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CHEMICAL AND BIOLOGICAL ACTIVITY STUDIES OF *ENTEROMORPHA FLEXUOSA* WULFEN

ELIAS AHMED, MOHAMMAD SHOEB*, MD. MIZANUR RHAMAN, MD. NAZRUL ISLAM, RAFIZA ISLAM AND NILUFAR NAHAR

Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh

Abstract

The marine algae *Enteromorpha flexuosa* Wulfen belongs in the Ulvaceace family, grows naturally on the Saint Martin's Island, Cox's Bazar, Bangladesh. *E. Flexuosa* was collected from the Island for isolation of secondary metabolites and investigation of their biological properties. The dried algae was grounded and was extracted with a mixture of dichloromethane (DCM) and methanol (1:1). The parent extract was suspended in water and was partitioned successively with n-hexane, DCM and 1-butanol. From DCM soluble part myristic acid, palmitic acid, β-sitosterol and stigmasterol were isolated by repeated column chromatography and their structure were determined by FT-IR, ^{1}H and ^{13}C NMR spectroscopic studies The oil of the algae was found to contain palmitoleic acid (45%), *cis*-9-oleic acid (19%), linoleic acid (15%), palmitic acid (15%) and myristic acid (6%). Cytotoxicity assay of different sub-fractions of parent extract was carried out on the HeLa cell line, a human cervical carcinoma. DCM soluble part was found to be significantly cytotoxic. Antioxidant activity & capacity and phenolic content of the sub-fractions were also evaluated and DCM soluble part was more active than other sub-fractions. Palmitoleic acid (45%), *cis*-9-oleic acid (19%), linoleic acid (15%), palmitic acid (15%) and myristic acid (6%) were found to be the major acids of the oils.

Keywords: *Enteromorpha flexuosa, β*-sitosterol, palmitic acid, stigmasterol, myristic acid, antioxidant activity, cytotoxicity

Introduction

The filamentous dark to light green marine algae *Enteromorpha flexuosa* Wulfen ex Roth belongs to the Ulvaceace family, distributed throughout the world. There are about 135 species of the genus Enteromorpha. *E. flexuosa* is widely used as food, feed (especially for fish and pigs), fertilizer and medicine. It is abundantly growing in costal seashore of Asia. Marine macro algae are rich with various source of bioactive natural products and were studied as potential bioactive and pharmacological agents.[2,3] In recent years, there are several reports of microalgae derived compounds that have a wide range of biological activities such as anti-fungal, antiviral, anti-neoplastic, antifouling, anti-inflammatory, anti-turmeric, cytotoxic, and anti-diabetic.[4] At present seaweeds constitute commercially important marine renewable resources which are providing valuable thoughts for the development of new drugs against cancer, microbial infections and inflammations.[5] Marine algae have been screened broadly to isolate lifesaving drugs or biologically active substance all over the world.[6] Various secondary metabolites including alkaloids, steroids, phenols, saponins and flavonoids were also isolated from the genus Enteromorpha.[7] It is commonly known as aonori and is used in the preparation of variety of dishes including soups, salads, meals, and condiments in China, Japan, USA, France and Chile.[8] There is no scientific evaluation of *E. flexuosa* is grown in Saint Martin's Island in Bangladesh. Therefore, objectives are to isolate secondary metabolites and to evaluate possible biological activities of *E. flexuosa* Wulfen algae naturally grown in Saint Martin's Island.

* *Corresponding Author: shoeb71@du.ac.bd & shoeb71@yahoo.com*

Materials and Methods

Instrument: A Shimadzu FT-IR 4800S spectrometer was used to record FT-IR. Ultra-violet (UV) spectra were recorded using the double beam UV/Visible spectrophotometer (UV-1800), Bruker spectrometer was used to record ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra. Shimadzu GC 2025 Gas Chromatograph having FID Detector was used for fatty acid analysis.

Collection of plants: *E. flexuosa* was collected from Saint Martin's Island, Cox's Bazar, Bangladesh and was identified by the Botanist, Department of Botany and University of Dhaka, Bangladesh (Voucher Specimen EF 001). The leaves were first dried in room temperature and then in the oven at 40°C and finally dried materials were grounded into power.

Extraction: The powdered algae was extracted with mixture of DCM and methanol (1:1). The crude extract (7.50 g) was suspended in water and was partitioned successively with hexane, dichloromethane (DCM) and finally with 1-butanol. All the extracts were separately concentrated using rotary vacuum evaporator at 40°C under reduced pressure, and n-hexane (1.02 g), DCM (4.0 g) and 1-butanol (1.87 g) extracts were obtained.

Isolation of secondary metabolites: The DCM soluble part (4.0 g) was fractionated by silica gel (Si-gel G-60; 0.063-0.200 mm, 70-230 mesh ASTM) column chromatography using n-hexane as column packing & equilibrating solvent, eluted with 0 to 100% DCM in hexane followed by 5 to 100% ethyl acetate (EtOAc) in DCM, then 100% EtOAc and nine fractions (F_1-F_9) were collected. One of the fractions (F_4) was re-fractionated by a Si-gel sub-column chromatography and five fractions (J_1-J_5) were obtained. The fraction J_4 was purified by crystallization with n-hexane to get compound **1** (5.0 mg) and another fraction J_3 yielded compound **2** (7.2 mg) after purification with n-hexane. The fraction, F_1 was subjected to fractionation by sub-column Si-gel chromatography and eight fractions (K_1-K_8) were obtained. The fractions K_4 and K_6 yielded compound **3** (6.5 mg) and compound **4** (3.4 mg) by purification with *n*-hexane, respectively.

Compound 1: Solid, soluble in DCM, FT-IR (KBr pellet) $\boldsymbol{\nu}_{max}$: 3426, 2960, 2938, 2865, 1465, 1382, 1174 and 1063 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$; δ values are in ppm) 5.34 (distorted multiplete), 3.51 (multiplete), 2.26, 1.99 (broad singlet), 1.82, 1.56, 1.48, 1.24, 0.96 (ss), 0.91 (d), 0.87 (t) 0.83 (d), 0.81 (d) and 0.67 (sharp singlet) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$; δ values are in ppm): 140.8, 121.7, 71.8, 56.8, 56.1, 50. 2, 45.8, 43.4, 42.4, 39.8, 37.2, 36.9, 36.1, 35.3, 33.9 , 31.9, 31.7, 30.3, 29.2, 26.1, 24.3, 23.1, 21.2, 19.8, 19.4, 19.0, 18.8, 13.7 and 12.5 ppm.

Compound 2: Solid. Soluble in DCM, FT-IR (KBr pellet) $\boldsymbol{\nu}_{max}$: 3437, 2934, 2859, 1645, 1462, 1376 and 1058 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$; δ values are in ppm): 5.62, 5.34, 5.15, 3.51, 2.27, 1.99, 1.85, 1.82, 1.48, 1.24, 1.18, 1.01, 0.99, 0.90, 0.83, 0.8 and 0.67 ppm. ^{13}C-NMR spectrum (100 MHz, $CDCl_3$; δ values are in ppm) 140.8, 138.3, 129.3, 121.7,71.9, 56.8 , 56.1, 51.2 , 50.1, 45.9, 42.3, 42.2, 39.8, 37.2, 36.6, 36.2, 31.9, 29.2, 28.2, 25.4, 24.3, 23.1, 21.2, 19.8, 19.4, 19.0, 18.7, 14.1 and 12.2 ppm.

Compound 3 Oily, soluble in hexane, FT-IR (KBr pellet) $\boldsymbol{\nu}_{max}$: 3434, 2918, 2849, 1705, 1487 and 1295 cm^{-1}. ^{1}H-NMR spectrum (400 MHz, $CDCl_3$; δ values are in ppm) 2.37 (t), 1.64 (m), 1.32 (bs), 1.27 and 0.90 (t) ppm. ^{13}C-NMR spectrum (100 MHz, $CDCl_3$; δ values are in ppm): δ 180.0, 34.0, 31.9, 29.6, 29.6, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 27.2, 24.7, 22.7 and 14.0 ppm.

Compound 4: Yellowish white oily, Soluble in hexane, FT-IR (KBr pellet) $\boldsymbol{\nu}_{max}$: 3445, 2919, 2850, 1704, 1464 and 1295 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$; δ values are in ppm): 2.37 (t), 1.65 (m), 1.28 (bs) and 0.91 (t) ppm.^{13}C-NMR spectrum (100 MHz, $CDCl_3$; δ values are in ppm): 178.2, 33.7, 31.9, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 24.7, 22.7 and 14.1 ppm.

Analysis of fatty acid compositions: Fatty acid compositions of oil from *E. flexuosa* was analyzed following the procedure described in earlier report.[8]

Cytotoxicity assay on cancer cell line: The cytotoxicity assay of *E. flexuosa* extracts was assessed against a human cervical carcinoma cell line, HeLa which was maintained in DMEM (Dulbecco's modified Eagles medium) containing 1% penicillin-streptomycin (1:1) and 0.2% gentamycin and 10% fetal bovine serum (FBS). Cells (4×104/200 µL) were seeded onto 48-well plate and incubated at 37ºC+5% CO_2. Next day, 50 µL sample (filtered) was added in each well. Cytotoxicity was examined under an inverted light microscope after 48 hour of incubation. Samples were dissolved in 5% DMSO and duplicate wells were used for each sample.[9]

Total Phenolic Content: The total phenolic contents of *E. flexuosa* extracts were determined by modified Folin-Ciocalteu method and expressed as mg gallic acid equivalents per gram of dry extract using the equation obtained from gallic acid calibration curve ($y = 0.003x - 0.001$, $r^2 = 0.996$).[10, 11]

Total Antioxidant Capacity: The total antioxidant capacity of *E. flexuosa* extracts was evaluated by the Phosphomolybdenum assay method.[11, 12] It was determined and expressed as mg ascorbic acid equivalents per gram of dry extract using the equation obtained from ascorbic acid calibration curve ($y=0.002x-0.006$, $r^2=0.991$).

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity: The stable DPPH radical-scavenging activity was measured using the modified method.[11-13]

DPPH radical-scavenging activity (I %), $\mathbf{= [(A_0 - A) / A_0] \times 100,}$ where A_0 is the absorbance of the control solution; A is the absorbance of the DPPH solution containing plant extract.

Results and Discussion

Silica gel column chromatography of the DCM extract of *E. flexuosa* afforded four compounds **1-4** (Fig: 1). The FT-IR spectrum of **1** showed absorption band at 3426 cm^{-1} assignable to O-H group and 2960 cm^{-1} which was due to the presence of aliphatic C-H stretching. The two peaks at 1465 cm^{-1} and 1382 cm^{-1} were indicative of -CH_2- and -CH_3 groups, respectively. The absorption band at 1174 cm^{-1} was due to C-O stretching and 1063 $cm^{-1}$was suggestive of>C=C-H. The ^{1}H-NMR spectrum of **1** had two sharp singlets (s) at δ_H 0.67 and 0.96 ppm typical for the presence of methyl protons. The spectrum had a multiplete at 3.51 ppm indicated the presence of oxymethine proton (H-3α). The distorted multiplete at δ_H 5.34 ppm was indicative of the presence of olefinic proton (H-6) at C-6. The three doublets at δ_H 0.91, 0.83 ppm and 0.81 ppm were due to the presence of three methyl protons. The spectrum had a triplet at δ_H 0.87 ppm was due to the presence of methyl group. The other signals between δ_H 1.24 and 2.26 ppm were due to the presence of different methylene (-CH_2-) and methine (>CH-) protons. The ^{13}C-NMR spectrum of **1** showed signals at δ_c 140.8 and 121.7 ppm which were due to two olefinic carbons and signals at δ_c 36.9 and 43.4 ppm were assignable to two quaternary carbons. The signals at δ_c 12.5, 13.7, 18.8, 19.0, 19.4 and 19.8 were due to the presence of six methyl carbons and the signals at δ_c 37.2, 33.9, 42.4, 31.9, 21.2, 39.8, 24.3, 23.1, 29.2, 35.3 and 26.1 ppm were due to the presence of eleven methylene carbons. The signals at δ_c 71.8, 31.7, 50.2, 56.8, 56.1, 36.1, 45.8 and 30.3 ppm were due to the presence of eight methine carbons. The signal at δ_c 71.8 was due to oxymethine carbon. ^{1}H- and ^{13}C-NMR data of **1** was compared with reported NMR data and found identified with *β*-sitosterol.[14] Compound **2** had similar spectral data of compound **1** with additional signals at δ_H 5.62 and 5.15 ppm and δ_c 138.3 and 129.3 ppm in the ^{1}H and ^{13}C NMR spectrum, respectively. The spectroscopic data of compound **2** was compared with the reported data of stigmasterol and found identical.[14] Thus, compound **2** was confirmed as stigmasterol. The FT-IR spectrum of compound **3** showed absorptions band at 3434, 2918, 2849, 1705, 1487 and 1295 cm^{-1} for -OH, -CH_3, CH_2, -C=O, C-H, and C-O cm^{-1}, respectively. The ^{1}H-NMR (400 MHz, in $CDCl_3$) spectrum of the isolated compound **3** had one peak at δ_H 0.90 ppm for methyl group and the other four peaks 2.37, 1.64, 1.32 and 1.27 ppm for methylene group. The ^{13}C-NMR spectrum of isolated compound showed the presence of 16 carbon signals. Among them, the peaks at δ_C 180.0 and 14.0 ppm were assigned for ester group (–COOH)

and methyl group (-CH_3), respectively. The other fourteen peaks were assigned form ethylene (-CH_2-) group. From all spectroscopic data, it is confirmed that compound **3** is palmitic acid. The compound 4 had similar ^{1}H and ^{13}C NMR data with lack of two carbons which confirmed **4** to be myristic acid.

Relative Fatty Acid Compositions

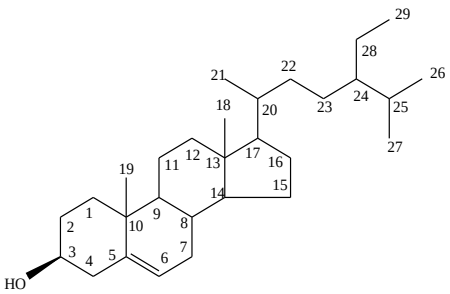
The fatty acids in the oil from hexane extract were made into their methyl ester by saponification with methanolic NaOH followed by esterification with BF_3-MeOH complex, and analysed by GC-FID. The relative percentage of methyl ester of fatty acids in oil was identified by comparing their retention time with that of methyl ester of fatty acid standard. It was found that palmitoleic acid (45%), *cis*-9-oleic acid (19%), linoleic acid (15%), palmitic acid (15%) and myristic acid (6%) were major acids in the extracts of *E. flexuosa* and palmitoleic acid was predominant among all. The present of unsaturated palmitic acid may be helpful for the consumer to use *E. flexuosa* as vegetable.

Cytotoxicity assay: Cytotoxicity assay of n-hexane, DCM and MeOH extracts of *E. flexuosa* were assessed against HeLa, a human cervical carcinoma cell line. Out of **4** different extracts only DCM, MeOH and 1-butanol extracts were found to be cytotoxic against Hela cell lines **(Fig. 2)**.

Total Phenolic Content: The total phenolic content of hexane, 1-butanol, DCM and methanol extract were 7.0±1.4, 3.0±1.03, 81.33±0.87 and 39.0±0.77 mg GAE/g of dry extract, respectively. The results showed that DCM extract exhibited the highest phenolic contents. The activity of the DCM extract might be due to the presence of phenolic or flavonoid type's compounds present in the extract **(Fig. 3-4)**.

Total Antioxidant Capacity: The total antioxidant capacity of n-hexane, DCM, methanol and 1-butanol extracts were 184±3.80, 312.5±7.9, 205.5±4.1 and 96.0±3.7 mg AAE/g of dry extract. The DCM extract showed the highest antioxidant capacity among all extracts **(Fig. 5-6)**.

Free Radical Scavenging Activity: Ascorbic acid was used as positive control. The IC_{50} value of n-hexane, DCM and MeOH extracts were 681.76±0.410, 79.29±0.20 and 387.53±0.38 µg/mL, respectively while that of ascorbic acid was 2.47±0.013µg/mL. A lower IC_{50} value is associated with a higher radical scavenging activity. The activity of DCM extract was higher which might be due to the presence of flavonoids, coumarine and phenolic compounds **(Fig. 7-10)**.



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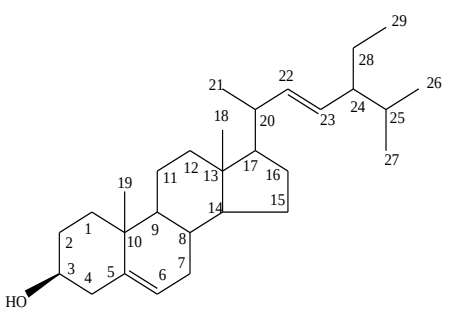

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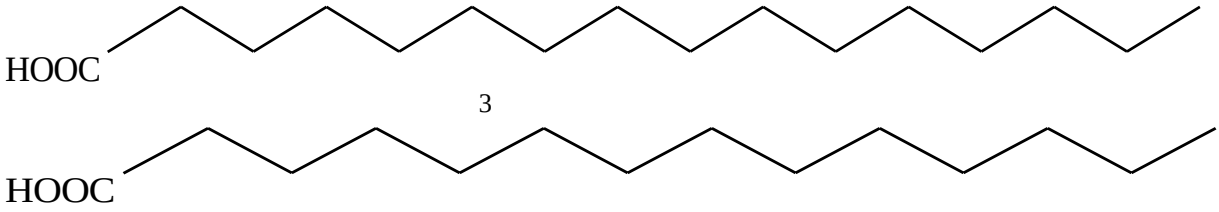

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Fig. 1: Structure of isolated compounds

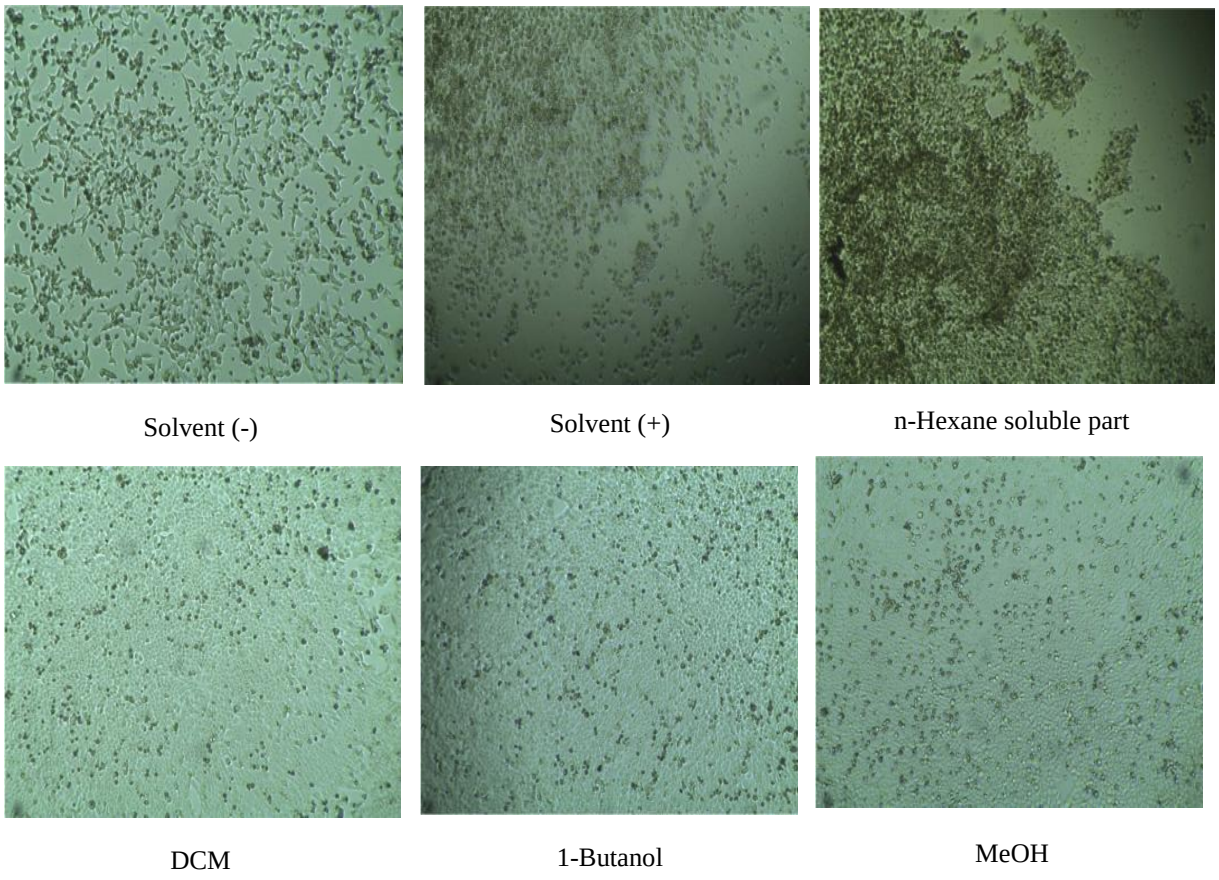
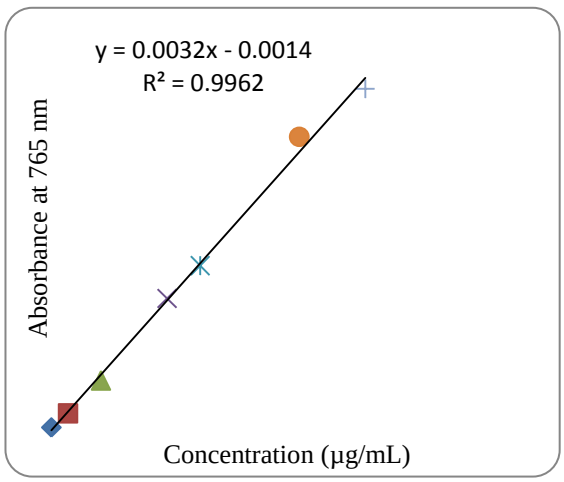


Fig. 2: HeLa cell line treated with samples



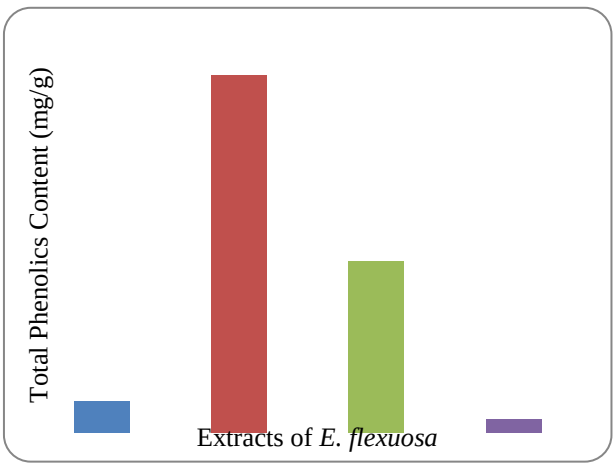


Fig. 3: Calibration curve of standard Gallic acid

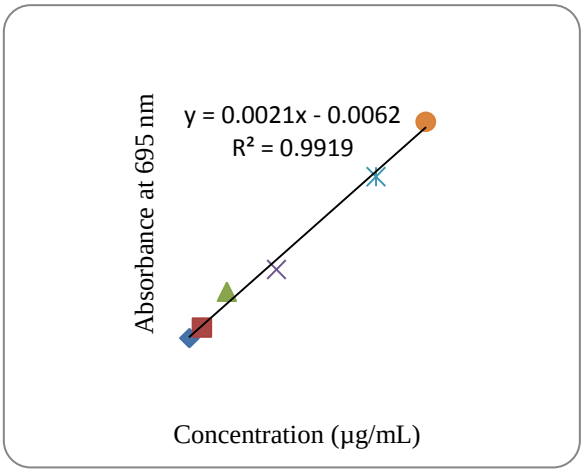


Fig. 4: Total phenolic content in the different Sub-fractions of *E. filexuosa* extract

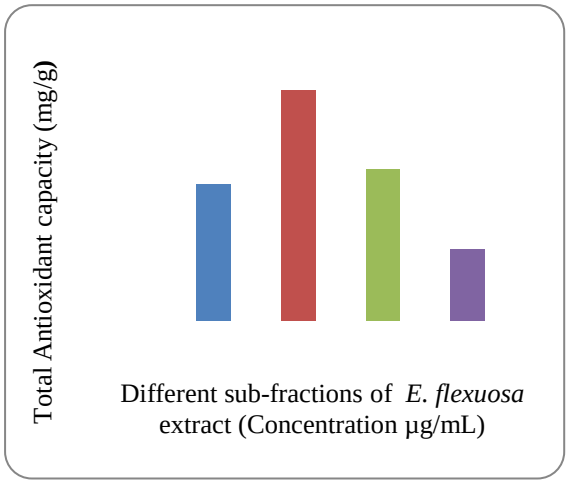


Fig. 5: Calibration curve of standard Ascorbic acid

Fig. 6: Total antioxidant capacity of different Sub-fraction of *E. filexuosa* Extract

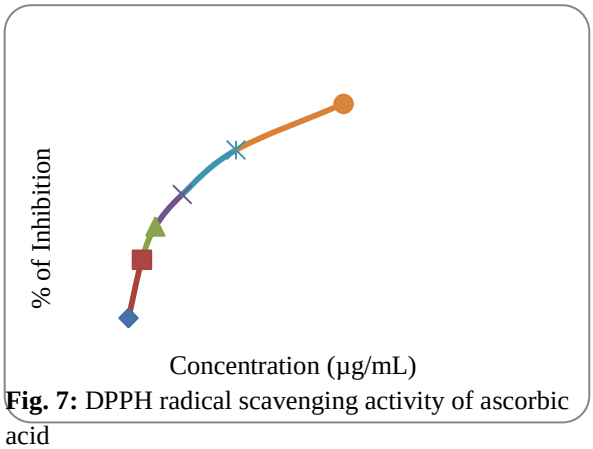


Fig. 7: DPPH radical scavenging activity of ascorbic acid

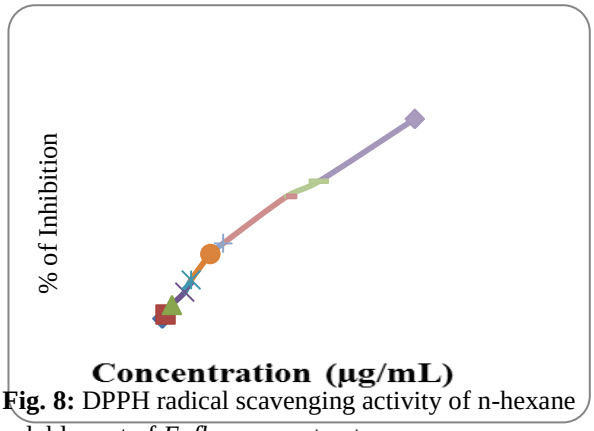


Fig. 8: DPPH radical scavenging activity of n-hexane soluble part of *E. flexuosa* extract

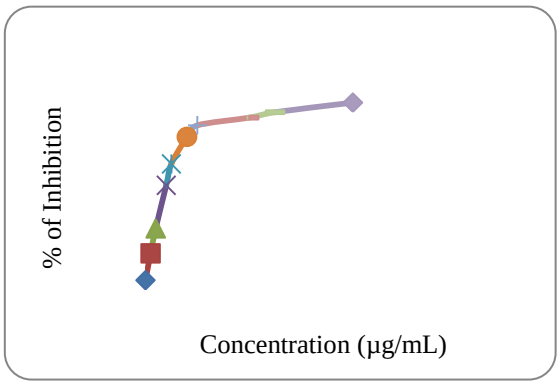


Fig. 9: DPPH radical scavenging activity of DCM soluble part of extract of *E. flexuosa*

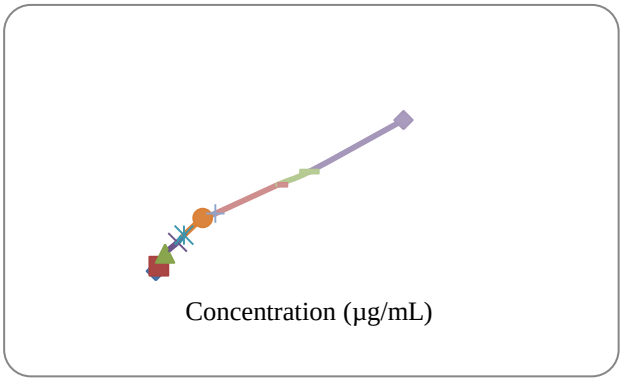


Fig. 10: DPPH radical scavenging activity of MeOH soluble part of the extract of *E. flexuosa*

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