ANTIBACTERIAL ACTIVITY AND IDENTIFICATION OF ISOLATED ORGANIC CONSTITUENTS FROM LEAVES OF *ACACIA CONCINNA* DC. (KIN- MUN- GYIN)

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Abstract

The leaves of *Acacia concinna* DC. (Kin-mun-gyin) are used as folk medicine for the treatment of jaundice, malarial fever, laxative, diarrhoea and boils. The Kin-mun-gyin leaves were chosen for determination of antibacterial activity and organic constituents. The plant sample was collected from Mingalardon Township, Yangon Region. The meso-tartaric acid and myristic acid were isolated from aqueous and ethyl acetate extracts of Kin-mun-gyin leaves by column and preparative thin layer chromatographic methods. It was identified by UV, FT IR, ^{1}H NMR, ^{13}C NMR and ESI MS spectroscopic methods. Then, *in vitro* screening of antibacterial activity was done on four crude extracts (PE, EtOAc, EtOH and H_2O) of Kin-mun-gyin leaves against 3 species of *Staphylococcus aureus* and 2 species of *Escherichia coli*, one species of *Bacillus subtilis*, one species of *Proteus morganii* and one species of *Vibrio cholerae* by employing agar disc diffusion method. Except PE extract, all of the crude extracts were found to exhibit the inhibition zones against all of the organisms tested. Minimum inhibitory concentration (MIC) values of two isolated compounds were also determined by microplate dilution method on above 8 species of bacteria. The lowest MIC values of meso-tartaric acid and myristic acid from *A. concinna* leaves were respectively found to be 0.0058 mg mL^{-1}, 0.0937 mg mL^{-1} against *S. aureus* ws. From this study, it may be concluded that meso-tartaric acid and myristic acid possess antibacterial activities useful for the medicinal purposes for the curing of diseases caused by the microorganisms tested.

Keywords: *Acacia concinna* DC. (Kin-mun-gyin), antibacterial activity, meso-tartaric acid, myristic acid, agar disc diffusion method, MIC values

Introduction

Genus *Acacia* belongs to the subfamily Mimosoideae in Leguminosae which contains 600 genera and about 12000 species. It has 750-800 species (Dastur, 1962). Among them, 34 species grow in Myanmar (Kress and Yin Yin Kyi, 2003). Most members of subfamily Mimosoideae are trees or shrubs; leaves usually bipinnate; flowers regular; calyx usually gamosepalous; stamens equal in number to the petals or twice as numerous; fruit a legume (Figure 1) (Evans, 2002).

Figure 1 Photographs of the plant and leaves of *A. concinna* DC.

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Materials and Methods

Sample Collection

The leaves of *Acacia concinna* DC. (Kin-mun-gyin) were collected from Sein Shwe Gyone Ywar, Htauk Kyant, Mingalardon Township, Yangon Region, Myanmar during the months of September and October, in the 2006. All the fresh samples were washed with distilled water. After cleaning, the leaves were air-dired at room temperature for three weeks. The dried samples were cut into pieces and then ground in a grinding machine to powder. The dried powdered samples were stored separately in air-tight containers to prevent moisture changes and other contamination. The following instruments were used for the determination of physical data: melting point; Gallenkamp melting point apparatus, UV spectra; Shimadzu UV-240, UV-Visible spectrophotometer, IR spectra; Perkin Elmer GX FT IR spectrophotometer, ^{1}H (300 MHz), ^{13}C (300 MHz) and ^{1}H (400 MHz) NMR spectra; were measured on a Bruker 300 and JEOL JNM-GM 400 spectrometer were used to record the spectra. ESI mass spectra was recorded at the Department of Organic Chemistry, Goettingen University, Germany.

Preparation of Crude Extracts

The dried powdered sample of Kin-mun-gyin leaves (100 g) were extracted with 70 % ethanol (200 ml× 4) at room temperature for two weeks. After two weeks, the extract was filtered and concentrated by rotatory evaporator at 45 °C. The concentrated extracts were combined and then partitioned with PE (60-80 °C) to remove the fat. In this way two layers, namely PE soluble layer (upper layer) and PE insoluble layer (low layer) (or) aqueous layer were obtained. Then the aqueous layer was partitioned with ethyl acetate in a separating funnel. The aqueous layer was concentrated under vacuum rotatory evaporator to obtain the aqueous extract. The EtOAc extract was dried over anhydrous Na_2SO_4, filtered and the total filtrate was concentrated under vacuum rotatory evaporator to obtain EtOAc crude extract.

The dried powdered samples (100g) were extracted with (250 mL) of, pet-ether, ethyl acetate, 70 % ethanol and water in separate conical flask, respectively for at least 7 days and then filtered. The filtrates were evaporated by using rotatory evaporator and desiccated. Then the dried extracts were weighed. Each extract was stored in refrigerator for screening of antibacterial activity.

Isolation of Organic Compound from Aqueous Crude Extract of *A. concinna* Leaves

The aqueous crude extract was chromatographed on a silica gel column (2 cm × 55 cm) using EtOH:NH_3:H_2O (80:5:15) (v/v) solvent system. Compound A separated out as a white powder. Recrystallization thrice from EtOH gave pure compound A (0.007 %) as white crystalline powder.

Isolation of Organic Compound from EtOAc Crude Extract of *A. concinna* Leaves

The EtOAc crude extract (1 g) was subjected to silica gel chromatography by using a gradient system of $ClCH_2CH_2Cl$-MeOH. After combining together similar fractions, 4 fractions were obtained. Fraction F_3 was rechromatographed eluting with $ClCH_2CH_2Cl$-MeOH (93:7) (v/v) solvent system to yield pure compound B (0.006 %) as colourless powder.

Structural Elucidation

The structures of isolated compounds were elucidated and identified by modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR and ESI MS spectroscopies.

***in vitro* Screening of Antibacterial Activity of Different Crude Extracts by Agar Disc Diffusion Metho**

The antibacterial activity of different crude extracts (PE, EtOAc, EtOH and H_2O) was determined by agar disc diffusion method (Mar Mar Nyein *et al.*, 1991) at Department of Medical Research (Lower Myanmar), Dagon Township, Yangon Region, Myanmar.

Procedure

The filter discs (6 mm diameter) were made by punched No.1 Whatmann filter paper. The disc were sterilized by autoclaving and following by dry heating at 60 °C for 1 hour. It was then impregnated with concentrated extracts to obtain approximately 20 µg/disc. Prior to adherence on the culture plates, the discs were allowed to dry at 42 °C in incubator. The bacterial suspension from trypticase soy broth was streak evenly onto the surface of the trypticase soy agar plate with sterile cotton swab. After the inoculums had dried (5 min), the dried discs were placed on the agar with flamed forceps and gently pressed down to ensure proper contact. One disc, impregnated individually with solvent was placed along the test disc for control and comparing purpose. The plates were incubated immediately or within 30 min after inoculation. After overnight incubation at 37 °C, the zones of inhibition diameter including 6 mm discs were measured (Finegold *et al.*,1978).

Determination of Minimum Inhibitory Concentration (MIC) of the Isolated Compounds by Microplate Dilution Method

The sample was first screened at a concentration of 100 µg mL^{-1}. For every experiment, a sterility check was done on broth medium and the extract.

For the determination of minimum inhibitory concentration (MIC) values of the tested sample. Prior to the performing of MIC, the 100 µmL of trypticase soy broth was first introduced in 96 wells. Then 100 µmL of sample was introduced into the first well to obtain 200 µg mL^{-1}. By using multi-channel pipetter (8-channels) and Titertek micro-titration equipment, 100 µmL of the mixture was used for downstream serial dilutions up to 12 consecutive wells, each already containing 100 µmL of media. The last 100 µmL was discarded.

While transferring the content of each well, the mixture was mixed thoroughly with a multi-channel pipetter. Then 20 µmL of the already prepared inoculum was introduced to its respective wells and the microplates were incubated at 37 °C for overnight. The last well with no growth of the microorganism was taken to represent the MIC of the isolated compounds.

The MIC values of the two isolated compounds were tested with 3 species of *S. aureus*, 2 species of *E. coli*, one strain *P. morganii*, one strain *B. subtilis* and one strain *V. cholerae* by using microplate dilution method.

Results and Discussion

Identification of Isolated Compound A from Aqueous Extract of *A. concinna* Leaves

The compound A was isolated from the 70 % EtOH extract of the leaves of *A. concinna*, after successively removing the petroleum ether and ethyl acetate soluble parts of the extract, that is, it comes from the remaining very polar part of the extract; in fact, compound A was eluted from the silica gel column with the polar solvent system of EtOH-ammonia-water (80:5:15), indicating that it must be a very polar compound. On TLC plate, it is invisible under UV 254 or 365 nm lights, but it is detectable as a brown spot by heating after dipping the plate in Schwepps reagent; these observations suggests an organic acid containing no system of conjugated double bonds.

The UV spectrum (Figure 2) of compound A also shows no absorptions between 200 and 400 nm range given by conjugated systems, in agreement with the above observation on TLC plate. In the FT IR spectrum (Figure 3) of compound A, the strong broad O-H stretching band at 3600-2700 cm^{-1}, which is characteristic of a carboxylic acid, and the strong C=O stretching band at 1732 cm^{-1} can be observed.

Concerning the ^{1}H NMR (Figure 4, Table 1) and ^{13}C NMR (Figure 5, Table 2) spectra of compound A, one is inclined at first sight to dismiss it as nothing significant, since the spectra were so simple with only one or two signals. However, the carbon signal at δ 174.88 is in accord with the carbonyl group of a carboxylic acid, a fact already seen in FT IR spectrum above. The only other signals observed in ^{13}C NMR are the two oxygenated sp^3 carbons at δ 73.88 and δ 73.50 ppm; and the corresponding proton on such carbon appears in ^{1}H NMR spectrum at δ 4.51 ppm, the only observed proton signal for compound A.

At this point, it may thus be supposed that there are two CHO and one COOH groups in compound A, totaling to 103 mass units; there seems to be only 3 carbons. Now, the peaks at m/z 149.0 (98 %) and 299.0 (53 %) in the ESI MS (Figure 6, Table 3) of compound A suggest, respectively, M-H and 2M-H peaks, with M having 150 mass units. Thus there remains 150 - 103 = 47 mass units. From the m/z 150(4.5 %) in mass spectrum, the maximum possible number of carbons is (4.5/98)x100/1.1= 4.2, i.e., 4. Therefore the isolated compound A can be deduced as meso tartaric acid.

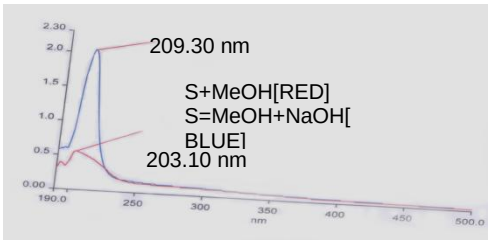


Figure 2 UV spectrum of compound A

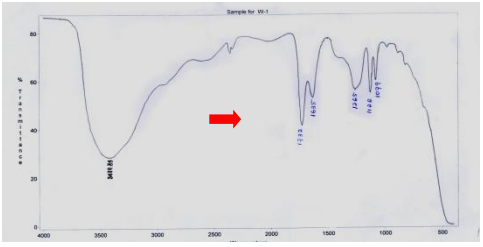


Figure 3 FT IR spectrum of compound A

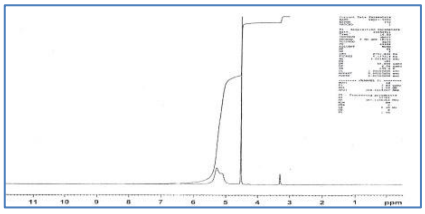


Figure 4 ^{1}H NMR spectrum of compound A

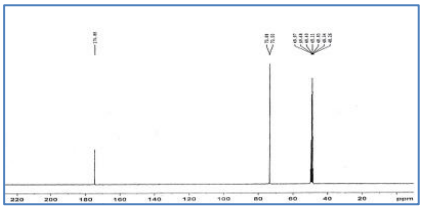


Figure 5 ^{13}C NMR spectrum of compound A

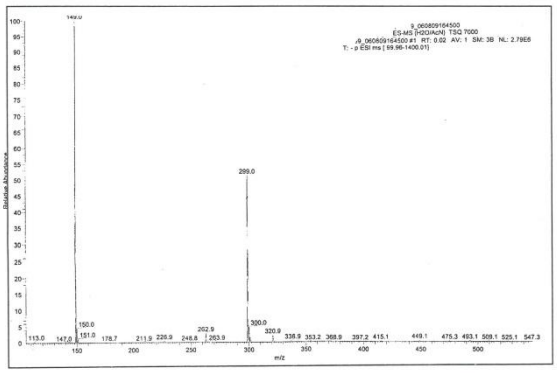


Figure 6 ESI MS spectrum of compound A

Table 1 ^{1}H NMR (300 MHz, MeOH-d_6) Spectral Data of Compound A

Chemical shift (δ)	Number of protons	Multiplicity	Structural unit
4.51	2	Singlet	HOOC(OH)C**H**-C**H**(OH)COOH

Table 2 ^{13}C NMR (150 MHz, MeOH-d_6) Spectral Data of Compound A

Chemical shift (δ)	Number of carbons	Structural unit
174.88	2	-**C**OOH, two numbers
73.88	1	-**C**H(OH)COOH, one number
73.50	1	-**C**H(OH)COOH, one number

Table 3 ESI-MS Spectral Data of Compound A

m/z	Relative abundance (%)	Remarks
149.0	98	$(M-H)^-$
150.0	4.5	$(M-H+1)^-$
299.0	53	$(2M-H)^-$

Identification of Isolated Compound B from EtOAc Extract of *A. concinna* Leaves

Compound B was isolated from the fraction 3 of ethyl acetate extract after rechromatographed on silica gel G 60 using $ClCH_2CH_2Cl$: MeOH (93:7). It gave a brown colour spot on TLC after spraying and heating with 5% H_2SO_4. Its melting point is 57 °C. The maximum absorption spectrum of UV (Figure 7) showed at 207 nm in MeOH and at 195 nm in MeOH and 2 drops 2 N NaOH.

The IR spectrum (Figure 8) showed a broad absorption band at 3500-3000 cm^{-1} due to OH- stretching vibration of carboxylic acid group. The strong absorption bands due to the CH stretching vibration of $–CH_2$ and $–CH_3$ group showed at 2919 cm^{-1} and 2852 cm^{-1}. The absorption band at 1709 cm^{-1} was assigned as carbonyl group.

The ^{1}H NMR spectrum ($CDCl_3$) (Figure 10) showed signals assignable to a saturated fatty acid at δ 2.35 (H –2), 1.64 (H –3), 1.30 (10 CH_2, H –4-13) and 0.90 (H –14)ppm (Table 4). The integration ratio of proton signals suggested the presence of 27 protons. The proton signals of compound B are also similar with that of myristic acid obtained from ACD labs (Figure 9). Therefore, according to above-stated spectral data, it was found to be myristic acid.

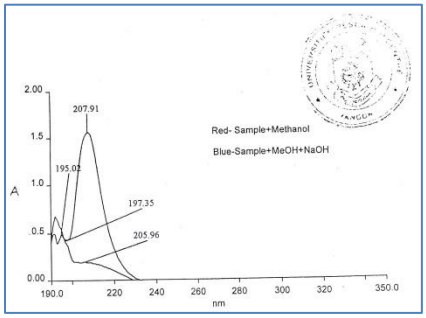


Figure 7 UV spectrum of compound B

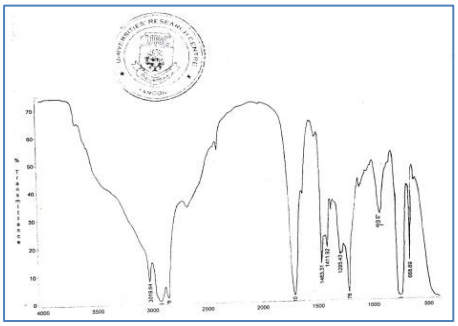


Figure 8 FT IR spectrum of compound B

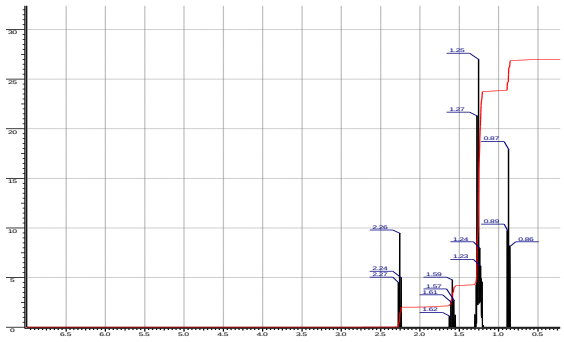


Figure 9 ^{1}H NMR spectrum of myristic acid predicted by ACD Labs software

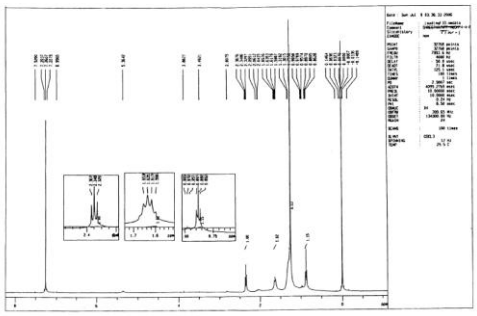


Figure 10 ^{1}H NMR spectrum of compound B

Table 4 ^{1}H NMR (300 MHz, $CDCl_3$) Spectral Data of the Isolated Compound B

Atom	δ /ppm	Integration	Assignment
H-2	2.35	2 H	- CH_2 (methylene)
H-3	1.64	2 H	- CH_2 (methylene)
H-(4-13)	1.30	20 H	- CH_2 (methylene)
H – 14	0.90	3 H	- CH_3 (methyl)

***In vitro* Screening of Antibacterial Activity of Different Crude Extracts from *A. concinna* Leaves**

The inhibition zone diameters for different crude extracts of *A. concinna* leaves are illustrated in Table 5. It can be obviously seen that PE extract (inhibition zone diameters ranged 0 mm), EtOAc extract (inhibition zone diameters ranged between14 mm - 23 mm), EtOH extract (inhibition zone diameters ranged between 15 mm - 25 mm) and H_2O crude extract (inhibition zone diameters ranged between 16 mm - 24 mm) exhibited the antibacterial activity against 3 species of *Staphylococcus aureus*, 2 species of *Escherichia coli*, one species of *Bacillus subtilis*, one species of *Proteus morganii* and one species of *Vibrio cholerae*. It was observed that pet-ether extracts of *A. concinna*

leaves did not show any antibacterial activity against all organisms tested. Therefore, EtOAc, EtOH and H_2O crude extracts of *A. concinna* leaves can be considered to be biologically active.

Table 5 Antibacterial Activities of Different Crude Extracts of *A. concinna* leaves on Different Species of Bacteria by Agar Disc Diffusion Method

No.	Type of Bacteria	Inhibition Zone Diameter (mm)			
		I	II	III	IV
1.	*Staphylococcus aureus* ws	-	23	25	24
2.	*Staphylococcus aureus* ns	-	18	18	20
3.	*Staphylococcus aureus* w sepsi	-	18	18	20
4.	*Escherichia coli* (STLT)	-	17	15	20
5.	*Escherichia coli* (ATCC)	-	17	15	16
6.	*Bacillus subtilis*	-	18	16	20
7.	*Proteus morganii*	-	14	16	18
8.	*Vibrio cholerae*	-	16	16	18

I = PE extract of *A. concinna* leaves
II = EtOAc extract of *A. concinna* leaves
III = EtOH extract of *A. concinna* leaves
IV = H_2O extract of *A. concinna* leaves

Determination of Minimum Inhibitory Concentration (MIC) of Isolated Compounds by Microplate Dilution Method

The MIC value of isolated compound A and B were determined by microplate dilution method with optical density and growth a nutrient agar (Figure 11). Three species each of *S. aureus* ws, two species each of *E. coli*, one strain *P. morganii*, one strain *B. subtilis* and one strain *V. cholerae*. The MIC values of isolated compound A and B were determined by micro plate reader at the wavelength 490 nm are shown in Table 6, Figure 12(a) and Table 7, Figure 12(b). It was found that the lowest MIC values of isolated compound A and B were respectively found to be 0.0058 mg mL^{-1} and 0.0937 mg mL^{-1} against with *S. aureus* ws.

Compound A Compound B

Figure 11 Photograph showing falcon 3072 sterile pack containing 96 wells used for the determination of MIC values of compound A and B isolated from *A. concinna* leaves by microplate dilution method

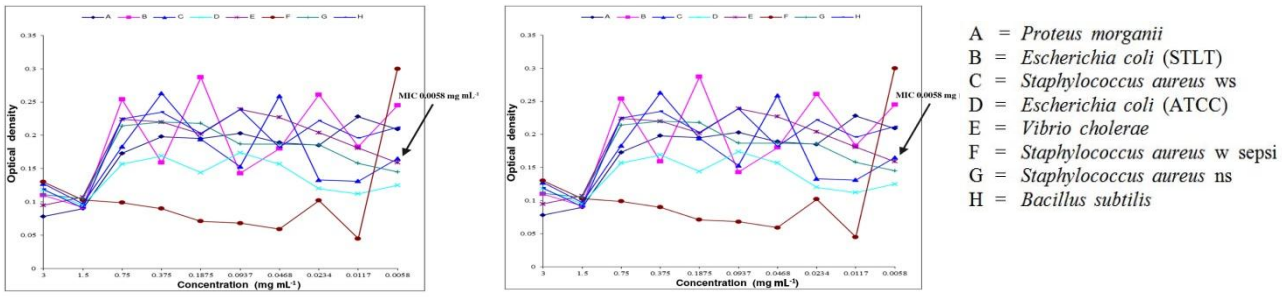
Table 6 Optical Density and Various Concentrations of Compound A from *A. concinna* (Kin-mun-gyin) on *P. morganii,* 2 species of *E.coli,* 3 species of *S. aureus, V. cholerae* and *B. subtilis* by Microplate Reader measured at the wavelength 490 nm

Concentration (mg mL^{-1})	Optical Density							
	A	B	C	D	E	F	G	H
3.0000	0.119	0.111	0.130	0.095	0.118	0.127	0.110	0.078
1.5000	0.091	0.105	0.103	0.107	0.095	0.097	0.092	0.090
0.7500	0.225	0.214	0.099	0.224	0.157	0.183	0.254	0.173
0.3750	0.235	0.220	0.090	0.220	0.169	0.263	0.159	0.198
0.1875	0.203	0.218	0.071	0.202	0.144	0.194	0.287	0.195
0.0937	0.239	0.187	0.068	0.239	0.174	0.153	0.143	0.203
0.0468	0.182	0.187	0.059	0.227	0.157	0.259	0.180	0.189
0.0234	0.222	0.186	0.102	0.204	0.120	0.133	0.261	0.185
0.0117	0.196	0.158	0.045	0.181	0.112	0.131	0.183	0.228
0.0058	0.212	0.145	0.300	0.159	0.125	0.165	0.245	0.209
0.0029	0.278	0.208	0.048	0.181	0.146	0.290	0.246	0.225
0.0014	0.031	0.043	0.027	0.018	0.039	0.060	0.101	0.061

Table 7 Optical Density and Various Concentrations of Compound B from *A. concinna* (Kin-mun-gyin) on *P. morganii,* 2 species of *E. coli,* 3 species of *S. aureus, V. cholerae* and *B. subtilis* by Microplate Reader measured at the wavelength 490 nm

concentration (mg mL^{-1})	Optical Density							
	A	B	C	D	E	F	G	H
3.0000	0.291	0.253	0.113	0.207	0.226	0.139	0.136	0.214
1.5000	0.268	0.258	0.105	0.303	0.233	0.348	0.164	0.195
0.7500	0.258	0.229	0.091	0.312	0.231	0.375	0.194	0.231
0.3750	0.291	0.260	0.089	0.271	0.212	0.206	0.189	0.202
0.1875	0.299	0.208	0.102	0.256	0.213	0.170	0.193	0.208
0.0937	0.271	0.252	0.066	0.226	0.254	0.117	0.185	0.215
0.0468	0.241	0.193	0.061	0.226	0.179	0.316	0.187	0.203
0.0234	0.235	0.178	0.059	0.208	0.186	0.295	0.181	0.187
0.0117	0.307	0.157	0.072	0.196	0.160	0.322	0.164	0.213
0.0058	0.210	0.179	0.090	0.204	0.203	0.120	0.150	0.222
0.0029	0.268	0.243	0.160	0.212	0.195	0.171	0.184	0.181
0.0014	0.025	0.030	0.008	0.029	0.060	0.039	0.021	0.025

A = *Proteus morganii*
B = *Escherichia coli* (STLT)
C = *Staphylococcus aureus* ws
D = *Escherichia coli* (ATCC)
E = *Vibrio cholerae*
F = *Staphylococcus aureus* w sepsi
G = *Staphylococcus aureus* ns
H = *Bacillus subtilis*



(a) (b)

Staphyloccoccus aureus ws
MIC value of compound A 0.0058 mg mL^{-1}

Staphyloccoccus aureus ws
MIC value of compound B 0.0937 mg mL^{-1}

Figure 12 Plots showing variation of optical density with concentration of (a) compound A and (b) compound B for each of the eight tested bacterial strains in the determination of the MIC values by microplate dilution method

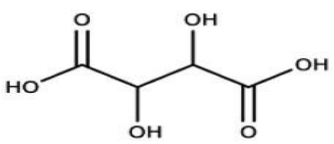
Conclusion

The following inferences could be deduced from the overall assessment of the chemical investigation on the leaves of *A. concinna* DC .(Kin-mun-gyin). On silica gel column chromatographic separation, two compounds were isolated from aqueous and ethyl acetate crude extracts of the *A. concinna* leaves. The compounds isolated from the aqueous and ethyl acetate extracts from *A.concinna* leaves were identified to be (one dicarboxylic acid, meso-tartaric acid) (m.pt. 140 ºC, 0.007 %) and (one saturated fatty acid, myristic acid) (m.pt 57 ºC, 0.006 %). The isolated compound A and B were characterized by some physical and chemical properties and structurally identified by the combination of UV, FT IR, ^{1}H NMR , ^{13}C NMR and ESI MS spectroscopic methods and also by comparing with the reported data.

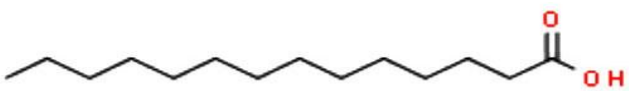
The antibacterial activity of crude extracts (PE, EtOAc, EtOH and H_2O) of *A. concinna* leaves was screened by using agar disc diffusion method on eight bacterial strains including 3 species of *S. aureus*, 2 species of *E. coli*, one *B. subtilis*, one *P. morganii* and one *V. cholerae*. Except PE extracts, all of the crude extracts were found to exhibit antibacterial activity against the organisms tested.

The lowest MIC values of isolated compound A (meso tartaric acid) and compound B (myristic acid) isolated from *A. concinna* leaves determined against *S.aureus* ws by micro plate dilution method were respectively found to be 0.0058 mg mL^{-1} and 0.0937 mg mL^{-1}.

In conclusion , it was found that crude extracts (EtOH, EtOAc and H_2O) of *A. concinna* leaves can be effective in the formulation of medicine for the treatment of diseases such as diarrhoea, fever, inflammation, laxative and boils.



meso-tartaric acid



myristic acid

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