SMU Medical Journal

Indexed in SIS (USA), ASI (Germany), I2OR & i-Scholar (India), SJIF (Morocco) and Cosmos Foundation (Germany) databases. Impact Factor: 3.835 (SJIF)

ISSN : 2349 – 1604 (Volume – 4, No. 2, July 2017) Research Article

Isolation and Characterization of a Compound from the Leaves of *Amaranthus spinosus* linn.

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Manuscript received: 08.05.2017
Manuscript accepted: 01.06.2017

Abstract

By solvent extraction, acid hydrolysis, chromatography followed by crystallization, a compound was isolated from the leaves of *Amaranthus spinosus* Linn. Infra red spectroscopy, mass spectroscopy and nuclear magnetic resonance studies showed that the isolated compound was chemically 3,4 dihydroxy benzoic acid.

***Keywords*:** *Amaranthus spinosus* Linn., chromatographic techniques, 3,4 dihydroxy benzoic acid

Introduction

Amaranthus spinosus Linn. (*A. spinosus* L.), a medicinal plant under the family of amaranthaceae, is distributed in lower to middle hills (3000–5000 ft) of entire north eastern Himalayas. The plant grows in cultivated areas as well as in waste places. Leaves of *A. spinous*

L. are stacked and alternate. The plant is known as "prickly amaranthus" in English and "ban lure"or "dhuti ghans" in Nepali. Medicinal uses of *A. spinosus* L. as mentioned in Ayurvedic text [1,2] are: Leaf infusion is diuretic and used in anemia. Root paste is used in gonorrhea, eczema, menorrhea etc. Besides, *A. spinosus* L. is used as laxative, diuretic, digestive and anti pyretic. It is also used to treat anorexia, leprosy, blood diseases, burning sensation, bronchitis, piles and leucorrhoea. The plant is further reported having anti-inflammatory properties, immunomodulatory activity and has effect on hematology [3-7]. Recent studies showed antidiabetic property of *A. spinosus* L. [8-10]

Ethnic use of *A. spinosus* L. is mainly with village-people of Sikkim who use leaf infusion of the plant in stomach disorder specially in case of indigestion and peptic ulcer [10]. Hussain et al (2009) showed that ethanol extract of whole plant of *A. spinosus* L. has anti diarrheal and anti ulcer activity in experimental animals [4]. Recently we confirmed anti ulcer activity of *A. spinosus* L. in ethanol induced gastric ulcer and cysteamine induced duodenal ulcer in albino rats [11].

Considering the medicinal importance of *A. spinosus* L. phytochemical studies of the plant were extensively undertaken. Phenol, sitosterol, stigmasterol, essential oil, friedolin, and unidentified esters were found as active components of *A. spinosus* L.[12,13].

Recently we have isolated and characterize a compound from the leaves of *A. spinosus* L. Results are being reported in this communication.

Materials and methods

Plant Material

Leaves of *A. spinosus* L. were collected from the medicinal plants garden of the University of North Bengal and authenticated by the experts of the department of Botany of the said University. A voucher specimen was kept in the department of Medical Biotechnology, Sikkim Manipal Institute of Medical Sciences of Sikkim Manipal University, Gangtok, Sikkim,

Fig. 1: *Amaranthus spinosus* L. Leaves

India for future references. Leaves were shade dried and powdered. The powder was used for extraction and isolation studies.

Extraction and Isolation

First step: 50g of powdered leaves of *A. precatorius* L. were extracted with 500 ml of 10 : 1 (v/v) chloroform – ethyl alcohol mixture in a soxhlet machine for ½ h at room temperature (15-18 ^{0}C). It was then centrifuged. Supernatant was collected and evaporated to dryness. Dry brown mass was obtained.

Second step*:* Dry brown mass was refluxed with 100 ml of 1(N) HCl for 10 minutes on a water bath at 100 ^{0}C. It was cooled and centrifuged. Supernatant was evaporated to dryness .

Third step: Dry brown mass thus obtained from the supernatant was extracted with 20 ml of ethyl acetate on a rotary shaker for ½ h. It was evaporated to dryness.

Fourth step: Brown mass obtained was dissolved in 10 ml chloroform and subjected to

column chromatography using silica gel G as adsorbent. 5 bands were separated. Bands were collected in separate beakers . Elution was done by 50% acetone – chloroform mixture.

Fifth step: Second band was separately evaporated to dryness. The dry mass was extracted with 10 ml ethyl acetate for 10 minutes. It was then filtered. With filtrate polyamide column chromatography was done. Elution was made by ethyl formate : formic acid mixture (60 : 40 v/v). Five bands were separated.

Sixth step: The third band was evaporated to dryness. Repeated crystallization was done from ethyl acetate–formic acid (60:40, v/v) mixture. Crystals obtained. Yield was 5.3 mg.

Homogeneity of the active compound

This was ascertained by silica gel- G thin layer chromatography by using the following solvent systems; Acetone : methanol : water - 50 : 25 : 25; n-butanol : acetic acid : water - 60 : 20 : 20; Chloroform : methanol : water - 40 : 30 : 30

Structure determination

FT-IR spectrum of the sample was taken in KBr pellets using Shimadzu FT-IR 8300 Spectrophotometer. NMR spectrum was taken using Bruker AVH 300 Spectrometer operating at 300 MHz (for ^{1}H) and 75 MHz (for ^{13}C) and in solvent, as indicated. ^{13}C NMR spectrum was run in ^{1}H-decoupled mode. The High Resolution Mass Spectral data for the compound was obtained in Mass Spectrometer (Model: Micromass Q-Tof Micro), run under Electron Spray Ionization (ESI) Positive Mode. Melting point was observed in an open sulfuric acid bath and is uncorrected.

Results and Discussion

Homogeneity of the active compound

In all cases of thin layer chromatographic experiments using three different solvent systems single spot was obtained. Thus, it was a single compound.

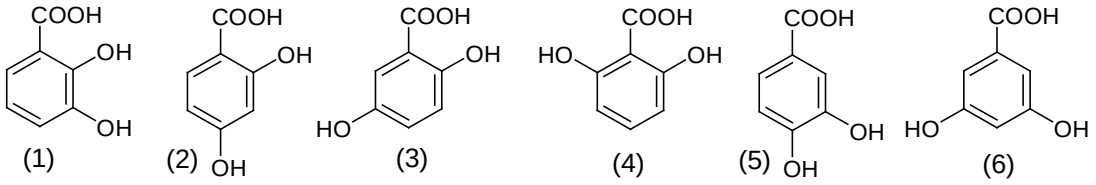
Structure elucidation

The isolated compound was colorless solid, mp. 200-202 ^{o}C

The ^{1}H-NMR in D_6-DMSO displayed as: δ 6.80 (d, 1H, J = 8.1 Hz), 7.31 (dd, 1H, J = 8.1 & 2.1 Hz), 7.36 (d, 1H, J = 2.1 Hz), 9.30 (br. s, 1H), 9.67 (br. s, 1H), 12.36 (br. s, 1H) ppm.

^{13}C-NMR (D_6-DMSO): δ 115.5, 116.9, 122, 122.3, 145.2, 150.3, 167.7 ppm.

The ^{1}H-NMR spectral data displayed three aromatic hydrogens suggesting that other three positions of a benzene ring might be substituted. On the other hand, three broad singlets could be assigned for hydrogens attached with heteroatoms. By comparison, it may be suggested that these three broad singlets correspond to one carboxylic acid proton and two hydroxyl protons. Therefore the compound could be a dihydroxy benzoic acid. Based on three substituents in one aromatic ring, following isomers of dihydroxy benzoic acid are possible:



Two aromatic protons appearing at δ 7.36 and 7.31 ppm suggest that there might be some electron-withdrawing “deshielding” effect of the carboxylic acid group on the *ortho*-hydrogens. It is therefore presumed that the *ortho* positions of the carboxylic acid are un-substituted. As such, isomers (1) – (4) may be ruled out and we are left with the structures (5) or (6). In compound (6) i.e. 3,5-dihydroxybenzoic acid, two *ortho* Hs are chemically and magnetically

equivalent and they should have same chemical shift and be displayed as *meta* coupled two Hs having coupled by the non-equivalent H at C-4. On the other hand, in compound (5), all three Ar-Hs are magnetically non-equivalent and would be displayed at different chemical shift (δ)

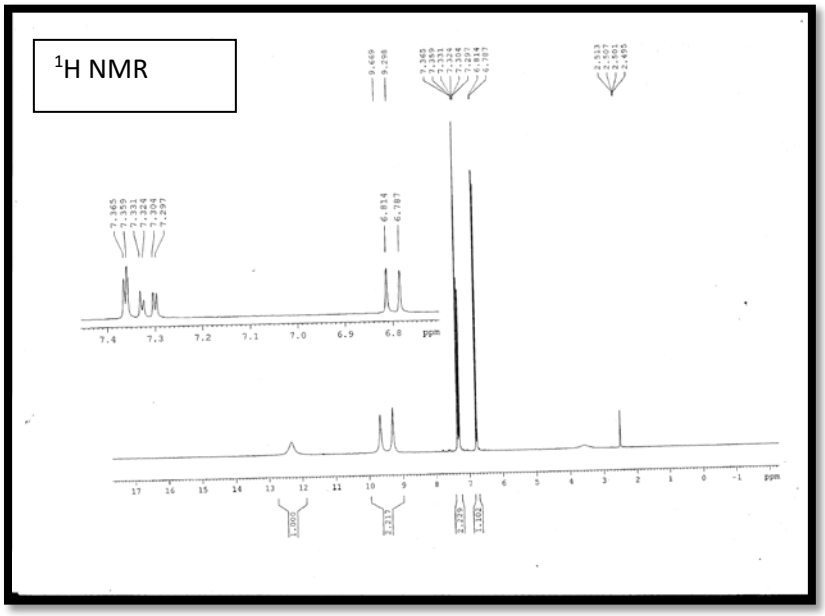


Figure 2. 1 H NMR spectrum of the isolated compound

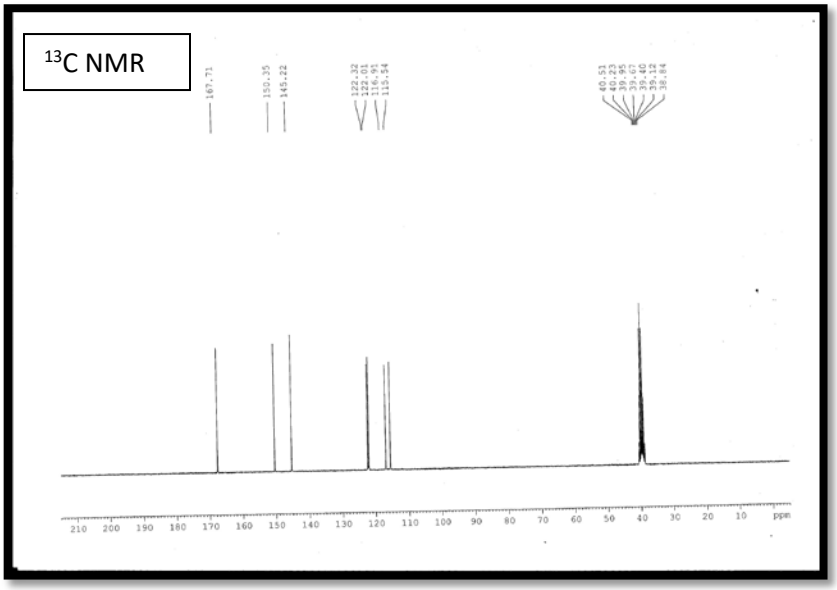


Figure 3. 13C NMR spectrum of the isolated compound

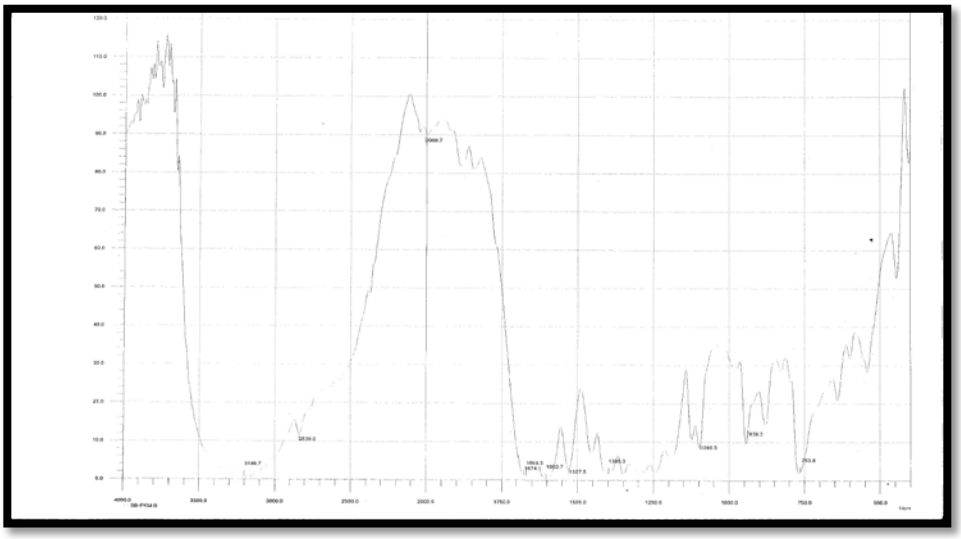


Figure 4. IR spectrum of the isolated compound

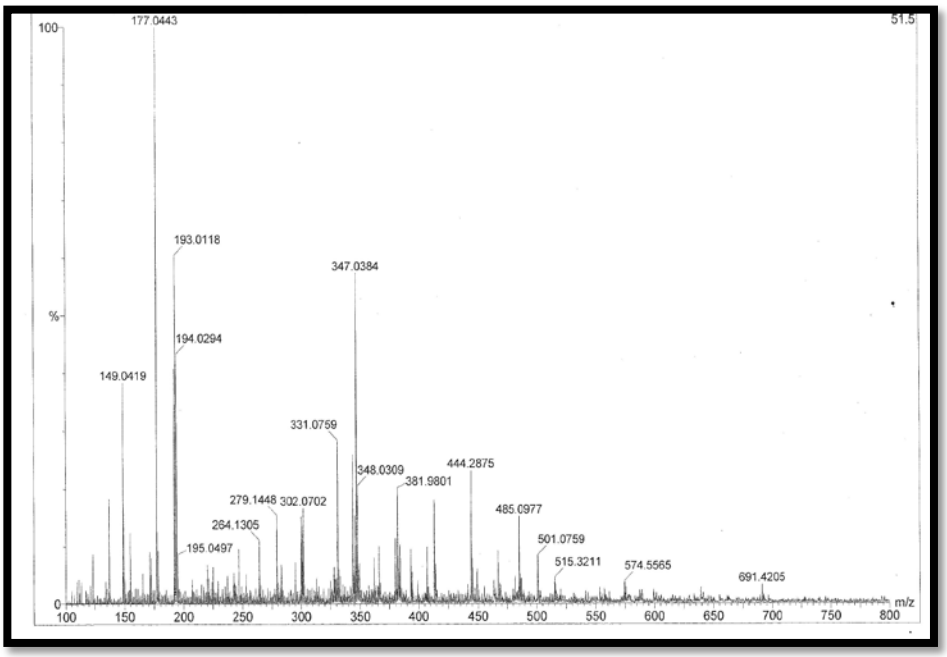
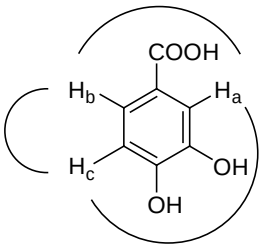


Figure 5. Mass spectrum of the isolated compound

values and with different splitting patterns. We therefore assign our compound as the compound (5), i.e. 3,4-dihydroxy benzoic acid. The Ar-Hs are indicated as follows:



(5)

H_a is *meta* coupled by H_b with J = 2.1 Hz,
H_b is *ortho* and meta coupled by H_c and H_b respectively with J = 8.1 & 2.1 Hz,
H_c is *ortho* coupled by H_b with J = 8.1 Hz

Figure 6. Spin-spin couplings between hydrogen in the structure of the isolated compound.

Thus, H_a would be coupled by H_b (*meta*, J = 2.1 Hz) and appears as a doublet. H_b would be coupled by H_a (*meta*, J = 2.1 Hz) and by H_c (*ortho*, J = 8.1 Hz) and appears as a doublet of a doublet (dd). H_c would appear as a doublet, only coupled by H_b as *ortho*-couple (J = 8.1 Hz).
In the H-decoupled ^{13}C-NMR spectrum of compound, it is expected that all six aromatic carbons would be magnetically non-equivalent and adding the carboxylic carbon, there should be total seven different carbons. Indeed, there are seven peaks in the H-decoupled ^{13}C-NMR spectrum. The assignment of various carbons may be made based on possible substituent effect: 115.5 (C-5), 116.9 (C-2), 122 (C-6), 122.3 (C-1), 145.2 (C-3), 150.3 (C-4), 167.7 (COOH),
FT-IR: (KBr) ν_{max} 3200, 2839, 1674, 1603 cm^{-1}.

The OH groups and COOH having conjugated with the benzene ring in its IR spectrum are displayed at ν_{max} 3200 and 1674 cm^{-1}, respectively, while the benzene ring double bonds showed absorption at 1603 cm^{-1}. Such decrease in absorption frequency for the carboxyl carbonyl function is acceptable because it is conjugated to the benzene ring.
HRMS: The exact mass for compound with mf $C_7H_6O_4Na$ [M^+Na] calculated to be 177.0164 and observed as 177.0443 again confirm for the compound. Hence the structure of Compound is 3,4-dihydroxybenzoic acid.

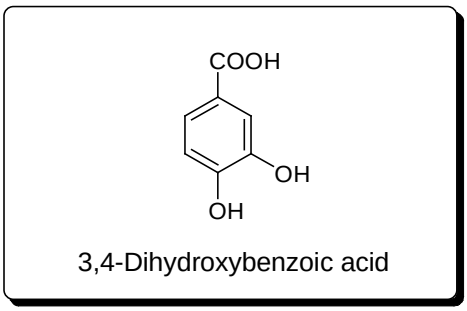


Figure 7. Name and structure of the isolated compound

Conclusion

A compound was isolated from the leaves of *A. spinosus* L. From spectral data the compound was characterized as 3,4 dihydroxy benzoic acid. In the list of phytochemicals of *A. precatorius* L. leaves, 3,4 dihydroxy benzoic acid will now be included.

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Prof. (Dr.) Prasanta Kumar Mitra is a very senior medical teacher and researcher. He has about 40 years experience in medical teaching and research. His research area is 'Medicinal plants of India'. He has four Ph.D.s to his credit and published one hundred fifty nine research papers in peer reviewed national & international journals of repute. 15 students did Ph.D. work under his guidance. He was co-supervisor of the research projects of 5 MD students.

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