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Phytochemical Investigation, Fatty Acid Analysis and In Vitro Membrane Stabilizing Activity of the Roots of *Amaranthus spinosus* L.

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Abstract

Ethylacetate extract of the roots of *Amaranthus spinosus* L. was subjected to phytochemical investigation and three compounds stigmasterol, 1-Eicosanol and oleic acid were isolated in pure state. The n-hexane extract was analysed for fatty acid with GC-FID and four saturated fatty acids; caprylic acid, stearic acid, arachidic acid and behenic acid, and four unsaturated fatty acids; palmitoleic acid, oleic acid, linoleic acid and erucic acid were identified and quantified. Different extracts were assessed to explore their *in vitro* membrane stabilizing activity using standard protocol. Methanol extract of *A. spinosus* showed 68.13% inhibition in hypotonic solution-induced hemolysis and 74.53% inhibition in heat induced hemolysis which was the highest than its other Kupchan fractions. Acetyl salicylic acid was used as standard that showed 42.00% inhibition of hemolysis at normal condition.

Keywords: Secondary metabolites, Membrane stabilizing activity, Fatty acids.

I. Introduction

Amaranthus spinosus L. is a medicinal plant of Amaranthaceae family found in tropical, subtropical and Himalayan region[1]. In Bangladesh it is known as katanotey (Chittagong), khuira kata (Mymensingh) according to the Bangladesh Ethnobotany online database[2]. It is an erect, monoecious herb, up to 100–130 cm tall grows annually as a weed in cultivated as well as fallow lands[3]. This plant is used as food in different tropical countries, especially tribal and mass people[4]. This plant has been reported to have some pharmacological properties. It's used in traditional medicines are very effective, have lower side effects, are accessible and affordable with low cost. It is used as antipyretic, laxative, stomachic, febrifuge traditionally[5]. The seed of it is used as a poultice for broken bones and the root of it is used to treat gonorrhoea[6]. It has been reported in modern scientific analysis that it has anti-depressant activity, anti-diabetic properties, anti-inflammatory activity, immunomodulatory activity, antibacterial property, anti-hyperlipidemic, spermatogenic activity, anti-malarial and effect on hematology[7-12]. *A. spinosus* is a good source of fatty acids such as stearic acid, oleic acid, palmitic acid, linoleic acid[4,5]. The plant contains some bioactive phytochemicals like n-alkanes, octacosanoate, hentriacontane, sterols including fatty acids, free alcohols, α-spinasterol, β-sitosterol, campesterol, cholesterol, stigmasterol, proteins and mixture of saponins, D-glucose and D-glucuronic acid. It is a good source of calcium and also contains phosphorous, iron, nicotinic acid, ascorbic acid and protein[5,13].

Previously less attention was observed on membrane stabilizing activity of *A. spinosus*. As they contain significant antioxidant[10] properties, they might have membrane stabilizing activity. Therefore, based on the evidence found from literature survey the present study was taken to investigate the phytochemicals and fatty acid analysis of the roots of *A. spinosus* and *in vitro* membrane stabilizing activity.

II. Experimental

Collection of the plant materials

The roots of the plant were collected from Gazipur, Bangladesh and taxonomic identification was made by the Department of Botany, University of Dhaka. The collected roots were cleaned to remove mud and dust particles. The roots were dried at room temperature followed by in an oven below 40°C. A grinder (Cyclotec 200 meshes) was used to grind the dried roots to powder. The root powder was stowed in an airtight bottle and used during the investigations.

Phytochemical screening

To identify the phytoconstituents such as tannin, phlabotannins, alkaloid, saponin, flavonoid, steroid, terpenoid, and cardiac glycoside etc. different phytochemical tests were done using standard protocols[14].

Extraction

The powdered roots of *A. spinosus* (360 g) were extracted with n-hexane followed by ethyl acetate (EtOAc). Using filter paper through a funnel these extracts were filtered separately and the filtrates were evaporated to dryness with a rotary evaporator (Stuart, UK) under reduced pressure temperature of 40^{o}C. Ethyl acetate extract (~3.10 g) was used for phytochemical investigation and n-hexane extract was kept for fatty acid analysis.

Isolation of phytochemicals from EtOAc extract

The crude EtOAc extract was exposed to TLC screening and it demonstrated several spots in iodine chamber and vanillin sulfuric acid spray on TLC plate. The dry mass of EtOAc extract (3.10 g) was subjected to column chromatography over column grade silica gel (Kiesel gel 60G). At first the column was eluted with 100% hexane and then eluted with mixtures of hexane with increasing amount of dichloromethane, and finally with increasing methanol. The effluents were collected in 250 mL conical flask where

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12 fractions marked as P_1, P_2, P_3, P_4, P_5, P_6, P_7, P_8, P_9, P_{10}, P_{11}, and P_{12} were obtained according to TLC pattern. Among the fractions P_8 was observed as a single spot. So, the fraction P_8 was allowed to stand for hours and white crystalline compound was obtained which was marked as **1**. The fraction P_9 appeared to contain three spots. The fraction P_9 was further subjected to sub column for re-fractionation by column chromatography. A total of seven fractions were collected based on their TLC pattern and marked as F_1, F_2, F_3, F_4, F_5, F_6 and F_7. Among them, F5 produced another white crystalline compound which was marked as **2**. The fraction F_6 was observed two spots with distinct R_f value. From F_6 a compound was separated and purified by preparative thin layer chromatography (PTLC) and was marked as **3**.

Analysis of fatty acids

n-hexane extract of *A. spinosus* was subjected to fatty acid (FA) analysis. Both free fatty acids (FFAs) and bound fatty acids (BFAs) were extracted from the plant and converted into their corresponding methyl ester to make the volatile to be capable of being analyzed by gas liquid chromatography (GC).The prepared methyl ester of FFA and BFA along with standard fatty acids ester samples were analyzed by GLC (Shimadzu 9A, Column-BP-50, Detector-FID, 105°C-5°C/min-150°C-2°C/min-280) and their retention time was recorded. The relative percentages of the FFAs and BFAs were calculated from peak area.

Membrane stabilizing activity

200 g dried powder of *A. spinosus* root was soaked in 1000 mL of methanol for 7 days with occasional shaking and filtered through a cotton plug followed by Whatman filter paper number 1. The filtrate was dried using a rotary evaporator under reduced pressure evaporator at low temperature. 5 g of the dried extract of *A. spinosus* was subjected to solvent-solvent partitioning following the modified Kupchan method[15] to yield n-hexane, dichloromethane, chloroform and aqueous soluble fractions. Then the crude methanol extract and its concentrated Kupchan fractions were evaluated for membrane stabilizing activity.

III. Results and Discussion

Three compounds were isolated (**1-3**) from the EtOAc extract of the roots of A. spinosus by column chromatography and preparative thin layer chromatography.

*Characterization of compound-**1***

Physical appearance of the compound-**1** was colorless, crystalline solid having R_F value of 0.80 in (98% DCM: 2% MeOH) and its melting point was found to be 158-160 °C. It dissolved in dichloromethane, chloroform. The ^{1}H-NMR (400 MHz, in $CDCl_3$) spectrum of the compound-**1** showed two sharp singlets (s) at δ 0.58 and 1.04 ppm typical for the presence of methyl protons at C-18 and C-19 respectively and multiplet at δ 3.62 ppm indicated of the presence of oxymethine proton (H-3). Two downfield signals at δ 5.04 and δ 5.15 ppm revealed the presence of olefinic protons at C-22 and C-23 respectively. A broad singlet at δ 1.28 ppm was due to the presence of methyl proton at C-29. The other signals of the spectrum between δ 1.05-2.06 ppm were due to the presence of different methylene (-CH_2-) and methine (>CH-) protons. The ^{13}C-NMR spectrum of compound-**1** showed the presence of twenty-nine (29) carbon signals. The signals at δ 139.58, 138.16, 129.47 and 117.47 ppm were due to for olefinic carbons and signals at δ 40.81 and 37.17 ppm were assignable to two quaternary carbons. The signals at δ 71.08 ppm give indication of oxymethine carbon. From the physical characteristics and spectral analysis (^{1}H-NMR and ^{13}C-NMR) data of the compound-**1** and comparing the reported value[16] of ^{1}H-NMR and ^{13}C-NMR spectral data of stigmasterol, the structure of the compound was established as stigmasterol.

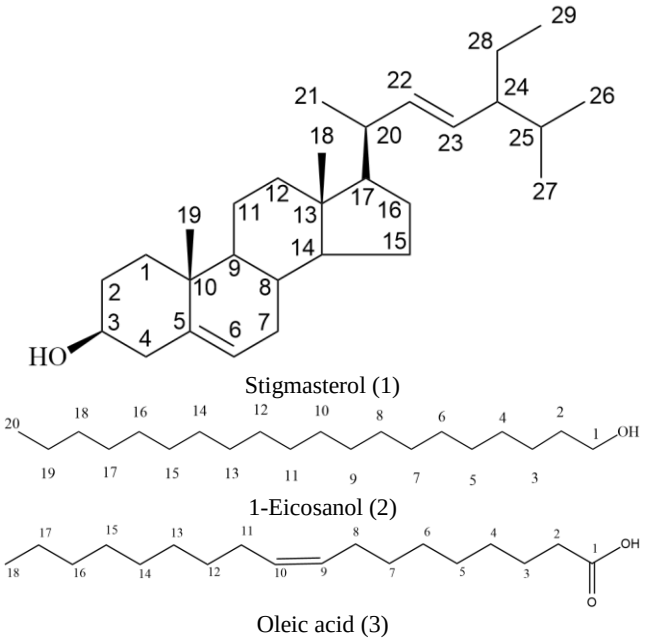
*Characterization of compound-**2***

Compound-**2** (~ 3.5 mg) was a colorless semi solid having R_F value 0.65 (in 70% DCM: 30% Hexane) and its melting point was found to be 58-60 °C. It was soluble in dichloromethane. The ^{1}H-NMR (400 MHz, in $CDCl_3$) spectral peak of the isolated compound-**2** at δ 0.90 ppm was indicative of methyl group. A strong sharp peak at δ 1.28 ppm was due to the presence of methylene protons at C-3 to C-19 [H-3 to H-19]. The signal at δ 1.59 ppm was due to the presence of proton at C-2 (H-2). The signal at δ 3.66 ppm was due to the methylene proton adjacent to C-1 containing –OH group. The ^{13}C-NMR spectrum of the compound-**2** showed the presence of 20 carbons. The signal at δ 63.12 ppm was due to the alcoholic carbon (>CH-OH) at C-1. The signal at δ 32.83 ppm is assignable to methylene carbon attached to C-1. Six signals between δ 22.69- 31.93 ppm were due to the presence of methylene (-CH_2-) carbon and the signal at δ 14.10 ppm was assignable to methyl carbon at C-20. All these ^{1}H-NMR and ^{13}C NMR spectral data of compound-**2** was in good agreement with the reported data[17,18] of 1-Eicosanol and the compound-**2** was established as 1-Eicosanol.

*Characterization of compound-**3***

Physical state of the compound-**3** (~ 3.2 mg) was a white-semi solid having R_F value 0.75 (in 70% DCM: 30% Hexane). The compound was soluble in dichloromethane. On spraying with vanillin-sulfuric acid spray reagent, followed by heating at 110 °C for several minutes and violet color was appeared. The IR spectrum of the compound-**3** revealed absorption band at 2954, 2849, 1707, 1631, 1472, 1434 and 719 cm^{-1}. IR absorption band at 2954 cm^{-1} and at 1707 cm^{-1} were assignable to O-H stretching and carbonyl group (C=O stretching), respectively. The absorption band at 1434 cm^{-1} was indicative to O-H bending. The ^{1}H-NMR (400 MHz, in $CDCl_3$) spectrum of the isolated compound-**3** had a peak at δ 0.90 ppm indicative of three protons of one methyl group. A strong sharp peak at δ 1.28 ppm was due to the presence of methylene protons at C-4 to C-7 and C-12 to C-16. The band at δ 2.37 ppm was due to the presence of proton at C-2

(α-carbon). The band at δ 5.38 ppm was due to the proton attached with unsaturated carbon. All these FT-IR and ^{1}H-NMR data was good agreement with the reported data[19] of oleic acid. Hence the compound-**3** was confirmed as oleic acid.



Stigmasterol (1)

1-Eicosanol (2)

Oleic acid (3)

Fig. 1. Structure of isolated compounds

Fatty acid analysis

431 mg of n-hexane extract was dissolved in n-hexane followed by extraction with 5% sodium bicarbonate solution. The mixture was taken in a separatory funnel & shaken vigorously and two layers were obtained. The lower layer (aqueous) and the upper layer were separated for the analysis of FFA and BFA respectively. Both types of FAs of the roots were converted to their methyl esters for analyzing by gas liquid chromatography (GLC). The prepared methyl ester of FFAs and BFAs along with standard fatty acids ester samples were subjected to GLC and their retention time was recorded. In the plant, four saturated fatty acids (SFAs); caprylic acid, stearic acid, arachidic acid and behenic acid, and four unsaturated fatty acids (USFAs); palmitoleic acid, Oleic acid, Linoleic acid and erucic acid were identified. USFAs were higher amount than the SFAs. Similarly, total amount of BFAs (117 mg) were higher than the total amount of FFAs (29 mg). The analysis of BFAs showed that *A. spinosus* contains highest proportion of stearic acid (26.45 %), and lowest proportion of arachidic acid (1.84 %) and others as palmitoleic, oleic, erucic, linoleic, caprylic behenic acids are present with intermediate percentage of 24.12, 19.02, 11.20, 7.82, 5.10 and 4.43 % respectively. The analysis of FFAs disclosed that palmitoleic acid is the most abundant (30.40 %) FA present in free form in the roots of *A. spinosus.* and erucic, stearic, behenic, oleic, linoleic, caprylic, arachidic acids are the other FAs present with an intermediate percentage of 23.45, 12.83, 10.19, 8.38, 8.37, 4.44 and 1.94 % respectively.

Evaluation of membrane stabilizing activity

Anti-inflammatory activity of methanolic extracts and their different fraction of the roots of *A. spinosus* were assessed by following the method[20] of hypotonic and heat induced hemolysis of human erythrocyte using aspirin as standard. The maximum level of membrane stabilizing activity was observed by methanol extract in both hypotonic solution-induced hemolysis (68.13%) and heat induced hemolysis (74.53%). In hypotonic solution-induced hemolysis, n-hexane fraction (HF) inhibited 39.94%, chloroform fraction (CHF) inhibited 32.65%, dichloromethane fraction (DCMF) inhibited 27.80%, and aqueous fraction (AQF) inhibited 16.88% of hemolysis of RBC whereas acetylsalicylic acid exhibited 61.90% inhibition of hemolysis at normal condition. Again, in heat induced hemolysis, HF inhibited 65.21%, CHF inhibited 61.47%, DCMF inhibited 51.22%, and AQF inhibited 22.68% of hemolysis of RBC while the acetylsalicylic acid showed 42.00% inhibition of hemolysis at normal condition.

IV. Conclusions

The ethylacetate extract of the roots of *A. spinosus* was subjected to different chromatographic separation techniques to isolate secondary metabolites and the isolated compounds were namely stigmasterol, 1-Eicosanol, and oleic acid. The structures of the isolated compounds were elucidated by analyzing their different spectroscopic data (FT-IR, ^{1}H-NMR, and ^{13}C-NMR). FA analysis of the plant material revealed that USFAs were higher than SFAs. The results of In Vitro membrane stabilizing activity have provided a reasonable indication that methanol extract of roots of *A. spinosus* possess significant anti-inflammatory activity. However, further investigations are required to isolate and characterize the active therapeutics accountable for this property.

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