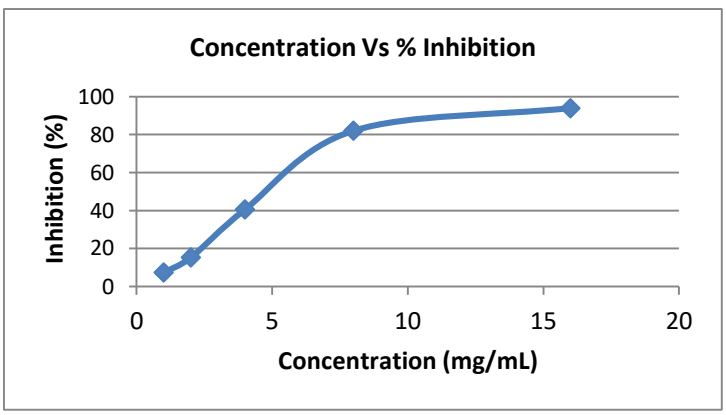


Figure 3 Inhibition percentage of standard ascorbic acid in different concentrations

Table 3 % Inhibition of Methanolic Extract in Different Concentrations and IC_{50} Value

Sample	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Methanolic Extract	11.25	25.96	47.90
	22.5	30.7	
	45	47.63	
	90	78.1	
	180	95.26	

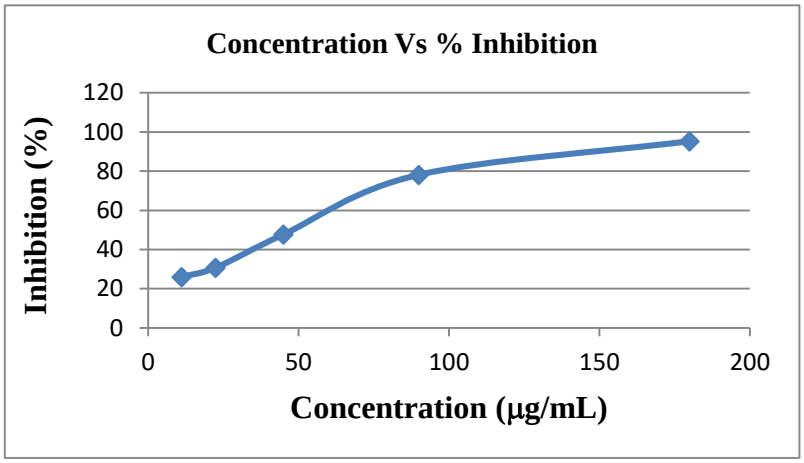


Figure 4 Inhibition percentage of methanolic extract in different concentrations

Table 4 % Inhibition of Compound 1 in Different Concentrations and IC_{50} Value

Sample	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
Pure Compound (1)	25	29.25	59.02
	50	47.46	
	100	59.26	
	200	77.61	
	400	78.81	

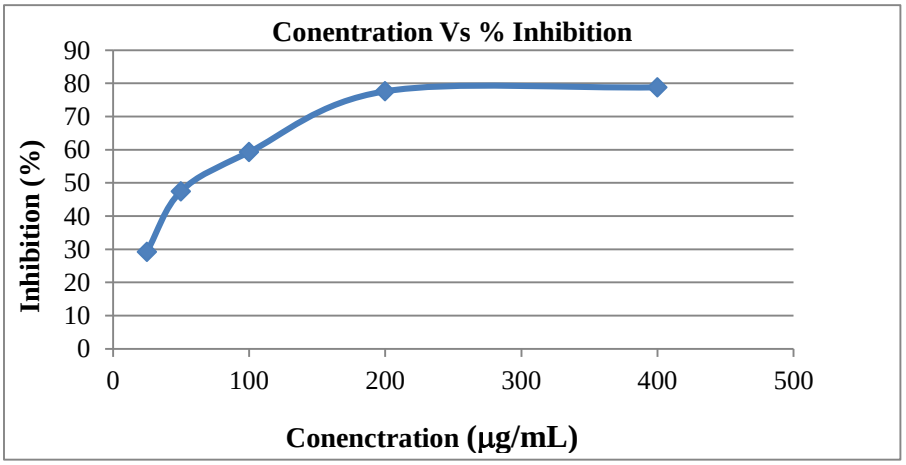


Figure 5 Inhibition percentage of pure compound 1 in different concentrations

Table 5 IC_{50} Values of Standard Ascorbic acid, Methanolic Extract and Compound 1

No.	Sample	IC_{50} (µg/mL)
1	Ascorbic acid	4.05
2	Methanolic Extract	47.90
3	Pure compound 1	59.02

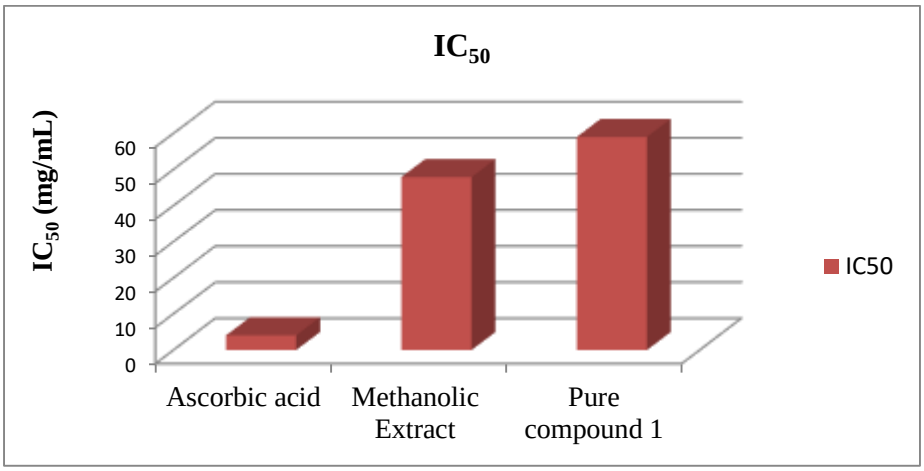


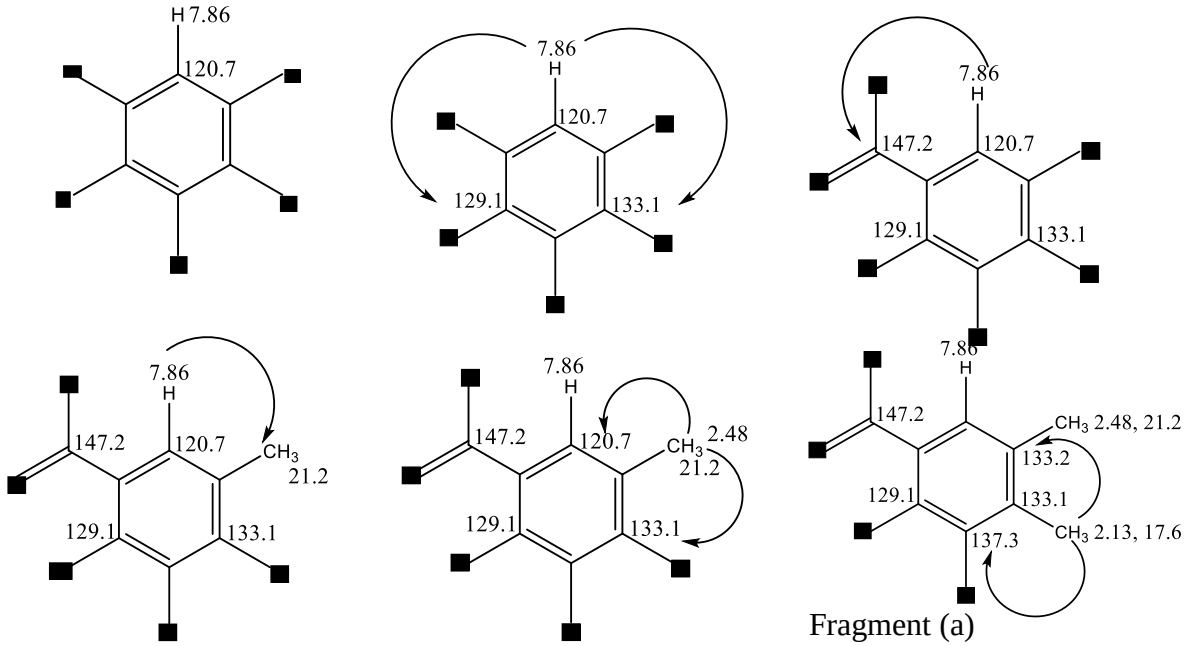
Figure 6 Comparison of IC_{50} values of standard ascorbic acid, methanolic extracts and compound 1

Structure Elucidation

The structure elucidation of compound 1 was determined by spectroscopic methods such as ^{1}H NMR, ^{13}C NMR, DEPT, DQF-COSY, HSQC and HMBC respectively. According to ^{1}H NMR spectrum (Figure 7.1), compound 1 contained 25 protons. By the analysis of ^{13}C NMR (Figure 7.2), together with DEPT (Figure 7.3), total of 23 carbon signals were detected which comprised of twelve sp^2 quaternary carbons at δ 122.7, 129.1, 131.8, 133.1, 133.2, 133.5, 137.3, 140.1, 147.2 (three quaternary carbons), 151.9 ppm, four sp^2 methine carbons at δ 101.6, 106.7 (two equivalent methine carbons), 120.7 ppm, two methyl carbons at δ 17.6, 21.2 and five methoxy carbons at δ 55.7, 56.4 (two methoxy carbons), 61.1 and 61.4 ppm.

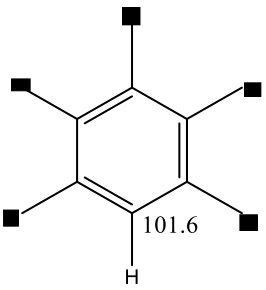
In the downfield aromatic region of ^{1}H NMR spectrum, (Figure 7.1), a singlet at δ 7.86 ppm was ascribed to one aromatic methine proton. In the HMBC spectrum, (Figure 7.6), the aromatic methine proton at δ 7.86 ppm showed β-correlation with two sp^2 quaternary carbons at δ 133.1 ppm and 129.1 ppm. Moreover, it showed strong correlation with another sp^2 quaternary carbon at δ 147.2 ppm.

In addition, the aromatic methine proton at δ 7.86 ppm showed HMBC strong correlation with one methyl carbon at δ 21.2 ppm. Moreover, the methyl singlet at δ 2.48 ppm which is attached to carbon at δ 21.2 ppm showed strong correlation with one sp^2 methine carbon at δ 120.7 ppm and one sp^2 quaternary carbon at δ 133.1 ppm. Furthermore, the methyl singlet at δ 2.13 which is attached to carbon at δ 17.6 showed β-correlation with two sp^2 quaternary carbons at δ 133.2 ppm and 137.3 ppm. Therefore, fragment (a) could be assigned.

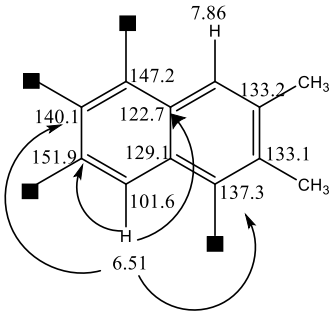


Fragment (a)

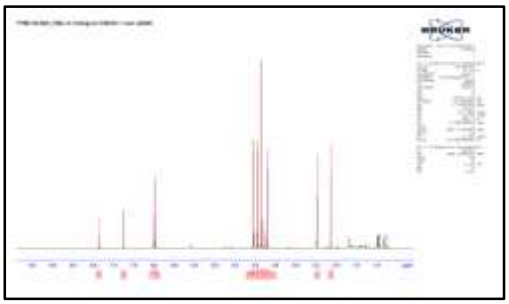
In the aromatic region of ^{1}H NMR spectrum (Figure 7.1), a singlet at δ 6.51 ppm was ascribed to another one aromatic methine proton and fragment (b) could be assigned. In the HMBC spectrum, (Figure 7.6), the singlet methine proton at δ 6.51 ppm which is attached to carbon at δ 101.6 ppm showed β-correlation with three sp^2 quaternary carbons at δ 140.1 ppm, 122.7 ppm and 137.3 ppm from the fragment (a). Furthermore, the methine proton at δ 6.51 ppm also showed weak correlation with one sp^2 quaternary carbon at δ 151.9 ppm. Therefore, fragment (a) and (b) could be connected as shown in fragment (c).



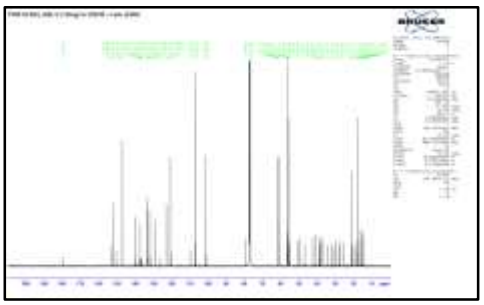
Fragment (b)



Fragment (c)



(7.1)



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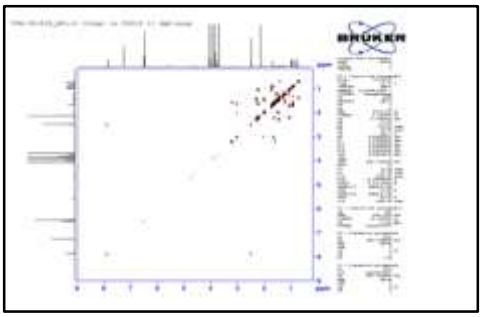

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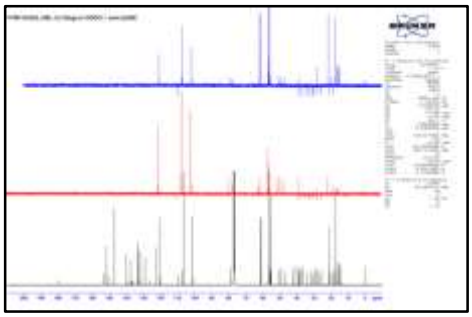

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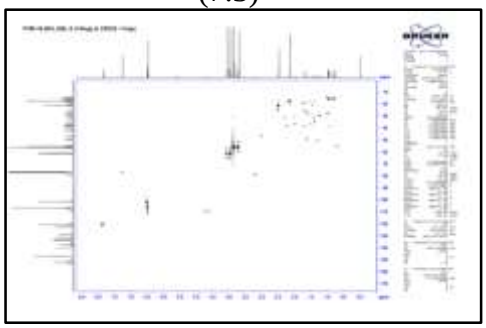

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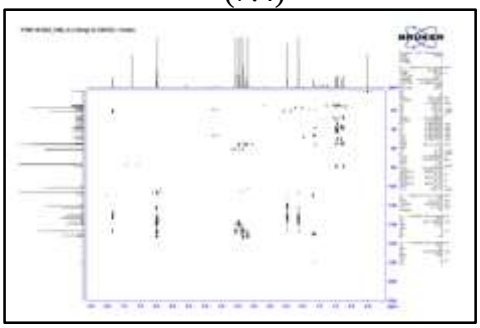

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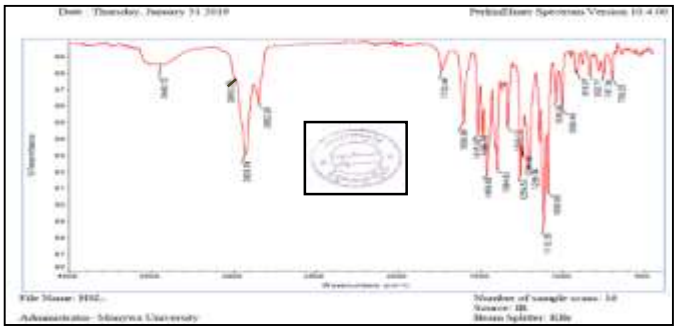
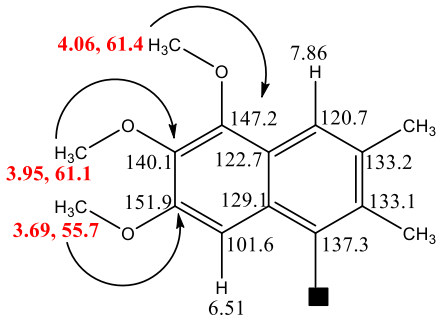


(7.7)

Figure 7 (7.1) ^{1}H NMR, (7.2) ^{13}C NMR, (7.3) DEPT, (7.4) DQF-COSY, (7.5) HSQC, (7.6) HMBC , (7.7) FT-IR of isolated pure compound (1)

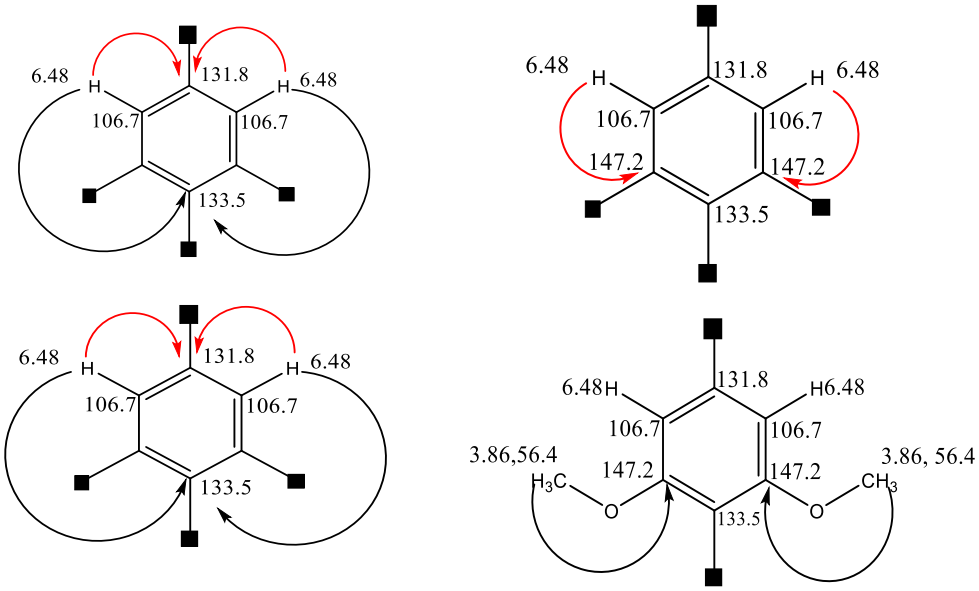
Moreover, the three methoxy signals at δ 4.06 ppm (δ_C 61.4), 3.95 ppm (δ_C 61.1) and 3.69 (δ_C 55.7) showed HMBC cross signals to three sp^2 quaternary carbons at δ 147.2, 140.1 and 151.9 ppm respectively. Therefore, fragment (d) could be assigned.



Fragment (d)

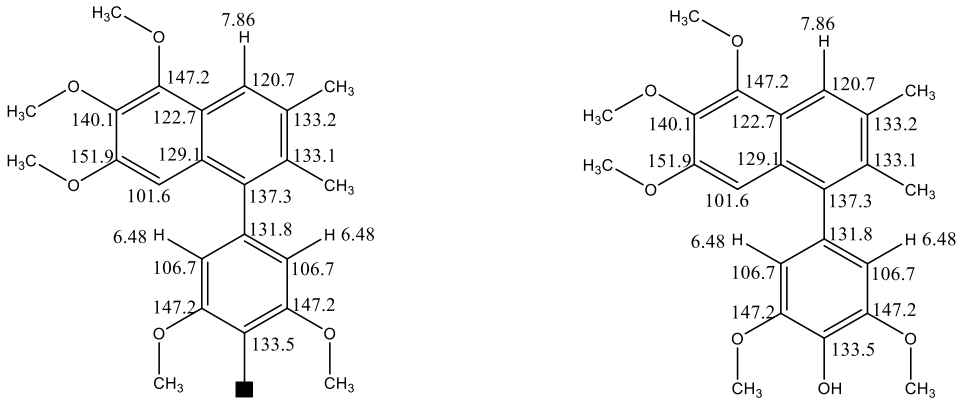
Furthermore, in the up field aromatic region of ^{1}H NMR spectrum (Figure 7.1), two chemical shift equivalent methine protons at δ 6.48 ppm (δ_C 106.7) was ascribed to 1, 2, 3, 5-

tetrasubstituted benzene ring. In the HMBC spectrum (Figure 7.6), these two methine protons showed HMBC cross signals between each other and coupled additionally to sp^2 quaternary carbons at δ 131.8, 147.2 and 133.5 ppm. Therefore, the atoms in the benzene ring could be assigned. Furthermore, two methoxy signals at δ 3.86 ppm which are attached to carbons at δ 56.4 showed β-correlation with two sp^2 quaternary carbons at 147.2 ppm. Therefore, fragment (e) could be assigned.



Fragment (e)

Moreover, the two equivalent methine protons at δ 6.48 ppm showed strong correlation with one sp^2 quaternary carbon at δ 137.3 ppm from fragment (d). So, fragment (e) could be connected to fragment (d) as shown in partial structure. Furthermore, FT-IR spectrum displayed the presence of OH group. Therefore, the complete structure of compound 1 could be elucidated with the molecular formula of $C_{23}H_{26}O_6$ and degree of unsaturation was 11.



Partial structure of compound 1 Complete structure of compound (1)

The isolated compound was assigned as 1 (4-hydroxy-3,5-dimethoxyphenyl) -2,3-dimethyl-5,6,7-trimethoxy naphthalene (1). According to literature survey and search in database such as Chemspider and Reaxys, the isolated compound was a new phenylnaphthalene derivative.

Conclusion

In this research work, one medicinal plant, *Orthosiphon rubicundus* was selected for chemical and biological characterization. From ethyl acetate extract, phenylnaphthalene derivative, 1-(4-hydroxy-3,5-dimethoxyphenyl)-2,3-dimethyl-5,6,7-trimethoxy naphthalene (1) was isolated and characterized by NMR studies. Moreover, the methanolic extract and compound (1) showed the antioxidant activity on DPPH with IC_{50} values of 47.90 µg/ mL and 59.02 µg/mL, respectively but lower than that of ascorbic acid solution, standard antioxidant. The result of the present study suggested that the selected plant can be used as a source of antioxidant and antimicrobial for pharmacological preparations which is very well evidenced by the present work.

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