ISOLATION, STRUCTURE ELUCIDATION OF FLAVONOID DERIVATIVE FROM THE BARK OF *ALBIZIA PROCERA* (ROXB.) BENTH. AND STUDY ON ITS ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

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Abstract

In the present research, the bark of *Albizia procera* (Roxb.) Benth. (Local name = Thit-phyu) was selected for chemical investigation. Preliminary phytochemical investigation was carried out according to the standard procedures. Antimicrobial properties was evaluated by using agar-well diffusion method on six test microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Candida albican and Mycobacterium* species. Moreover, a pure compound, pale yellow needled shape crystals, was isolated by using separation techniques such as thin layer and column chromatography. The isolated compound was assigned as flavonoid derivative, namely, 2(R), 3(S)-2-(2, 4-dihydroxy phenyl) chroman-3,5,7-triol by using spectroscopic methods, such as FT-IR, ^{1}H NMR, ^{13}C NMR (125 MHz), DEPT, DQF-COSY, HMQC, HMBC, NOESY, and EI mass spectral data. The isolated compound was further confirmed by phytochemical test which gave rise to positive for flavonoid test. The yield percent was calculated as (33.87 mg), (1.07%) based upon the ethyl acetate crude extract and the melting point was 191°C–192°C. Moreover, the antioxidant activity of isolated flavonoid compound was measured by DPPH (1,1–Diphenyl-2-picryl-hydrazyl) assay. The isolated compound showed the high antioxidant activity.

Keywords: chromatography, flavonoid, DPPH

Introduction

In the last decade, there has been a global upsurge in the use of traditional medicine and complementary and alternative medicines in both developed and developing countries. Hence, the safety and efficacy, as well as the quality control of traditional medicine have become important concerns for both health authorities and the public. Herbal medicines are the most widely used traditional medicines. Before manufactured drugs came into widespread use, herbal medicines played an important role in human health (WHO, 2005). Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products.

Different medicinal plants and herbs grow in Myanmar as there are different seasons and various geographical features. Among them, one traditional indigenous medicinal plant, *Albizia procera* Benth., which is locally known as Thit-phyu and applying as indigenous medicine (Aye, 2004). No chemical investigation has been done on this plant species in Myanmar. In India, leaves are poultice onto ulcers. Bark is useful in pregnancy and stomachache. Bark extract was given with salt as a medicine for water buffalo. It can prevent the phlegm and the diseases of the bile. So, the bioactive compound from bark of *Albizia procera* Benth. was investigated in the present research work.

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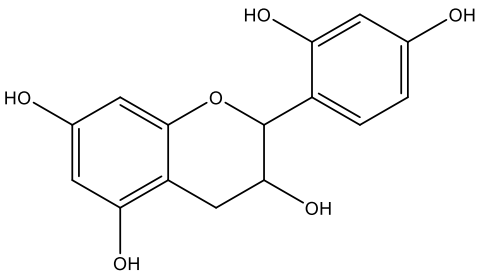


Figure 1 Structure of isolated compound

Figure 2 Plant, stem, leaf and fruit of *Albizia procera* (Roxb.) Benth.

Botanical Classification

Botanical name	- *Albizia procera* (Roxb.) Benth.
Family	- Mimosaceae
Myanmar name	- Sit, Thit-phyu
Distribution	- Native to tropical Asia and Australia

Materials and Methods

General Experimental Procedures

Commercial grade reagents and solvents were used without further purification. Analytical preparative thin layer chromatography was performed by using silica gel. (Merck Co. Inc, Kieselgel 60 F_{254}). Silica gel (60, 70 to 230 mesh ASTM) was used for Column Chromatography. The advanced instruments which were used in the characterization of samples and elucidation of pure compound were shown below.

1. UV lamp (Lamda-40, Perkin-Elmes Co. England)
2. UV 1601 PC (P/N 206.675) spectrophotometer
3. FT-IR spectrometer (Shimadzu, Japan)
4. ^{1}H NMR spectrometer (500MHz)
5. ^{13}C NMR spectrometer (125 MHz)
6. EI-Mass spectrometer

Plant Materials

The bark of *Albizia procera* were collected from Shwebo Township, Sagaing Region, Myanmar. The collected plant materials were screened and taxonomically identified by authorized botanist from Botany Department, University of Mandalay. The plant materials were dried at room temperature and ground into powder.

Preliminary Phytochemical Analysis

The preliminary phytochemical screening of *Albizia procera* was determined using standard method of Harborne (Harborne, 1995).

Antimicrobial Assay

Antimicrobial tests were performed at Pharmaceutical Research Department (PRD), Insein Township, Yangon Region. Antimicrobial activities of plant extracts were tested by agar-well diffusion method on six test microorganisms such as *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillius pumilus*, *Candida albicans* and *Mycobacterium* species.

Extraction and Isolation of Pure Compound

The air dried bark powder of *Albizia procera* (800 g) was percolated with 95 % ethanol (1500 mL) for two months. The ethanol extract was filtered and evaporated. The residue was dissolved in 500 mL of ethyl acetate. Then, ethyl acetate solution was evaporated and to obtain crude extract (5.64 g). The ethyl acetate crude extract (3.15 g) was chromatographed on a silica gel (70-230 mesh) column, eluting with the solvent system n-hexane: ethyl acetate with various ratios from non-polar to polar. About 1 mL of each portion was collected in a small bottle and followed by TLC detection of each portion. Totally 178 fractions were collected. The fractions with the same R_f value were combined and altogether (11) combined fractions were obtained. The fraction (K) which showed only one spot on TLC, was concentrated and recrystallized by using (50%) ethyl acetate in n-hexane for two times. The pure pale yellow needled shape crystals (33.87 mg) were obtained. The total yield percent of pure compound was (1.07 %) based upon the crude ethyl acetate extract. This pure compound was reconfirmed by phytochemical test which gives rise to positive for flavonoid test.

Determination of Melting Point

A few pale yellow needled shape crystals of pure isolated compound were inserted into the capillary tube and the melting point was determined by using the electric melting point apparatus. The melting point of pure compound was found to be 191°C-192°C.

Measurement of Radical Scavenging Activity by UV Spectrophotometric Method

Preparation of 60 μM DPPH Solution

DPPH powder (2.36 mg) was thoroughly and gently dissolved in 100 mL of 95% ethanol and stored in brown coloured volumetric flask at 5 °C (no longer than 24 hours) before use.

Preparation of Test Sample Solution

Pure isolated compound 2.0 mg and 10 mL of 50% ethanol were gently mixed by vortex mixer to obtain the stock solution. It was diluted with 50% ethanol in various ratios to obtain five ranges of concentrations, such as, 0.625 μg/mL, 1.25 μg/mL, 2.50 μg/mL, 5.0 μg/mL and 10.0 μg/mL respectively. Then, 5.0 mL of ethanol solution was prepared for each concentration.

Preparation of Standard Ascorbic Acid Solution

Furthermore, the solution of standard ascorbic acid was also prepared as the same concentrations of isolated compound in 50% ethanol and the same volume (5 mL) of standard ascorbic solution was prepared for each concentration.

Measurement of DPPH Radical Scavenging Activity by Spectrophotometric Method

The control solution was prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95% ethanol by vortex mixer. Moreover, the blank solution could be prepared by mixing 1.5 mL of test sample solution and 1.5 mL of 50% ethanol thoroughly in the vortex mixer. Furthermore, the sample solution was also prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of test sample solution gently by applying vortex mixer. After that, the solutions were allowed to stand at room temperature for 30 min. Then the absorbance value of each solution was measured at 517 nm by UV-1601 PC (P/N 206-675) spectrophotometer (Kiattsin *et al*., 2016).

These measurements were performed in triplicate for each solution. The absorbance values obtained were applied to calculate percent inhibition by formula:

$$\%\,\text{inhibition} = \frac{\text{Abs}_{\text{DPPH}} - [\text{Abs}_{\text{sample}} - \text{Abs}_{\text{Blank}}]}{\text{Abs}_{\text{DPPH}}} \times 100\,,$$

where % inhibition = percent inhibition of test sample, Abs_{DPPH} = absorbance of control solution, $\text{Abs}_{\text{sample}}$ = absorbance of test sample solution, $\text{Abs}_{\text{blank}}$ = absorbance of blank solution.

Results and Discussion

Phytochemical Analysis

Preliminary phytochemical analysis of the extract of *Albizia procera* bark revealed the presence of phytochemicals such as flavonoid, sugar, glycoside, phenol and polyphenol.

Antimicrobial Assay

The antimicrobial activities of plant extracts were tested by applying agar-well diffusion method on six test microorganisms. According to antimicrobial assay, the n-hexane extract of *Albizia procera* did not inhibit the growth of test microorganisms. But other four solvents extracts (chloroform, acetone, ethyl acetate, ethanol) of *Albizia procera* exhibit the medium activities on five organisms and high activities on *Mycobacterium* species.

Table 1 Antimicrobial Activities of Selected Medicinal Plant

		Organisms/mm					
Samples	**Solvents**	**I**	**II**	**III**	**IV**	**V**	**VI**
Albizia procera Bark	n-hexane	–	–	–	–	–	–
	$CHCl_3$	14	17	13	16	19	20
	Acetone	15	15	14	16	18	20
	EtOAc	15	15	14	16	18	20
	EtOH	13	15	13	15	17	17

Agar-well – 10 mm

10 mm ~14 mm (+) weak active
15 mm ~19 mm (++) medium active
20 mm above (+++) strong active

(I) *Bacillus subtilis*
(II) *Staphylococous aureus*
(III) *Pseudomonas aeruginosa*
(IV) *Bacillus pumilus*
(V) *Candia albicans*
(VI) *Mycobacterium* species

The antimicrobial activities of pure compound were rechecked by using agar-well diffusion method on six organisms. The isolated compound responded medium activities on five test microorganisms except mycobacteria.

Table 2 Antimicrobial Activities of Pure Compound

Name of Organisms	Inhibition Zone (mm)
Bacillus subtilis	15
Staphylococous aureus	15
Pseudomonas aeruginosa	15
Bacillus pumilus	15
Candia albicans	17
Mycobacterium species	-

Antioxidant Activity

The antioxidant activity of isolated pure compound was analyzed by DPPH (2,2-diphenyl-1-picryl-hydrazyl) method. The comparative absorbance values of isolated flavonoid compound and those values of standard ascorbic acid were tabulated in Table 2.

Table 3 Comparative Absorbance Values of Standard Ascorbic Acid Solution and Isolated Compound

No.	Concentration (µg/mL)	Absorbance	
		Ascorbic Acid	Isolated Compound
1	0.625	0.057	0.123
2	1.25	0.031	0.098
3	2.5	0.01	0.078
4	5	0.002	0.063
5	10	0.001	0.033

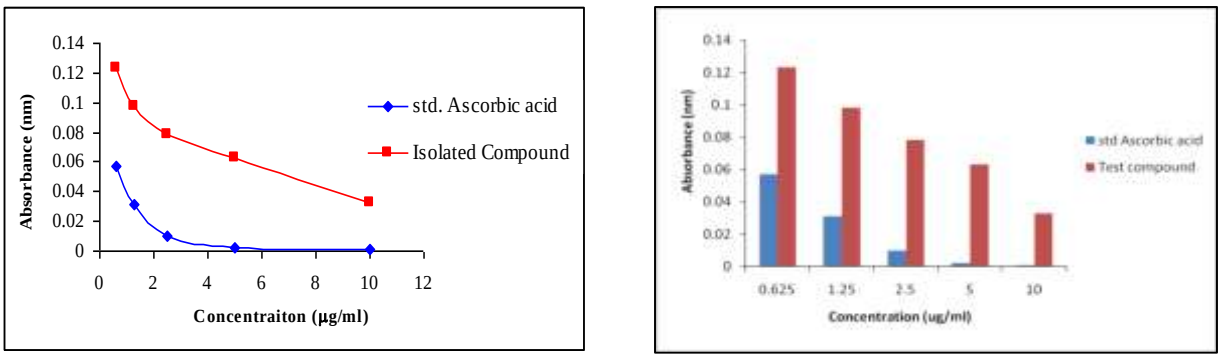


Figure 3 Plot of absorbance vs concentration of isolated compound and standard ascorbic acid

The inhibition percentage of isolated compound and standard ascorbic acid in various concentrations were described in Table 3.

Table 4 %Inhibition of Ascorbic Acid Solution and Isolated Compound in Various Concentrations

No	Concentration (µg/mL)	% Inhibition	
		Ascorbic acid (Standard)	Isolated Compound
1	0.625	69.0	50.2
2	1.25	83.2	60.3
3	2.5	94.6	68.5
4	5	98.9	74.5
5	10	99.5	86.6

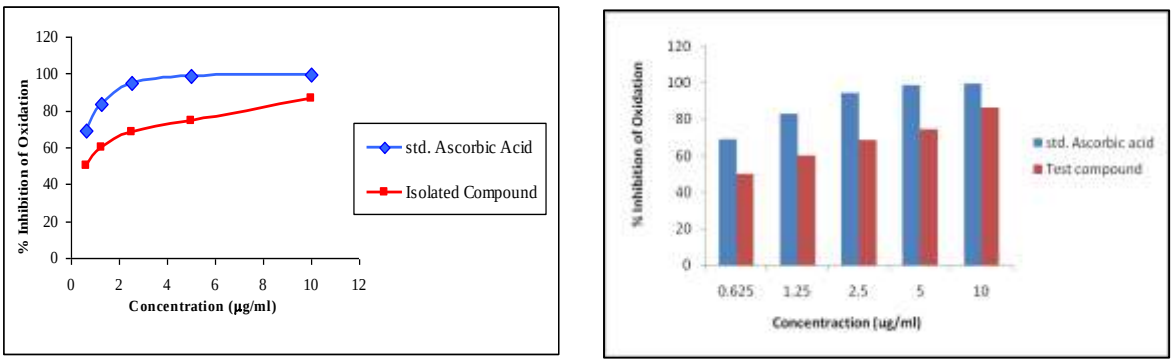


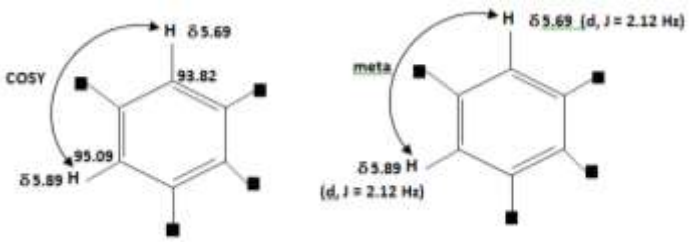
Figure 4 Plot of % inhibition vs concentration of isolated compound and standard ascorbic acid

As indicated in Figure 3, absorbance decreases as concentration increases in both of isolated compound and standard ascorbic acid. Decrease in absorbance implies increase in % inhibition of oxidation. In accordance with these figures, the absorbance values of isolated compound and standard ascorbic acid are considerably different at lower concentration (0.625 µg/mL). But at higher concentration level (10 µg/mL), the differences of absorbance between isolated compound and standard ascorbic acid become smaller. Hence at higher concentration level, the isolate compound responds high antioxidant activity.

Moreover, in Figure 4, % inhibitions of oxidation of isolated compound and standard ascorbic acid increase with the increase in concentrations of both of the two compounds. At higher concentration level (10 µg/mL), the isolated compound responds 85% inhibition of oxidation based upon the standard ascorbic acid.

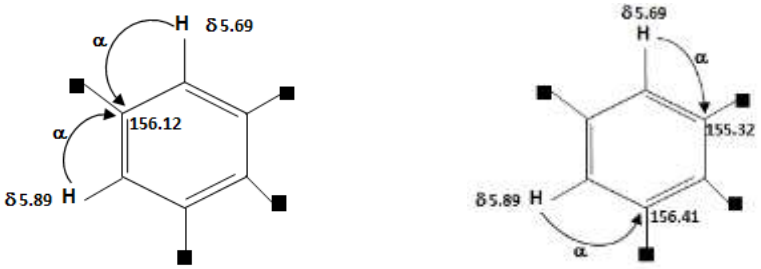
Structure Elucidation of Pure Compound

In the DQF-COSY spectrum, Figure 5, the observation of the small graphic area between the two aromatic protons at δ 5.69 ppm and 5.89 ppm leads to the following tetra substituted benzene fragment.

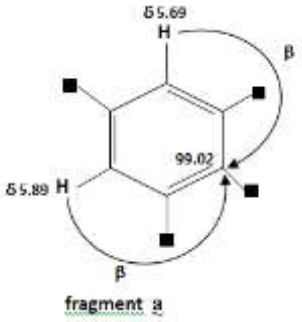


This fragment was supported by the splitting patterns and the coupling constants (J values) of these two aromatic protons at δ 5.69 ppm (d, J = 2.12 Hz) and δ 5.89 ppm (d, J = 2.12 Hz) in ^{1}H NMR spectrum, Figure 5, in which both protons should be oriented at meta position.

Moreover, the proton-carbon orientation of this tetrasubstituted benzene fragment could be determined by HMBC spectrum, Figure 5. In this spectrum, the occurrence of α ^{1}H-C long range signal of both of two aromatic protons at δ 5.69 and 5.89 ppm with the sp^2 quaternary carbon at δ 156.12 ppm and sp^2 quaternary carbons δ 155.32 and 156.41 ppm and indicated the following fragments.

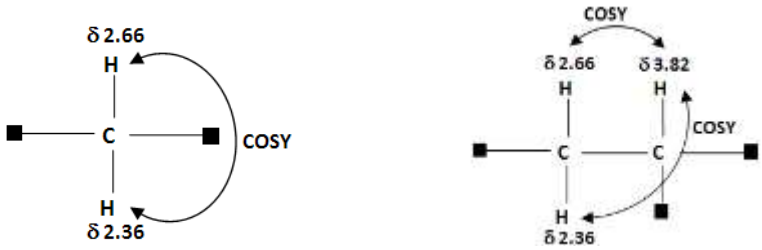


Furthermore, the fragment a could be assigned by the existence of β ^{1}H–C long range signal between both of two aromatic protons (δ 5.69 ppm and δ 5.89 ppm) and sp^2 quaternary carbon (δ 99.02 ppm) in HMBC spectrum, Figure 5, as shown below.



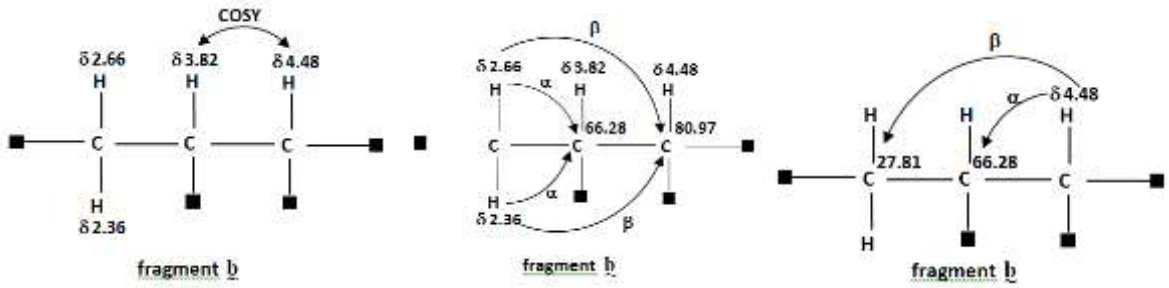
fragment a

On the other hand, the DQF-COSY spectrum, Figure 5, shows a large graphic area between the two geminal methylene protons (δ 2.36 ppm and δ 2.66 ppm) which produces the geminal fragment. In addition, the observation of medium graphic area of these methylene protons (δ 2.36 ppm and δ 2.86 ppm) with their adjacent sp^3 methine proton (δ 3.82 ppm) in the DQF-COSY spectrum, Figure 5, gives rise to the following fragment.

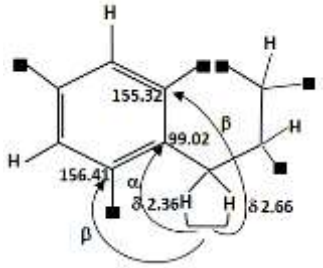


The extended fragment b could be determined by the observation of medium graphic area between sp^3 methine proton (δ 3.82 ppm) and another sp^3 methine proton (δ 4.48 ppm) in DQF-COSY spectrum, Figure 5. Moreover, this fragment b could be confirmed by HMBC spectrum, in Figure 5, in which the two geminal methylene protons (δ 2.36 ppm and δ 2.66 ppm) have α and

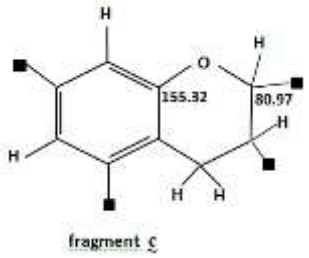
β ^{1}H-C long range coupling with sp^3 methine carbon (δ 66.28 ppm) and another sp^3 methine carbon (δ 80.97 ppm). Inversely, the determination of α and β ^{1}H-C long range signal of sp^3 methine proton (δ 4.48 ppm) with sp^3 methine carbon (δ 66.28 ppm) and methylene carbon (δ 27.81 ppm) in this HMBC spectrum.



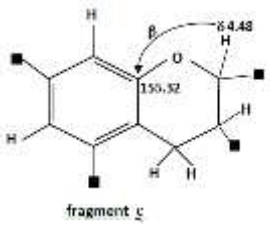
Furthermore, the connection between fragment a and fragment b could be done by HMBC spectrum, Figure 5. In HMBC spectrum, both of the two geminal methylene protons (δ 2.36 ppm and δ 2.66 ppm) have α and β ^{1}H-C long range coupling with three sp^2 quaternary carbons (δ 99.02 ppm, δ 155.32 ppm and δ 156.41 ppm) which leads to the following longer fragment.



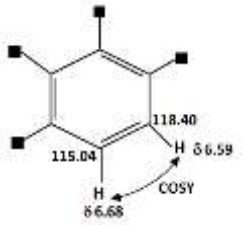
On the other hand, the FT-IR spectrum, Figure 5, give rise to the good evidence for the existence of ether functional group which appears at 1026.1 cm^{-1}. Thus logical correlation of ether oxygen atom to both of down field chemical shift sp^2 quaternary carbon (δ 155.32 ppm) and sp^3 methine carbon (δ 80.97 ppm) produces the most reliable fragment c.



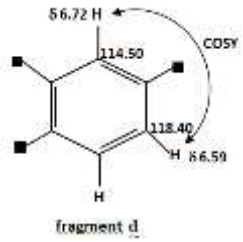
However, in HMBC spectrum, Figure 5, there is β ^{1}H-C long range signal between sp^3 methine proton (δ 4.48 ppm) and sp^2 quaternary carbon (δ 155.32 ppm) which confirms the fragment c as shown below.



Furthermore, in the DQF-COSY spectrum, Figure 5, the existence of medium graphic area between the two aromatic protons (δ 6.59 ppm and δ 6.68 ppm) implies the following another benzene fragment.

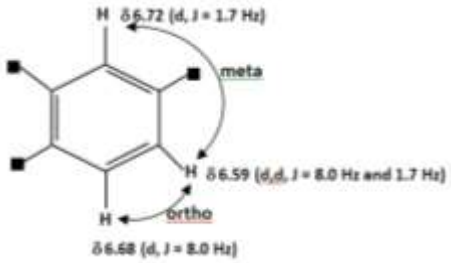


In addition, the occurrence of small graphic area between two aromatic protons (δ 6.59 ppm and δ 6.72 ppm) in the DQF-COSY spectrum, Figure 5, reveals the following trisubstituted benzene fragment d .



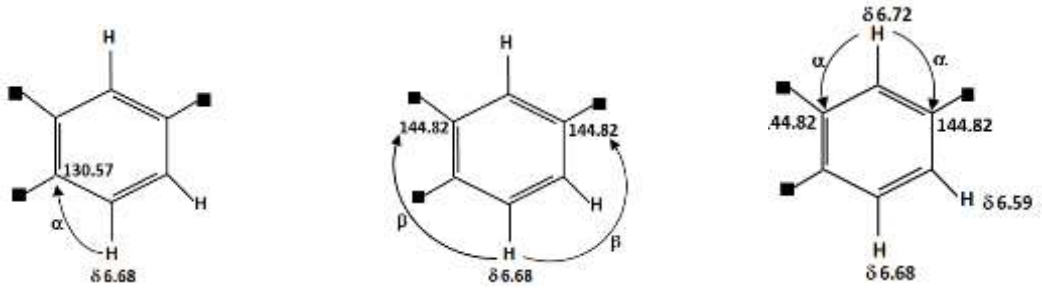
fragment d

The above trisubstituted benzene fragment could be confirmed by the splitting patterns and coupling constants (J values) of these three aromatic protons (^{1}H δ 6.59 ppm, d, d, J = 8 Hz and 1.7 Hz, ^{1}H δ 6.68 ppm, d, J = 8 Hz, and ^{1}H δ 6.72 ppm, d, J = 1.7 Hz) as shown below.

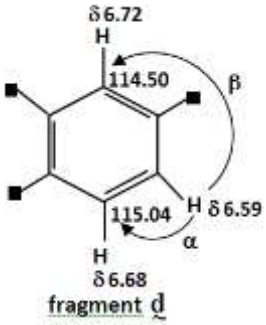


It means that both of these two aromatic protons (δ 6.59 ppm and δ 6.68 ppm) are ortho to each other and the two aromatic protons (δ 6.59 ppm and δ 6.72 ppm) are meta to each other.

The proton-carbon long range coupling of this trisubstituted aromatic benzene ring fragment could be also determined by HMBC spectrum, Figure 5. In this spectrum, the aromatic proton (δ 6.68 ppm) responds α ^{1}H-C long range signal with sp^2 quaternary carbon (δ 130.57 ppm). Moreover, in this spectrum, this methine proton (δ 6.68 ppm) also responds β ^{1}H-C long range signal with the two same chemical shift sp^2 quaternary carbons (δ 144.82 ppm). Furthermore, the HMBC spectrum, Figure 5, displays α ^{1}H-C long range coupling between the aromatic proton (δ 6.72 ppm) and both of two same chemical shift sp^2 quaternary carbons (δ 144.82 ppm) as shown below.

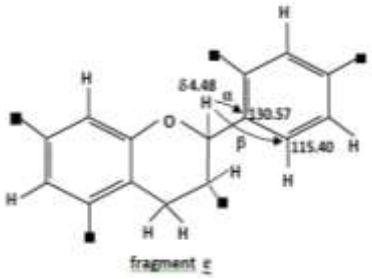


Moreover, the aromatic proton (δ 6.59 ppm) has α and β ^{1}H-C long range coupling with two aromatic carbons (δ 115.04 ppm and δ 114.50 ppm) in HMBC spectrum, Figure 5 which indicates the following fragment ḍ .



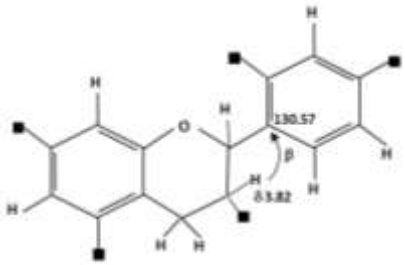
fragment ḍ

Meanwhile, the more longer fragment ḛ could be determined by the connection between fragment ḉ and fragment ḍ according to HMBC spectrum, Figure 5. In this spectrum, there is α and β ^{1}H-C long range signal of sp^{3} methine proton (δ 4.48 ppm) with two sp^{2} aromatic carbons (δ 130.57 ppm and δ 155.04 ppm) which leads to the following more longer fragment ḛ .

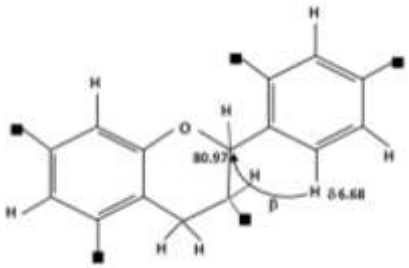


fragment ḛ

However, this HMBC spectrum, Figure 5, also responds the β ^{1}H-C long range coupling between sp^{3} methine proton (δ 3.82 ppm) and sp^{2} quaternary carbon (δ 130.57 ppm) which confirms the above fragment ḛ .

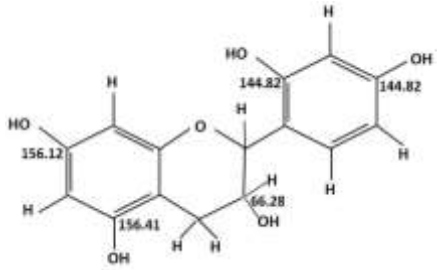


On the other hand, the correlation of β ^{1}H-C long range signal of aromatic proton (δ 6.68 ppm) with sp^{2} methine carbon (δ 80.97 ppm) in HMBC spectrum, Figure 5, also confirms this more longer fragment ḛ .

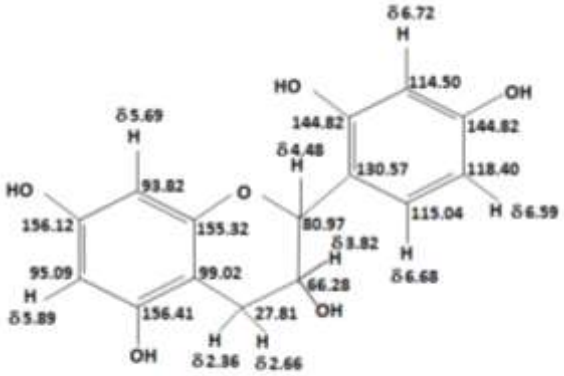


In this fragment ẹ , the remaining partial formula is calculated as H_5O_5, which are assumed to be five hydroxyl groups. It is supported by the FT-IR spectrum, Figure 5.

The complete structure of compound could be elucidated as described below, by the attachment of one –OH group to down field chemical shift of sp^3 carbinol methine carbon (δ 66.28 ppm) and the remaining four phenolic -OH functional groups to down field chemical shift of four aromatic quaternary carbons (δ 144.82 ppm, δ 156.12 ppm and δ 156.41 ppm) in two benzene rings.



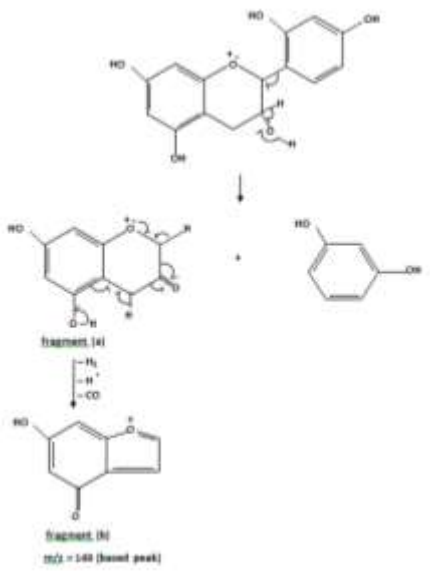
The complete planar structure of pure flavonoid compound could be assigned as follows.



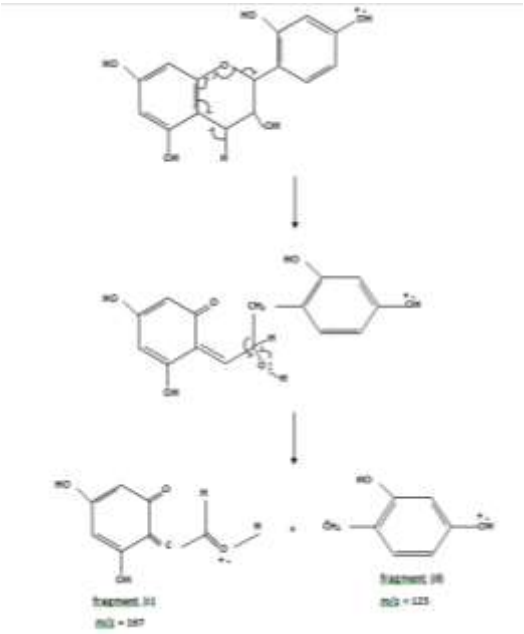
2– (2, 4-dihydroxy phenyl) chroman -3, 5, 7-triol

Confirmation of the Planar Structure of a Pure Flavonoid Compound by the Mass Fragmentation Behaviour

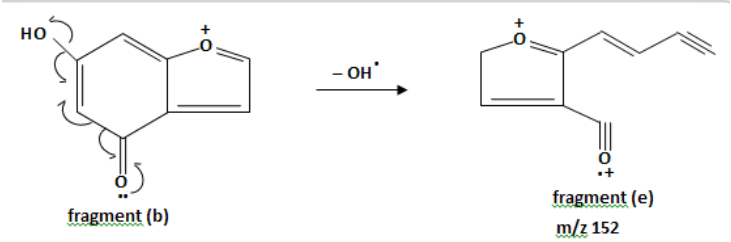
The proposed mechanism for the fragmentation pattern in EI mass spectrum was described below. The homolytic cleavage between $C_2 – C_{1'}$ leads to the fragment ạ and then liberation of carbon monoxide molecule, hydrogen molecule and hydrogen radical can give rise to the most intense base peak, fragment ḅ (m/z – 149).



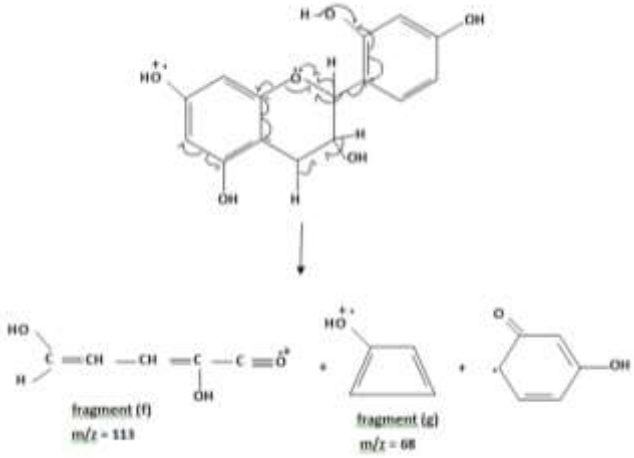
Moreover, the homolytic cleavages between C_2–O produces the intermediate fragment which also occurs the homolytic cleavage between C_2–C_3 give rise to the fragment ç (m/z 167) and the fragment d (m/z 123).



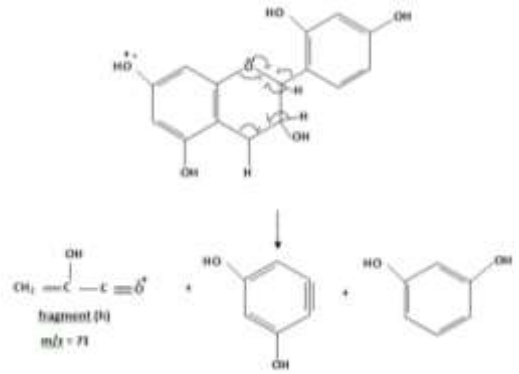
In addition, the abstraction of hydroxyl radical ($OH^{\bullet}$) from based peak, fragment b leads to the fragment e (m/z 152).



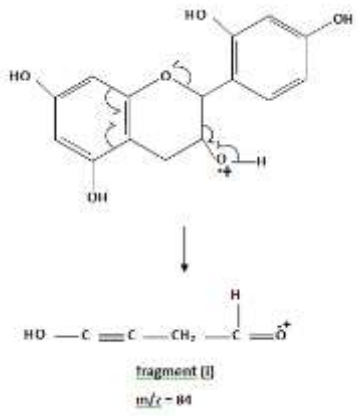
Then the fragment f (m/z 113) and fragment g (m/z 68) could be determined by the fragmentation between the $C_2 - C_{1'}$ and $C_6 - O$ of the molecular ion peak.



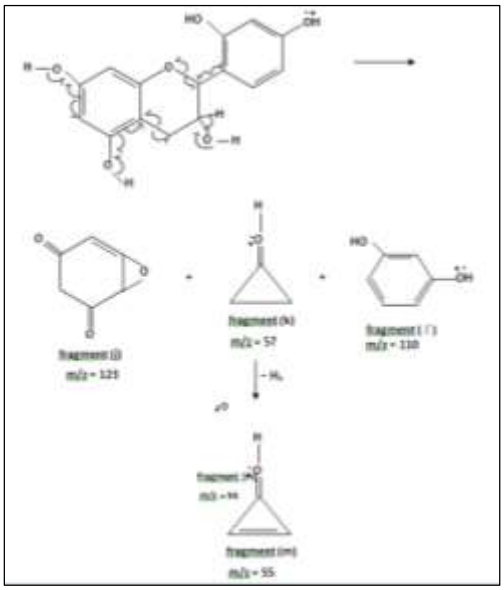
In addition, the fragment h (m/z 71) could be observed by the homolytic cleavages between $C_2 - C_{1'}$, $C_4 - C_{10}$ and $C_9 - O$ and liberation of hydrogen radical from the molecular ion peak.

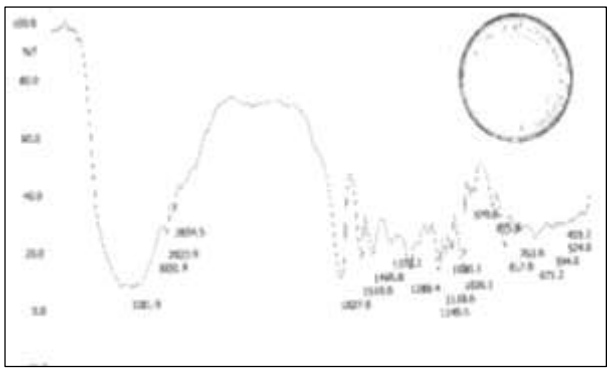


On the other hand, the homolytic cleavages between $C_2 - O$ and $C_2 - C_3$ from the molecular ion peak indicate the fragment i (m/z 84).

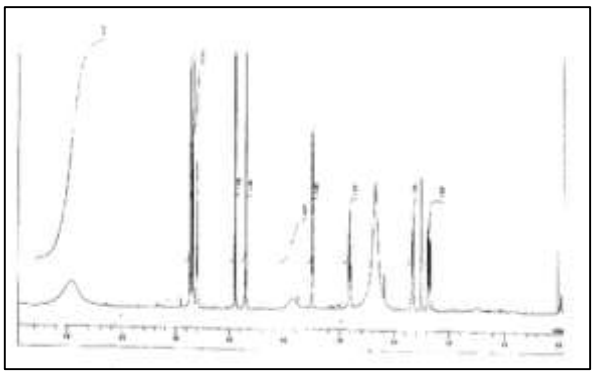


Furthermore, the three fragments j (m/z 123), k (m/z 57) and ℓ (m/z 110) could be observed by the homolytic cleavages between $C_2 - C_{1'}$, $C_4 - C_{10}$ and $C_2 - O$ from the molecular ion peak and then the liberation of hydrogen molecule from fragment k (m/z 57) occurs the fragment m (m/z 55).

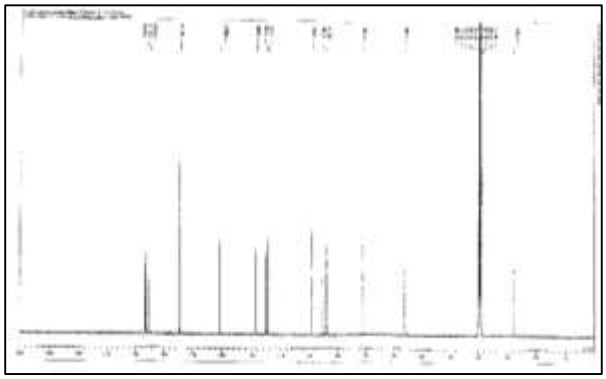




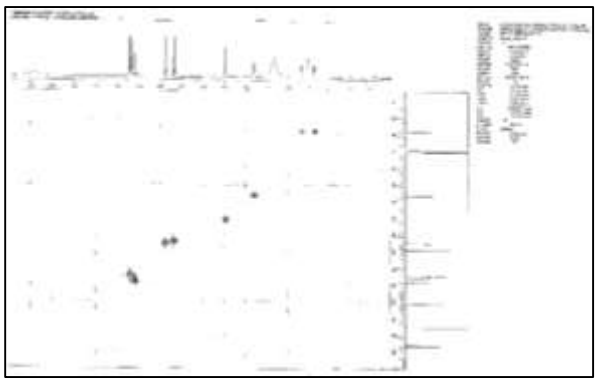
(a)



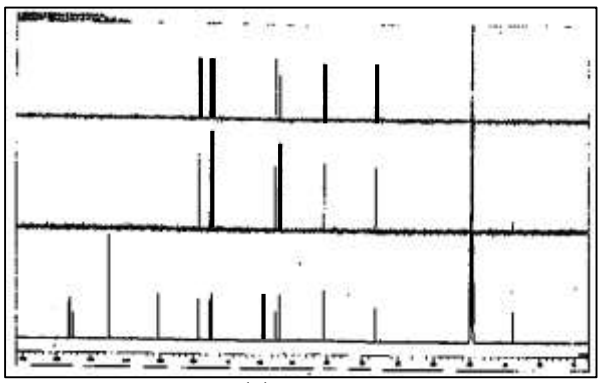
(b)



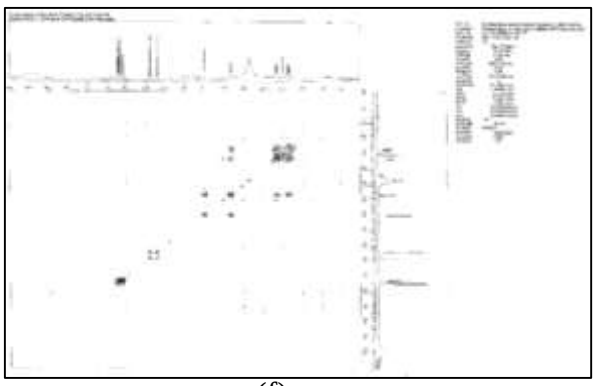
(c)



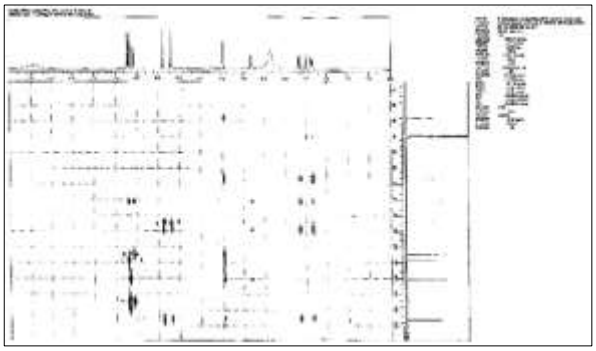
(d)



(e)



(f)



(g)

Figure 5 (a) FT IR spectrum (b) ^{1}H NMR spectrum (c) ^{13}C NMR spectrum (d) HSQC spectrum (e) DEPT spectrum (f) COSY spectrum (g) HMBC spectrum of isolated compound

Conclusion

In this research work, chemical investigation of the bark of *Albizia procera* was carried out. Pale yellow needle-shaped crystals, 2-(2,4-dihydroxy phenyl) chroman-3,5,7-triol was isolated from ethyl acetate extract and characterized spectroscopically. The isolated compound responded medium activities on five test microorganisms except mycobacteria. The isolated compound was further confirmed by flavonoid test. By DPPH assay, the antioxidant activity of isolated flavonoid compound showed the high antioxidant activity.

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