ACUTE TOXICITY OF THE ETHANOLIC PLANT EXTRACT AND STRUCTURE ELUCIDATION OF PURE ORGANIC COMPOUND FROM THE BARK OF *Cordia wallichii* G.DON. (THANAT-GYI)

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Abstract

In this research work, one Myanmar indigenous medicinal plant, *Cordia wallichii* G.Don. (Thanat-gyi) was selected for chemical analysis. It was collected in Pyin Oo Lwin Township, Mandalay Region in Myanmar. The phytochemical screening of this plant was determined which gave positive tests for flavonoids, terpenes, glycosides, reducing sugars, polyphenols, tannins, saponins, lipophilic and phenolic compounds. The antimicrobial activities of crude extract of this plant were tested in various solvent systems by Agar well diffusion method on six selected microorganisms: *Bacillus subtilis, Staphylococcus aureus, Bacillus pumilus, Pseudomonas aeruginosa, Candida albicans* and *E. coli*. In addition, the acute toxicity test of 95 % ethanolic extract of the bark of *Cordia wallichii* G.Don. was done by Organization of Economic Cooperation and Development (OECD) guideline 425 (2008) on albino mice. The pure organic compound (WH-1) was next isolated from the bark of Thanat-gyi by Thin Layer and Column Chromatographic methods. The pure yellowish brown crystal (0.62 %) was obtained based upon the ethyl acetate crude extract. Furthermore, the molecular formula ($C_{16}H_{12}O_7$), molecular mass (316) and complete structure of this pure compound was determined by FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT, HSQC, DQF-COSY, HMBC and EI-MS spectral data respectively. The IUPAC name of pure organic compound (WH-1) is (E)-4-(4-(2-carboxyvinyl)-2-hydroxyphenoxy)-3-hydroxy benzoic acid.

Keywords :*Cordia wallichii* G.Don., Thanat-gyi, acute toxicity, Chromatographic methods, NMR, EI-MS

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Introduction

Medicinal plants and plant derived medicine are widely used in traditional cultures all over the world and they are becoming increasingly popular in modern society. Plants have the ability to synthesize a wide variety of chemical compounds that are used to perform important biological functions and to defend against attack from predators such as insects, fungi and herbivorous mammals (Lemmens *et al*., 1999). The drugs are derived either from the whole plant or from different organs, like leaves, stem, bark, root, flower and seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex (Merck Index, 1983). Some important chemical intermediates needed for manufacturing the modern drugs are also obtained from plants. Not only, that plant-derived drug offers a stable market world wide, but also plants continue to be an important source for new drugs (Khare, 2007).

In Myanmar, most of the people prefer on traditional medicinal plants and tested medicines rather than modern medicines for the treatment of various human diseases. There is still many medicinal plants that are not well known for their medicinal values in our country. In future, biologically active plant derived chemicals can be expected to play an increasingly significant role in the commercial development of new agrochemicals and pharmaceuticals (Aye Than, 1996).

Before the medicinal plant could be used as medicines, it must be ensured to be safe. The Organization for Economic Cooperation and Development (OECD) defines acute toxicity as "the adverse effects occurring within a short time of (oral) administration of a single dose of a substance or multiple doses given within 24 h. The median lethal dose (LD_{50}) test is used to determine the acute toxicity of a substance. A key stage in ensuring the safety of drugs is to conduct toxicity tests in appropriate animal models. In Myanmar, most of the acute toxicity data comes from animals testing.

Fruits and barks of *Cordia wallichii* G.Don (Thanat-gyi) (Figure 1) were medicinally used in India and Myanmar. The fruits were used as an expectorant, astringent and demulcent. The fruits are also useful in treating coughs, the diseases of the chest and chronic fever (Parmar, and Kaushal., 1982). They remove pain from the joints and the burning of the throat and are

also effective in treating the diseases of the spleen. The bark is used in toothache and to expel bladder and kidney stones (Ah Shin Nagathein, 1967).

In this research, the acute toxicity of 95 % ethanolic extract of the bark of *Cordia wallichii* G.Don. was carried out based on the OECD guideline 425 on albino mice. A pure organic compound (WH-1) was isolated from the bark of *Cordia wallichii* G.Don. by applying Thin Layer and Column Chromatography. The complete structure of pure organic compound (WH-1) could be elucidated by using advanced spectroscopic methods such as FT IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, DQF-COSY, HSQC,

(a) (b) (c) (d)

Figure 1. (a)Plant, (b)fruits,(c) flowers and (d) barks of *Cordia wallichii* G.Don.

Materials and Methods

Sample Collection

The barks of *Cordia wallichii* G.Don. were collected from Pyin Sar Village, Pyin Oo Lwin Township, Mandalay Region. The samples were cut into small pieces and allowed to air dry. Then the dry samples were stored in a well-stoppered bottle and used throughout the experiment.

Study on Acute Toxicity of the Bark of *Cordia wallichii* G.Don.

Method for Acute Toxicity Study

Acute oral toxicity test on 95 % ethanolic extract of the bark of *Cordia wallichii* G.Don. was carried out according to OECD 425 guideline (2008). Therefore, the limit test at 5000 mg/kg was performed. Total number of adult albino mice, weighing (20-30 g) were selected. Since the route of administration selected should be the intended route for administration of the tested drug given to the human during therapy, the oral route was chosen for this test.

Figure 2. Administration of test substance suspension to the mice

Extraction and Isolation of Pure Compound (WH-1) from the Bark of *Cordia wallichii* G.Don.

Air dried sample (950 g) was percolated with 95 % ethanol (3.5 L) for two months. The extracted solution was filtered and evaporated in air. Then it was re-extracted with ethyl acetate (300 mL) and evaporated. The ethyl acetate crude extract (2.03 g) was obtained. Ethyl acetate crude extract was checked by TLC with n-hexane : EtOAc various ratios.

Column size	(1.5 × 40) cm
Adsorbent	SiO_2 (Silica Gel)
Flow rate	0.4 mL/min

The ethyl acetate crude extract (2.03 g) was fractionated by column chromatography over silica gel with various ratios of n-hexane and ethyl acetate from non-polar to polar. Totally (192) fractions were obtained. Each fraction was checked by TLC. The same R_f value fractions were combined and 6 combined fractions were obtained. Major combined fraction (V) gave only one spot on TLC and UV active. The pure yellowish brown crystal (12.6 mg)

was obtained. The yield percent of this pure compound (WH-1) was found to be 0.62 % based upon the ethyl acetate crude extract.

The molecular formula and the structure of this isolated compound were assigned by using advanced spectroscopic methods such as FT IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, DQF-COSY, HSQC, HMBC and EI-MS spectral data.

Results and Discussion

Phytochemical Tests for the Bark of *Cordia wallichii* G.Don.

The phytochemical screenings of the bark of *Cordia wallichii* G.Don. are carried out and these results are shown in Table 1.

Table 1. Phytochemical Test for the Bark of *Cordia wallichii* G.Don.

No.	Constituents	Reagent used	Observation	Results
1.	Alkaloid	Dragendorff's reagent	No orange ppt	–
2.	Flavonoid	EtOH, Mg ribbon, Conc: HCl	Pink colour solution	+
3.	Steroid	Petether, acetic anhydride, $CHCl_3$, Conc: H_2SO_4	No greenish blue colour solution	–
4.	Terpene	EtOH, acetic anhydride, $CHCl_3$, Conc: H_2SO_4	Reddish brown colour solution	+
5.	Glycoside	10 % lead acetate	Yellow ppt	+
6.	Reducing sugar	Benedict's solution	Red ppt	+
7.	Polyphenol	1 % $FeCl_3$, 1 % $K_3[Fe(CN)_6]$	Greenish blue colour solution	+
8.	Tannin	10 % $FeCl_3$, dil H_2SO_4	Yellowish brown ppt	+
9.	Saponin	Distilled H_2O shaken	Frothing	+
10.	Lipophilic	0.5 N KOH solution	Deep yellow colour solution	+
11.	Phenolic	H_2O, Δ, 10 min, 10 % $FeCl_3$	Greenish blue colour solution	+

(+) = presence of constituents, (–) = absence of constituents

According to this table, the bark of *Cordia wallichii* G.Don. contains flavonoid, terpene, glycoside, reducing sugar, polyphenol, tannin, saponin, lipophilic and phenolic compound.

Antimicrobial Activities of the Bark of *Cordia wallichii* G.Don.

The antimicrobial activities of the bark of *Cordia wallichii* G.Don. were tested in various solvent systems by using Agar well diffusion method. The results are tabulated in Table 2.

Table 2. Antimicrobial Activities of Crude Extract of Bark of *Cordia wallichii* G.Don.

Sample	Solvent	Diameter of Inhibition Zone (mm)					
		I	II	III	IV	V	VI
Thanat-gyi	n-hexane	–	–	–	–	–	–
	EtOAc	30 (+++)	50 (+++)	28 (+++)	–	35 (+++)	43 (+++)
	EtOH	–	–	12 (+)	–	30 (+++)	–

Agar Well – 10 mm
(+) ~ 10 mm – 14 mm
(++) ~ 15 mm – 19 mm
(+++) ~ 20 mm above

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

According to this table, the ethyl acetate extract of Thanat-gyi responds high activities on *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *E. coli*. The ethanol extract of Thanat-gyi shows high activity on *Candida albicans* and low activities on *Pseudomonas aeruginosa*.

Result of Acute Toxicity Study for *Cordia wallichii* G.Don.

Acute toxicity test was done by using limit test according to OECD 425 guideline (2008). The test substance was non-toxic at the test dose 5000 mg/kg. There was no lethality of the mices up to 14 days observation period. Therefore, median lethal dose (LD_{50}) of the ethanolic extract of bark of *Cordia wallichii* G.Don. was supposed to be greater than 5000 mg/kg.

Clinical observations

For evaluation of toxicity, no significant changes were observed in toxic parameters. Skin and fur changes, eyes, mucous membrane, respiratory rate, motor activity and behavioral pattern were found to be normal. Salivation, convulsion, cyanosis, tremors and diarrhoea did not occur in all mices. There was no abnormality detected (Table 3).

Table 3. Acute Toxicity Study of the Bark of *Cordia wallichii* G.Don. Based on Mortality Record

Extracts	Groups	No: of mice/ group	Diet	Dose of extract (mg/kg)	Final volume given (mL/kg)	Observed Period (week)	Ratio of dead and tested	Death %
Extract of Bark of *Cordia wallichii* G.Don.	I	3	Stock diet and DW	175	10	2	0/3	0
	II	3	Stock diet and DW	550	10	2	0/3	0
	III	3	Stock diet and DW	2000	10	2	0/3	0
	IV	3	Stock diet and DW	5000	10	2	0/3	0
	Control	3	DW	0	10	2	0/3	0

After two weeks, all the mice were alive and did not show any toxic symptoms such as body weight loss, diarrhoea, inactivity, aggressiveness, restlessness, etc. and no death when compared with that of the control group.

Body Weight

According to this table, four different groups of mices were administered with four different doses. Individual mice was weighed daily thereafter for 14 days. There was no significant change in body weight before and after administration of the test drug (Table 4).

Table 4. Acute Toxicity Study of the Bark of *Cordia wallichii* G.Don. Based on Daily Body Weight Record

Groups	Marking	Sex	Dose in mg/kg Body Weight	Weight of mice (g) 1st day	7th day	14th day
I	Head	Female	175	26.1	27.0	27.8
	Head	Male		18.5	24.5	27.7
	Bark	Male		20.8	26.4	28.9
		Mean value		**21.8**	**26.0**	**28.1**
II	Bark	Female	550	24.3	25.2	25.2
	Tail	Male		21.2	27.0	29.5
	R-Hand	Male		19.8	23.1	27.4
		Mean value		**21.8**	**25.1**	**27.4**
III	Tail	Female	2000	27.4	28.1	28.9
	R-Hand	Female		30.8	28.3	28.8
	L-Hand	Male		26.6	25.7	28.1
		Mean value		**28.3**	**27.4**	**28.6**
IV	Head	Female	5000	24.0	26.7	27.4
	Bark	Female		22.4	24.6	25.2
	Tail	Female		23.0	25.2	24.2
		Mean value		**23.1**	**25.5**	**25.6**
Control	R-Hand	Female	Distilled Water	25.1	25.9	26.4
	R-Leg	Male		20.1	23.6	26.1
	L-Hand and L-Leg	Male		25.8	27.8	28.6
		Mean value		**23.7**	**25.8**	**27.0**

In accordance with the results summarized in Tables 3 and 4 this plant extract can be labeled unclassified in the hazard category according to Globally Harmonized System and can be considered relatively safe.

Molecular Formula Determination of Pure Compound (WH-1)

The molecular formula of the isolated compound (WH-1) was determined by FT IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, HSQC and EI-mass spectral data (Silverstein *et al*., 2005)

FT IR assignment of compound (WH-1)

FT IR spectrum of isolated compound (WH-1) was measured at the Department of Chemistry, University of Mandalay. It is described in Figure 3 a.

According to FT IR spectral data, compound consists of carboxylic acid group, sp^2 hydrocarbon, carbonyl group, aromatic benzene ring, C – C – O stretching vibration of alcohol group, C – O – C stretching vibration of ether group and trans or E and cis or Z alkenic group respectively. The assignments of FT IR spectral data are shown in Table 5.

Table 5. FT IR Assignment of Pure Compound (WH-1)

Frequencies (cm^{-1})	Assignment
3300-2750	O – H stretching vibration of carboxylic acid group
3078.68	= C – H stretching vibration of sp^2 hydrocarbon
1707.06	C = O stretching vibration of carbonyl group
1604.83, 1516.10	C ---- C stretching vibration of aromatic benzene ring
1276.92	C – C – O stretching vibration of alcohol group
1192.05, 1031.95	C – O – C stretching vibration of ether group
985.66	= C – H out of plane bending vibration of trans or E alkenic group
819.77, 767.69	= C – H out of plane bending vibration of cis or Z alkenic group

^{1}H NMR spectral data of compound (WH-1)

^{1}H NMR spectrum (CD_3OD, 500 MHz) of this isolated compound is indicated in Figure 3b. According to this spectrum, compound (WH-1) contains (8) protons.

^{13}C NMR spectral data of compound (WH-1)

^{13}C NMR (125 MHz) spectrum, Figure 3c , indicates the total number of carbons (16) in this compound.

According to ^{1}H NMR, ^{13}C NMR, HSQC (Figure 3d) and DEPT (Figure 3 e) spectral data, the partial molecular formula of compound (WH-1) is $C_{16}H_8$ and partial molecular mass is 200. From the FT-IR assignments, compound (WH-1) should consist of at least one – OH group, one carbonyl group and one ether oxygen atom. Therefore, the partial molecular formula becomes $C_{16}H_9O_3$ and extended partial molecular mass is 249. In the ^{13}C NMR spectral data, two carbonyl carbons could be observed at (δ 170.27 ppm and δ 171.15 ppm). Thus, the extended partial molecular formula is $C_{16}H_9O_4$ and its molecular mass is 265. In accordance with EI-mass spectrum (Figure 3 f), molecular ion peak m/z is 316 which indicates the molecular mass of compound. The remaining molecular mass is (316 – 265 = 51). It must be three – OH groups. Therefore, the real molecular formula of compound (WH-1) is $C_{16}H_{12}O_7$.

$$\text{Hydrogen Deficiency Index (HDI)} = C - \frac{H}{2} + 1$$

$$= 16 - \frac{12}{2} + 1 = 11$$

Confirmation of molecular formula of compound (WH-1)

Molecular formula of compound (WH-1) could be confirmed by FT IR spectrum (Figure 3 a) and DEPT spectrum (Figure 3e) as shown in Table 6.

Table 6. Results Given by FT-IR and DEPT Spectral Data of Compound (WH-1)

Assignments	No. of Carbon	No. of Proton	No. of Oxygen
DEPT Spectrum			
- Eight sp^2 methine carbons	8	8	–
- Six sp^2 quaternary carbons	6	–	–
- Two sp^2 carbonyl carbons	2	–	2
FT-IR Spectrum			
- One –OH group	–	1	1
- One ether functional group	–	–	1
Partial molecular formula	C_{16}	H_9	O_4

Partial molecular mass = 265

Molecular ion peak m/z = 316

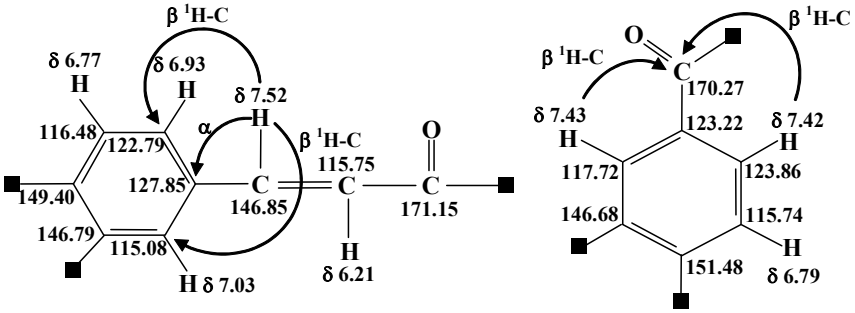
Remaining molecular mass = 316 – 265 = 51

It must be three –OH groups.

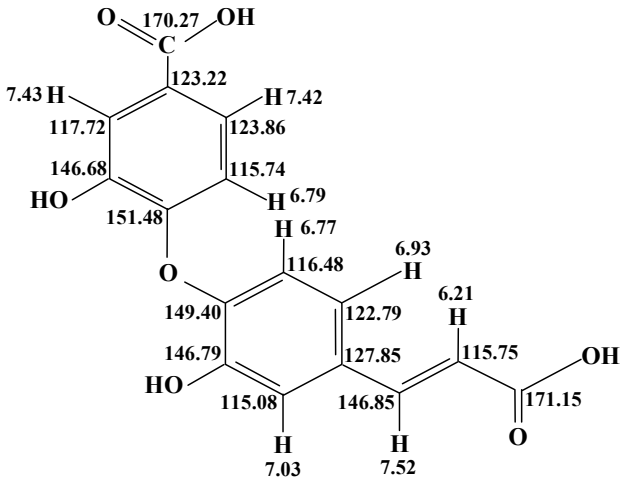
∴ Real molecular formula = $C_{16}H_{12}O_7$

Structure Elucidation of Pure Organic Compound (WH-1)

The structure of pure organic compound (WH-1) could be elucidated by applying DQF-COSY, ^{1}H NMR, HSQC and HMBC spectra respectively. The occurrence of medium graphic area in DQF-COSY spectrum (Figure 3 g) and HMBC spectrum (Figure 3h) lead to the following fragments.

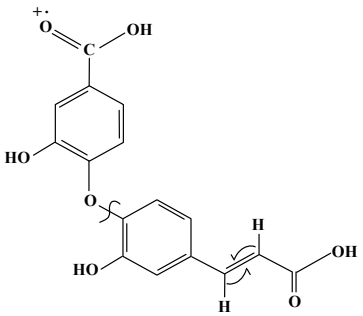


In accordance with HMBC spectral data, the complete structure of (WH-1) can be assigned.

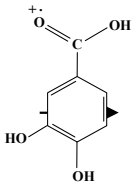


Mass Fragmentation Behaviour of Pure Compound (WH-1)

The elucidated structure of pure compound (WH-1) could be confirmed by EI-mass fragmentation behaviour. The proposed mechanisms for the fragmentation pattern in EI-mass spectrum (Figure 3f) are shown as follows.



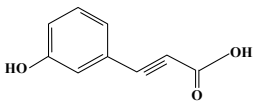
m/z – 316

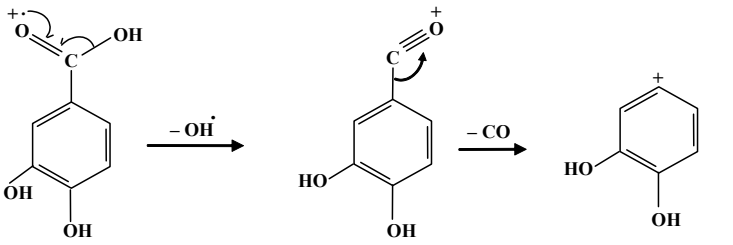


fragment a

m/z – 154 (54 %)

+

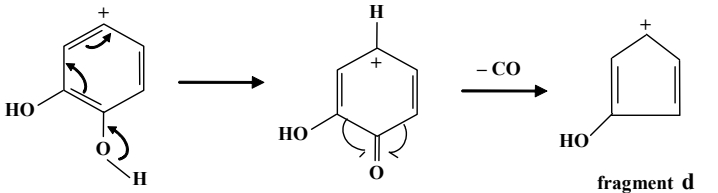




fragment a

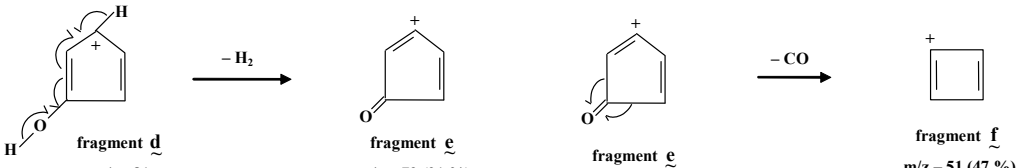
fragment b
m/z – 137 (100 %)
(base peak)

fragment c
m/z – 109 (33 %)



fragment c
m/z – 109

fragment d
m/z – 81 (37 %)

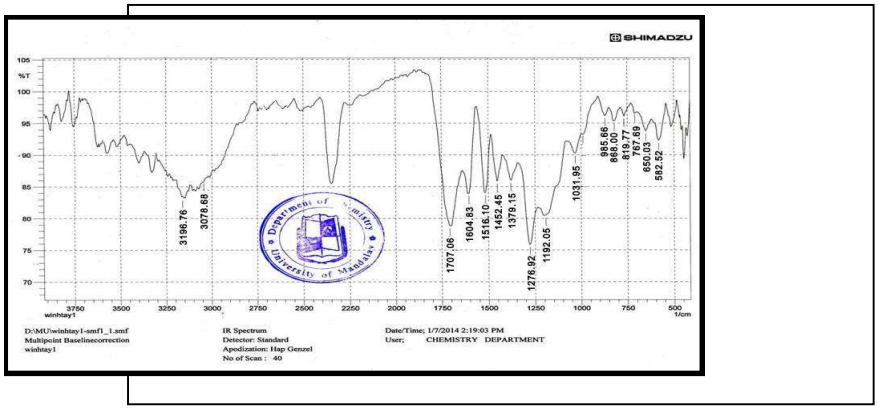


fragment d
m/z - 81

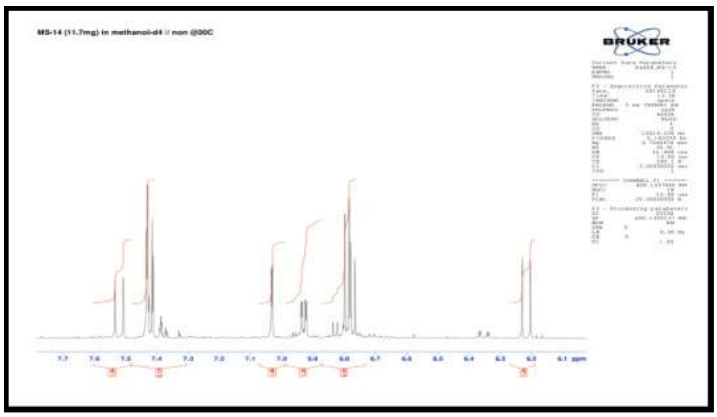
fragment e
m/z – 79 (21 %)

fragment e
m/z – 79

fragment f
m/z – 51 (47 %)



(a)


MS-14 (11.7mg) in methanol-d4 // non @00C
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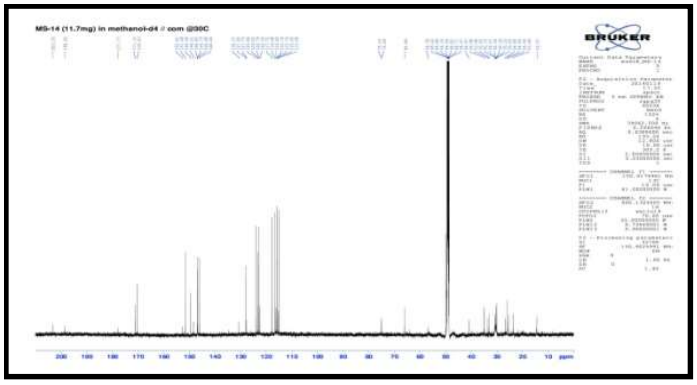
(b)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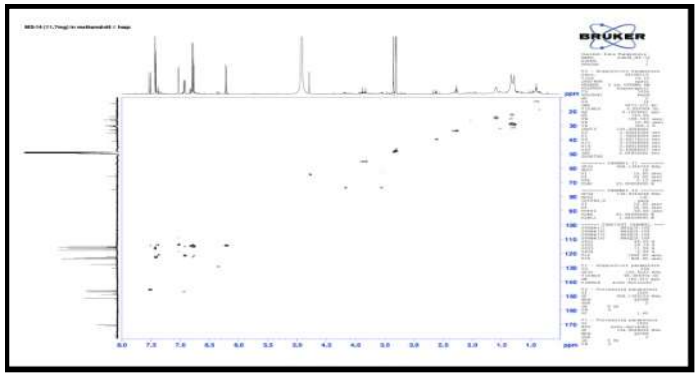
(c)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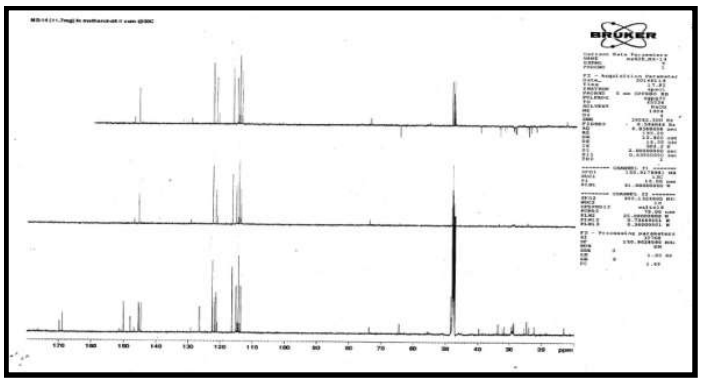
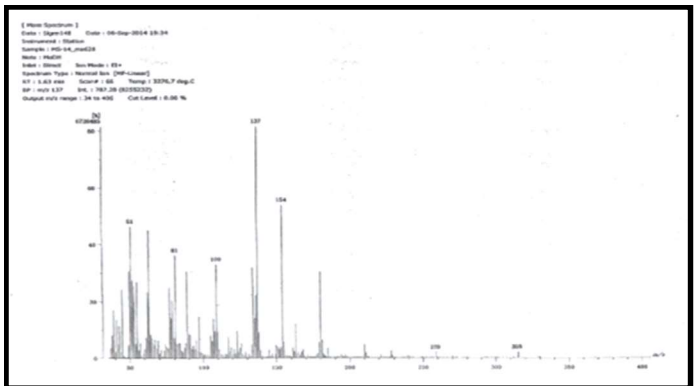
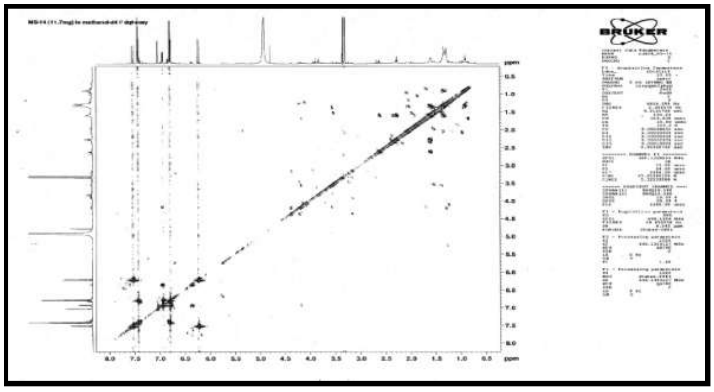
(d)



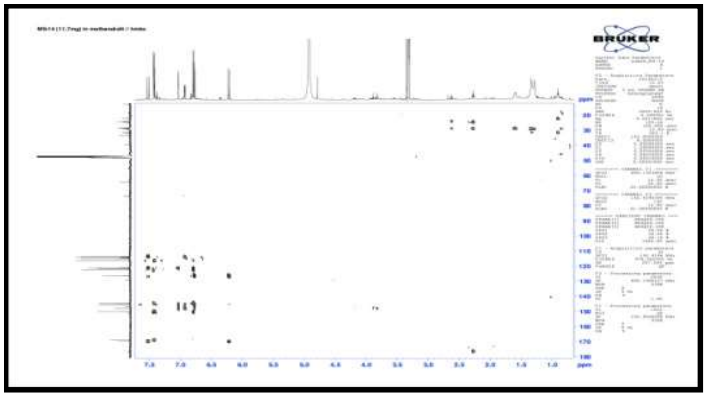
(e)



(f)



(g)



(h)

Figure 3. (a) FT- IR, (b) ^{1}HNMR, (c) ^{13}C NMR, (d) HSQC, (e) DEPT, (f) EI- Mass, (g) DQF-COSYand (h) HMBC-Spectra of Pure Organic Compound (WH-1)

Conclusion

In this research work, one Myanmar indigenous medicinal plant, *Cordia wallichii* G.Don. was selected for chemical idextification.

Firstly, phytochemical tests were examined and according to these results, the bark of Thanat-gyi contains flavonoids, terpenes, glycosides, reducing sugars, polyphenols, tannins, saponins, lipophilic and phenolic compounds.

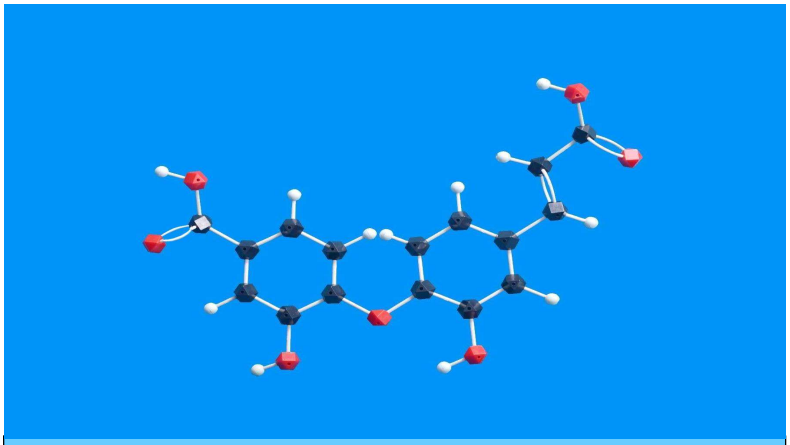
Moreover, the antimicrobial activities of various plant extracts were tested by Agar well diffusion method on six selected organisms. Ethyl acetate extract of Thanat-gyi responds high activities on five selected organisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans* and *E. coli*.

Regarding with the evaluation of toxicity, no significant change was observed in toxic parameters during 14 days. There was no significant change in body weight before and after administration of the test drug. After two weeks, all the mice survived no toxicity sign was observed up to the dose level

of 5000 mg/kg body weight. Hence, the tested substance (95 % ethanolic extract of the bark of *Cordia wallichii* G.Don.) can be labeled unclassified in the hazard category according to Globally Harmonized System and can be considered relatively safe.

Furthermore, pure compound (WH-1) was isolated from the bark of Thanat-gyi by using Thin Layer and Column Chromatographic techniques. The pure yellowish brown crystal (12.6 mg, 0.62 % yield) was obtained. The molecular formula of isolated pure compound was determined as ($C_{16}H_{12}O_7$) by applying spectroscopic methods such as FT-IR, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), DEPT, HSQC and EI-mass spectra. The structure of this isolated compound was elucidated by DQF-COSY, ^{1}H NMR, splitting patterns, coupling constant (J-values) and HMBC spectroscopic studies. Finally, the elucidated structure of isolated compound was confirmed by the fragmentation behavior of EI-mass spectrum.

The IUPAC name of isolated pure compound (WH-1) is (E)-4-(4-(2-carboxyvinyl)-2-hydroxyphenoxy)-3-hydroxy benzoic acid.



(E)-4-(4-(2-carboxyvinyl)-2-hydroxyphenoxy)-3-hydroxy benzoic acid

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