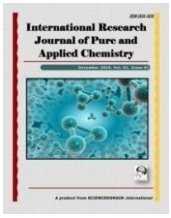

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Studies on the Isolation of 5, 3$^{/}$, 4$^{/}$-Trihydroxy, 7-Methoxy Isoflavone

Md. Siddiqur Rahman[1], Mst. Sarmina Yeasmin[1], A. S. M. Saem[2] and Md. Saifur Rahman[3*]

[1]BCSIR Laboratories, Binodpur Bazar, Rajshahi-6206, Bangladesh.
[2]Quality Assurance, Eskayef Bangladesh Limited, Dhaka, Bangladesh.
[3]Department of Applied Chemistry and Chemical Engineering, Rajshahi University, Rajshahi-6205, Bangladesh.

Authors' contributions

This work was carried out in collaboration between all authors. Author Md. Siddiqur Rahman designed the study and wrote the protocol. Authors Md. Siddiqur Rahman, MSY, ASMS and Md. Saifur Rahman preformed the statistical analysis, managed the literature search and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Studies were carried out on the leaves of *Cassia alata*. The leaf powder of *Cassia alata* was extracted with 80% ethanol defatted with n-hexane and resolved into water-soluble and water insoluble parts. The water-soluble part was then extracted with ethyl acetate and the extract was chromatographed over silica gel (70-230 mesh) column and successively eluted with a gradient mixture of organic solvent with increasing polarities. A new isoflavone 5, 3$^{/}$, 4$^{/}$-trihydroxy, 7-methoxy isoflavone was isolated by the elution of the column with ethylacetate and methanol (70:30 v/v) and the isolated compound was purified by preparative thin layer chromatography (PTLC). The compound was characterized on the basis of UV, IR, NMR, ^{13}C- NMR and Mass spectrometry.

**Corresponding author: E-mail: saifur_13bd@yahoo.com;*

Keywords: Cassia alata; isoflavone; ^{1}HNMR; ^{13}C NMR; mass spectrometry.

1. INTRODUCTION

Bangladesh being situated in the monsoon area of the world is a good treasure of indigenous plants of a large number of families; grow widely in forests, jungles, hillocks and gardens. *Cassia alata* (Dudmardan, *C. alata)* is one of such plant which had great potential as an important medicinal plant [1,2,3,4]. This plant is found in different parts of Bangladesh and the leaves are locally used for treating eczema and ringworm[2]. The leaves are taken internally as an aperient [5]. The use of medicinal plants for alleviating diseases originated from the activities of the most primitive man of the remote past. The use of plant materials to prevent and treat infectious diseases successfully over the years has attracted the attention of scientist worldwide [6].

C. alata (*Senna alata* (L.) Roxb.) is used in West Africa to treat parasitic skin diseases and pustular skin infections [7]. In the search for therapeutic agents from natural sources for the treatment of acquired immune deficiency syndrome (AIDS) patients the antibacterial and antifungal activities of water extracts of *C. alata* were investigated [8]. *C. alata* has wide range of medicinal uses in India and West Indies for the treatment of various ailments [9].

From the *C. alata* plant species both solvent and aqueous extracts were prepared and tested its toxicity against certain bio-molecules and metabolic enzymes which showed very high insecticidal activity [10].

C. alata has very high medicinal values like antimicrobial property particularly against fungal dermatophytes and traditionally it is used in the treatment of skin infections in man, leaf extract is also credited for the treatment of constipation, inguinal hernia, intestinal parasitosis, syphilis and diabetes [11]. In our previous studies, thirteen pathogenic fungi (6 human, 3 animal and 4 plant) were used in the fungicidal sensitivity test for the isolated pure compound 5, 3^{I}, 4^{I}-trihydroxy, 7-methoxy isoflavone which showed inhibition against most of the human pathogenic fungi; active against *Microsporum canis* and moderately active against *Trichophyton mentagrophytes* out of three animal pathogenic fungi and showed moderate activity against *Fusarium oxysporum* var. *lycopersici* and *Fusarium solania* var. *lycopersici* out of four plant pathogenic fungi [12].

Considering all the phenomena, the present work had, therefore been taken with a view to carry out a complete investigation of the plant *C. alata* for the isolation of some medicinally active compounds and accordingly a new isoflavone was isolated from the plant and the structure of the isoflavone was elucidated by using spectroscopic techniques. The previous compounds isolated from *C. alata* were 6, 8, 4′-trihydroxy isoflavone, 5, 7, 4′-trihydroxy flavanone, 3, 5, 7, 4′-tetrahydroxy flavone, 2, 5, 7, 4′-tetrahydroxy isoflavone, 5, 7- dihydroxy-3′, 4′-dimethoxy flavone and para-hydroxy benzoic acid.

2. MATERIALS AND METHODS

2.1 Materials

A reichart micro melting point apparatus was used for recording the melting point. UV spectra (MEOH) on a Shimadzu UV-240 spectrophotometer, IR spectra (KBr) on a Shimadzu IR-460 instrument, ^{1}HNMR spectra (CD_3OD) on a Bruker AM-500 FT NMR spectrometer (500 MHZ) using TMS as internal standard, ^{13}C-NMR spectra (CD_3OD) on a Bruker AM-500 FT NMR spectrometers (100 MHZ) and mass spectra on a Varian-MAT 112S spectrometer were recorded. Electron Impact (EI), Peak Matching experiments were performed on a MAT-312A mass spectrometer.

2.2 Extraction of Plant Sample

Fresh leaves were collected from the plants grown in the adjoining areas of BCSIR Laboratories, Rajshahi campus during August-September period. The leaves were washed with water to remove extraneous materials and then dried in shade. Care was taken to avoid exposure to sunlight. The dried material was crushed to powder. The air-dried *C. alata* leaf powder (6.6 Kg) was soaked in 80% ethanol for a week. The ethanolic extract was then filtered and the solvent was removed under reduced pressure to obtain a viscous residue (494 g). The crude residue was then defatted with n-hexane. The defatted mass was dried under reduced pressure to give a residue (162 g). The defatted extract was then treated with water, shaken well to resolve into water soluble and water insoluble parts. The water soluble part was extracted with ethyl acetate. The ethyl acetate soluble part was chromatographed over a silica gel (70-230 mesh)

column and successively eluted using a gradient of organic solvents with increasing polarity of n-hexane-ethyl acetate, ethyl acetate-methanol and methanol.

2.3 Isolation of Compound

The elution of the column was started with n-hexane: ethyl acetate (90:10, v/v 80:20, 70:30, 60:40, 50:50, 40:60, 20:80, v/v). Fractions of 500 mL were collected in conical flasks. It was then evaporated using a rotary vacuum evaporator. The temperature was not allowed to rise above 40°C. After the evaporation, the fractions were collected in glass vials with minimum volume of solvent. The same solvent system was continued until the fractions became colorless. Then the polarity of the solvent system was increased gradually using 80:20, 70:30, 60:40, 50:50, 40:60, 20:80 (v/v) of n-hexane: ethyl acetate and lastly with ethyl acetate. Every time the solvent system was continued until the fractions became colorless and after evaporation, the fractions were transferred into the glass vials with minimum volume of solvent.

The column was then eluted with ethyl acetate: methanol (90:10, v/v) and the polarity of the same solvent system was increased gradually to 80:20, 70:30, 60:40, 50:50, 40:60, 20:80 (v/v) of ethyl acetate: methanol and lastly with methanol to wash out of the column. Every time the solvent system was continued until the fraction became colorless. Evaporation of the solvents and collection of the fractions were done according to the previous case. Elution of the column with ethyl acetate and methanol (70:30 v/v) afforded a compound designated as compound-**1** along with minor impurities.

2.4 Purification of the Compound by Preparative Thin Layer Chromatography (PTLC)

The compound **1** with some impurities was applied to a PTLC card of silica gel 60GF254 (thickness 0.1mm) and eluted with ethyl acetate and methanol (9.2:0.8 v/v). A distinct single band (R_f= 0.66) was observed on the PTLC card. The band was collected and washed out with methanol to obtain light yellow powder (compound **1**, 10.5 mg, m.p. 252-254°C, R_f = 0.66).

2.5 Spectroscopic Analysis of Compound 1

UVλ_{max} (MeOH) (unit, nm): 346, 268, 249, 205, 195 (Fig. 2)
IR υ_{max} (KBr) (unit, cm^{-1}): 3400 (O - H), 2980 (C-H), 1660 (C = O), 1580 (C = C), 1270 (C = O).
EIMS m/z (rel. int %): 300 (100), 257 (8), 229 (8), 153 (25), 114 (11), 79 (16)
Peak matching m/z (formula): 300.06383 ($C_6H_{12}O_6$)
^{1}H-NMR δTMS (C_5D_5N): δ 6.95 (H-2, 1 H, s); δ 6.75 [H-6, 1 H, d, $J_{(H-6, H-8)}$ 2.07 Hz]; δ6.83 [H-8, 1 H, d, $J_{(H-8, H-6)}$ 2.07 Hz]; δ7.59 [H-2$^/$, 1 H, d, $J_{(H-2^/, H-6^/)}$ 2.07 Hz]; δ7.26 [H-5$^/$, 1 H, d, $J_{(H-5^/, H-6^/)}$ 8.3 Hz]; δ7.63 [H-6$^/$, 1 H, dd, $J_{(H-6^/, H-5^/)}$ 8.3 Hz, $J_{(H-6^/, H-5^/)}$ 2.07 Hz]; δ3.80 (3H, s, methoxy)
^{13}C-NMR (C_5D_5N): 148.0, 103.2, 181.7, 161.4, 98.9, 163.9, 94.0, 157.3, 103.6, 121.5, 129.5, 115.9, 150.7, 115.9, 129.5, 55.9 See (Table 1).

Table 1. ^{13}C-NMR (C_5D_5N, 100 MHz); Chemical shifts of 5, 3$^/$, 4$^/$-trihydroxy, 7-methoxy isoflavone

SL. No.	Multiplicity	^{13}C-NMR (δ)	^{1}H-NMR (δ)	$^1J_{HH}$ (Hz).
C-2	CH	148.0	6.95	S
C-3	C	103.2		
C-4	C	181.7		
C-5	C	161.4		
C-6	CH	98.8	6.75	d, j= 2.07
C-7	C	163.6		
C-8	CH	94.0	6.83	d, j= 2.07
C-9	C	157.3		
C-10	C	103.6		
C- 1$^/$	C	121.5		
C- 2$^/$	CH	129.5	7.59	d, j= 2.07
C- 3$^/$	C	115.9		
C- 4$^/$	C	150.7		
C- 5$^/$	CH	115.9	7.26	d, j= 8.3
C- 6$^/$	CH	129.5	7.63	dd, j= 8.3, 2.07
	OCH_3	55.9	3.80	s

SL. No. (Serial Number)

3. RESULTS AND DISCUSSION

The ethyl acetate triturate of the ethanolic extract of *C. alata* leaves yielded compound **1** (Fig. 1) as a light yellow solids after purification by preparative TLC. Compound **1** was suggested as flavonoid due to its light yellow appearance on silica gel card and deep yellow after spraying with ceric sulphate reagent.

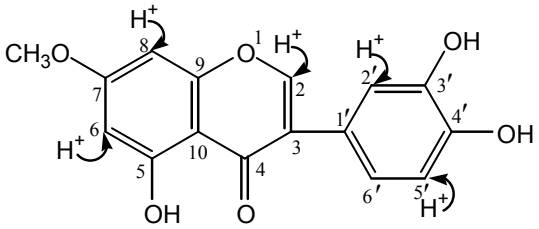

Fig. 1. Arrow Diagram of 5, 3′, 4′-trihydroxy, 7-methoxy isoflavone (1)

The absorptions at 3400 and 1660 cm^{-1} in the IR spectrum of (KBr) of Compound **1** were indicative of hydroxyl and carbonyl functions of the molecule, respectively. The IR spectrum also showed absorption bands at 2980 and 1580 cm^{-1} due to C-H and C=C functions, respectively.

The EI mass spectrum depicted in Figs. 3 and 4, showed the molecular ion as well as base peak at m/z 300.

The molecular formula was established with the help of ^{1}H NMR, ^{13}C NMR (Table 1) and peak matching experiments as $C_{16}H_{12}O_6$ corresponding to the mass m/z 300.06383 reported in Figs. 5, 6 and 7, respectively.

The broad band ^{13}C-NMR spectrum of copound-**1**, showed 16 signals including one methoxy, six methine and nine quaternary carbons. The multiplicities were determined with the help of DEPT experiment.

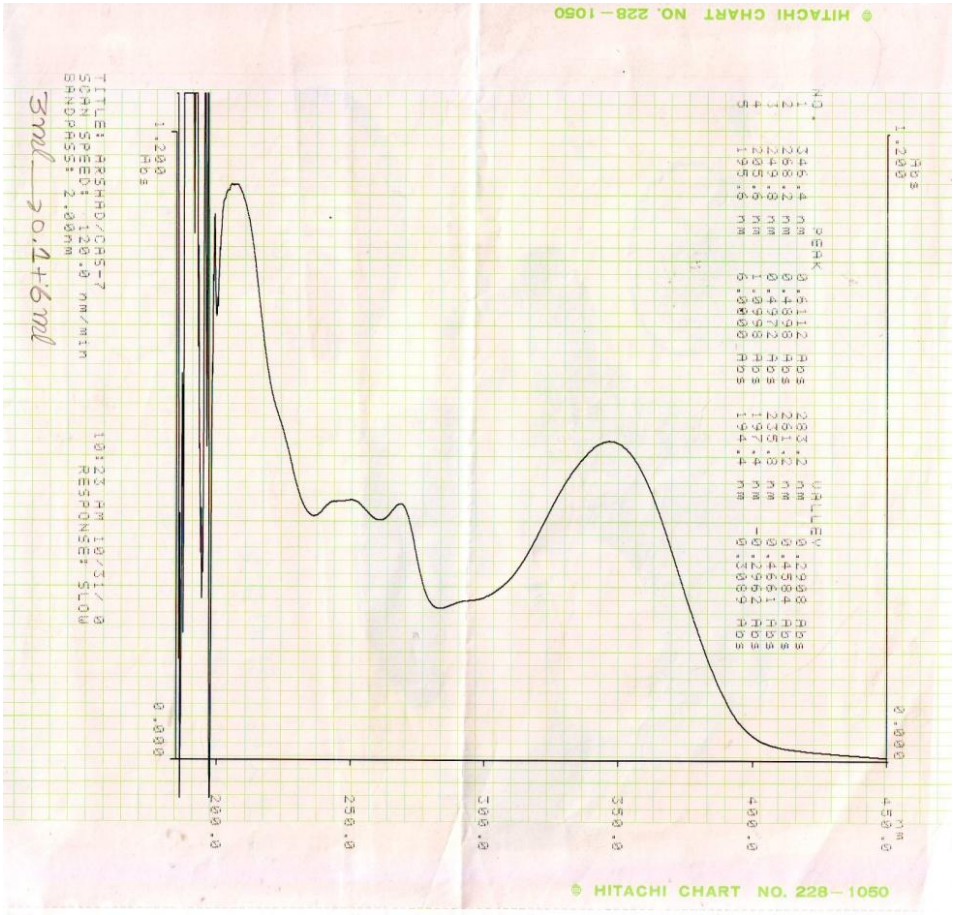

Fig. 2. UV spectra of 5, 3′, 4′-trihydroxy, 7-methoxy isoflavone (1)

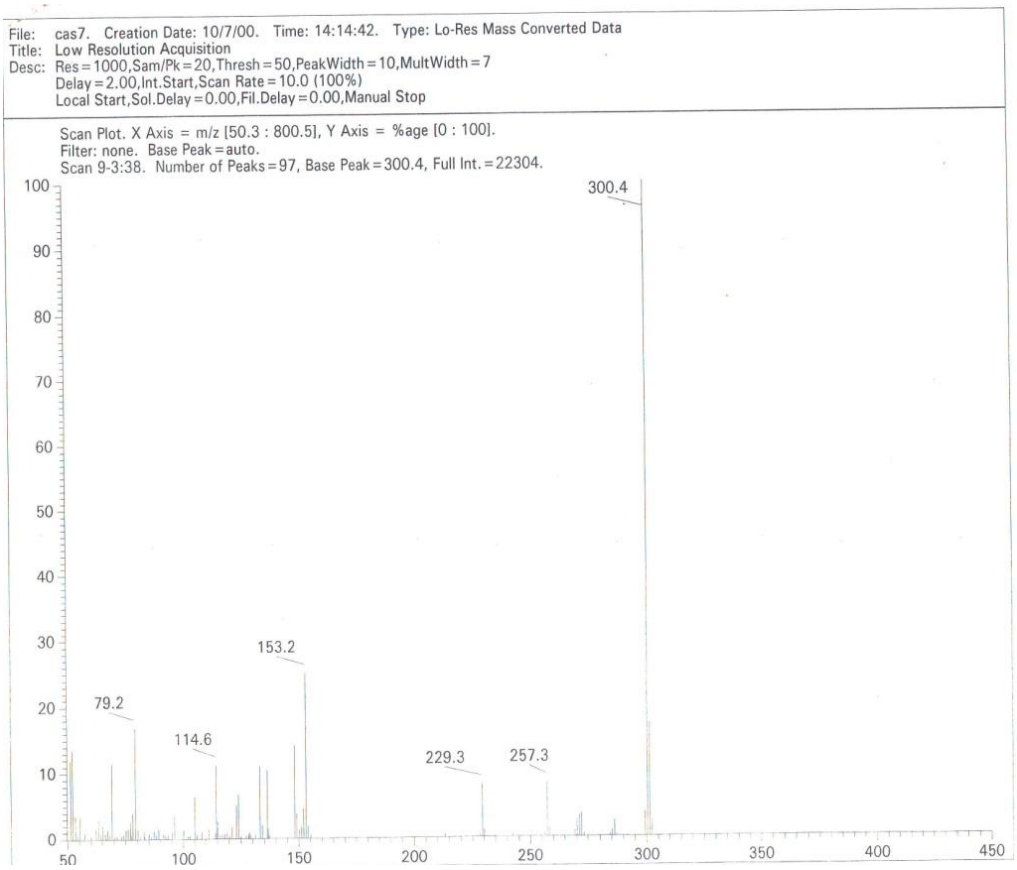

Fig. 3. Mass spectra of 5, 3′, 4′-trihydroxy, 7-methoxy isoflavone (1)

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m/z	Int.	%age	m/z	Int.	%age	m/z	Int.	%age
51.3	2650	11.88						
52.2	3013	13.51						
53.3	791	3.55						
55.3	754	3.38						
63.2	679	3.04						
65.2	450	2.02						
69.1	2569	11.52						
77.1	577	2.59						
78.1	892	4.00						
79.2	3748	16.80						
96.2	764	3.43						
105.2	1436	6.44						
114.6	2490	11.16						
115.1	572	2.56						
123.1	1127	5.05						
124.1	1494	6.70						
133.2	2452	10.99						
134.2	447	2.00						
136.2	2307	10.34						
148.2	3149	14.12						
149.2	833	3.73						
152.2	1004	4.50						
153.2	5584	25.04						
229.3	1788	8.02						
257.3	1834	8.22						
270.3	574	2.57						
271.3	715	3.21						
272.3	794	3.56						
286.3	546	2.45						
299.3	819	3.67						
300.4	22304	100.00						
301.4	3880	17.40						
302.4	539	2.42						

Fig. 4. Mass converted data of 5, 3′, 4′-trihydroxy, 7-methoxy isoflavone (1)

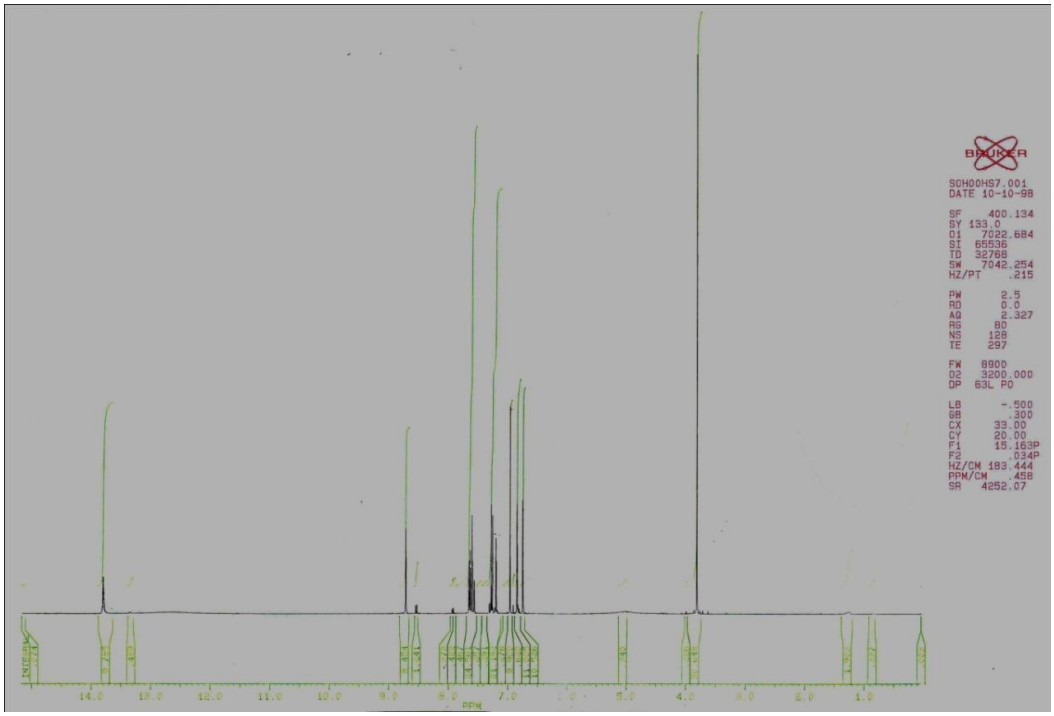

Fig. 5. ^{1}H-NMR spectra of 5, 3$^{/}$, 4$^{/}$-trihydroxy, 7-methoxy isoflavone (1)

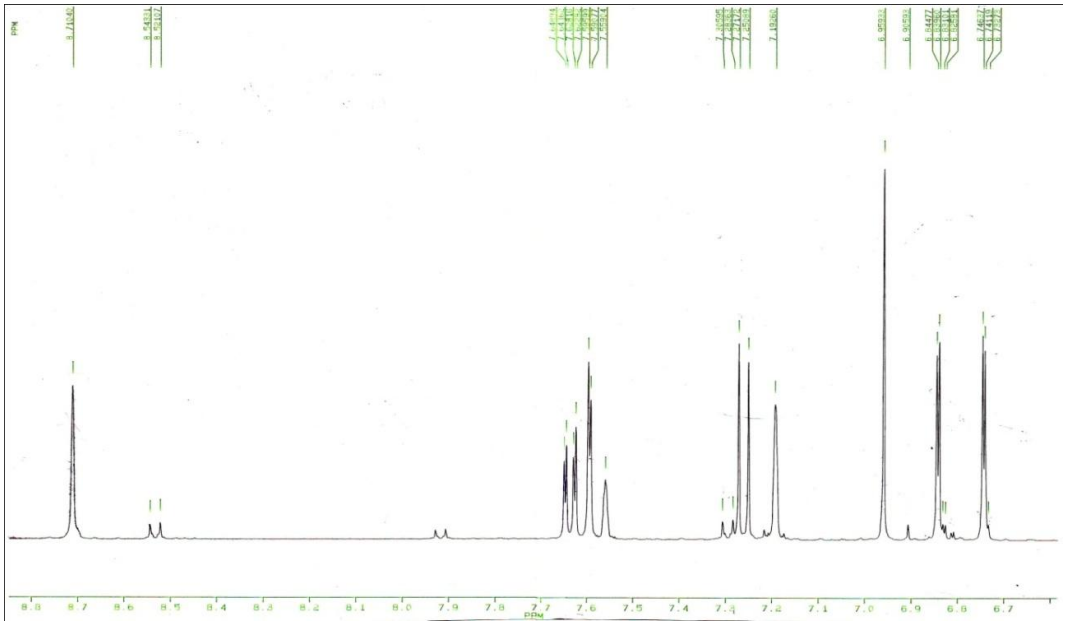

Fig. 6. ^{1}H-NMR spectra of 5, 3$^{/}$, 4$^{/}$-trihydroxy, 7-methoxy isoflavone (1) **(expanded)**

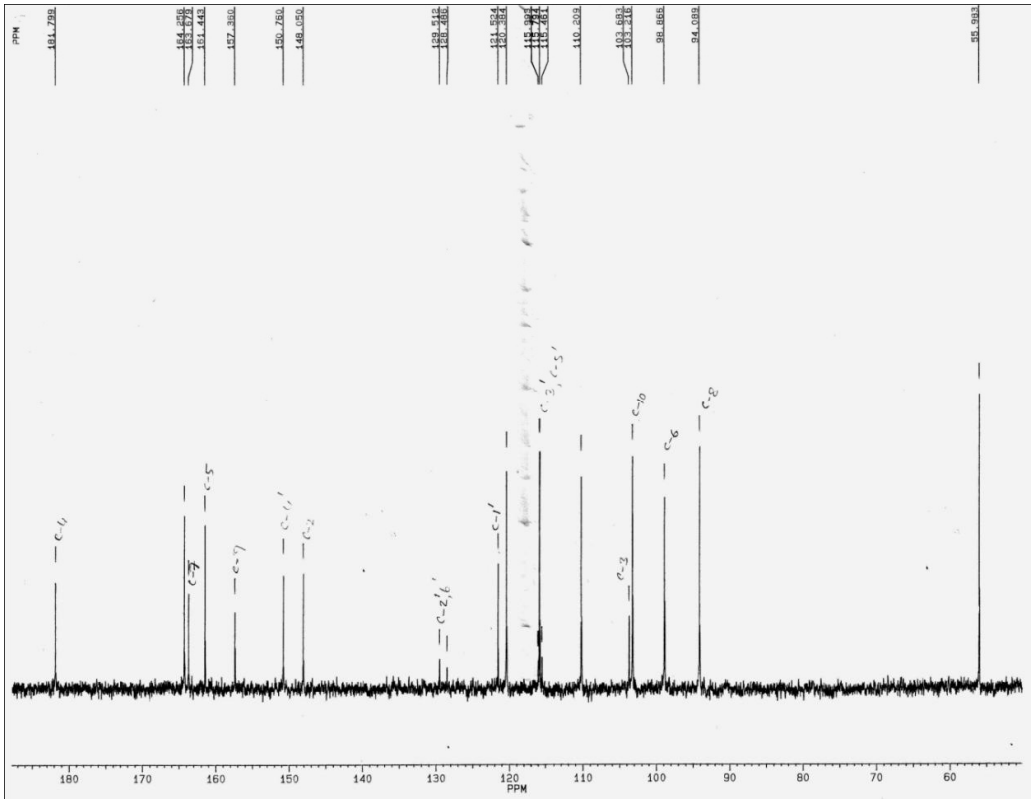

Fig. 7. ^{13}C **-NMR spectra of 5, 3$^{/}$, 4$^{/}$-trihydroxy, 7-methoxy isoflavone (1)**

The ^{1}H-NMR spectrum (Fig. 5) of compound **1** showed a broad singlet of one proton at δ6.95 (H-2). The same spectrum displayed a doublet of one proton at δ6.75 with a coupling constant 2.07 Hz for H-6 position. Another doublet of one proton resonated at δ6.83 having coupling constant 2.07 Hz was for H-8 position. The ^{1}H-NMR also displayed a doublet of one proton at δ7.59 with a coupling constant 2.07 Hz for H-2$^{/}$ proton. Another doublet at one proton resonated at δ7.26 having coupling constant 8.3 Hz was for H-5$^{/}$ proton. One proton at H-6$^{/}$ position gave a double-doublet at δ7.63 having coupling constants 8.3 and 2.07 Hz, respectively. A singlet of three protons at δ3.80 in the ^{1}H-NMR showed the presence of a methoxy group.

4. CONCLUSION

On the basis of the IR, ^{1}H NMR, ^{13}C NMR and EI Mass spectroscopic evidences, it became apparent that the compound 1 belonged to the isoflavone series and was characterized as 5, 3$^{/}$, 4$^{/}$-trihydroxy,7-methoxy isoflavone. As the flavonoids have bioactive compounds, so the isoflavone compound isolated from *C. alata* demands the investigation of its anti-microbial, antifungal, antibacterial and cytotoxic activities. It is assumed that these investigations may open up a new era in the public health sector of Bangladesh in particular and the world as a whole in protecting the people from the adverse effects of different infectious diseases.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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